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The Amazon River system as an ecological barrier driving genetic differentiation of the pink dolphin (*Inia geoffrensis*)

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The pink dolphin (*Inia geoffrensis*) is widely distributed along the Amazon and Orinoco basins, covering an area of approximately 7 million km^2. Previous morphological and genetic studies have proposed the existence of at least two evolutionary significant units: one distributed across the Orinoco and Amazon basins and another confined to the Bolivian Amazon. The presence of barriers in the riverine environment has been suggested to play a significant role in shaping present-day patterns of ecological and genetic structure for this species. In the present study, we examined the phylogeographic structure, lineage divergence time and historical demography using mitochondrial (mt)DNA sequences in different pink dolphin populations distributed in large and small spatial scales, including two neighbouring Brazilian Amazon populations. mtDNA control region (CR) analysis revealed that the Brazilian haplotypes occupy an intermediate position compared to three previously studied geographic locations: the Colombian Amazon, the Colombian Orinoco, and the Bolivian Amazon. On a local scale, we have identified a pattern of maternal isolation between two neighbouring populations from Brazil. Six mtDNA CR haplotypes were identified in Brazil with no sharing between the two populations, as well as specific cytochrome *b* (cyt *b*) haplotypes identified in each locality. In addition, we analyzed autosomal microsatellites to investigate male-mediated gene flow and demographic changes within the study area in Brazil. Data analysis of 14 microsatellite loci failed to detect significant population subdivision, suggesting that male-mediated gene flow may maintain homogeneity between these two locations. Moreover, both mtDNA and microsatellite data indicate a major demographic collapse within Brazil in the late Pleistocene. Bayesian skyline plots (BSP) of mtDNA data revealed a stable population for Colombian and Brazilian Amazon lineages through time, whereas a population decline was demonstrated in the Colombian Orinoco lineage. Moreover, BSP and Tajima's *D* and Fu's F_s tests revealed a recent population expansion exclusively in the Bolivian sample. Finally, we estimated that the diversification of the *Inia* sp. lineage began in the Late Pliocene (approximately 3.1 Mya) and continued throughout the Pleistocene. Published 2011. This article is a US Government work and is in the public domain in the USA. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 812–827.

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INTRODUCTION

The pink dolphin, *Inia geoffrensis* (de Blainvillei, 1817), also called 'boto' in Brazil, is a widely distributed freshwater river dolphin. It is found in the Amazon, Orinoco, and Araguaia/Tocantins (part of the Amazon basin) River systems of South America, an area of approximately 7 million km^2 (Best & da Silva, 1989). Current taxonomic classification considers *I. geoffrensis* as a single species with three geographically distinct subspecies: *Inia geoffrensis geoffrensis* from most of the Amazon and Araguaia/Tocantins

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basin; *Inia geoffrensis humboldtiana* from the Orinoco basin; and *Inia geoffrensis boliviensis* from the system of Bolivian and Brazilian rivers, with populations in the Madeira drainage area upstream of the Teotônio rapids in Brazil (Rice, 1998; Best & da Silva, 1989). Nevertheless, the taxonomic status of the *I. g. boliviensis* is currently under discussion and some studies report it as a separate species (Martínez-Agüero, Flores-Ramírez & Ruiz-García, 2006; Ruiz-García, 2010). In addition to the morphological differences first reported by Pilleri & Gihr (1977), studies with mitochondrial and nuclear DNA markers have suggested *I. g. boliviensis* to be a genetically distinct unit (Banguera-Hinestroza *et al*., 2002; Ruiz-García, 2010). Because of the recent proposal of *Inia boliviensis* as a separate species, we refer to this species complex as *Inia* sp. in the present study.

Geographical barriers to dispersal and gene flow, possibly leading to subspecies isolation, were identified along the *Inia* sp. distribution. The Amazon and Orinoco basins, where *I. g. geoffrensis* and *I. g. humboldtiana* are found, present an intermittent connection through the Casiquiare channel during the flood season (Rice, 1921; Best & da Silva, 1989). The Bolivian Amazon population (*I. g. boliviensis*) is isolated from the others by a stretch of 400 km of rapids in the Madeira drainage area, between Porto Velho and Guajará-Mirim in Brazil. The other main streams of the Amazon basin are unobstructed by physical barriers such as rapids or dams, and *Inia* occurs throughout its length with an apparent decline towards the mouth of the Amazon (Martin & da Silva, 2004b; Emin-Lima *et al*., 2007).

In the Orinoco and Amazon basins, *Inia* sp. is found in a variety of habitats including rivers, channels, and lakes. In the Central Amazon, seasonal variation in water levels may influence their distribution among habitats; high densities of *Inia* have been recorded in small channels and lakes, where the fish biomass is higher during the dry season (Martin & da Silva, 2004b). This pattern was also registered in Bolivia where *Inia* were seen in lakes more often during the dry season (Alliaga-Rossel, Mcguire & Hamilton, 2006). The high density of *Inia* found in such productive environments appears to correlate with the observation of increased entanglement by fishing nets employed by local fishermen in these areas (da Silva, 1994). Direct catch of *Inia* in the Brazilian Amazon has been forbidden by federal law since 1987, although this practice has intensified in recent years. Fishermen are currently using *Inia* as bait to catch catfishes (Estupinán *et al*., 2003), an activity that has become one of the main income sources in the Central Brazilian Amazon. Therefore *Inia* sp. overexploitation is currently a focus of major concern threatening the future of this species, along with other human-related activities, including habitat degradation as a result of expanding populations near its range area. Overall, the biological data are still in terms of allowing confident estimates of the conservation status for the Amazon River dolphin. Thus this species, which was previously listed as Vulnerable by the World Conservation Union (IUCN), is now considered as Data Deficient (Reeves *et al*., 2010).

Understanding the processes underlying the genetic diversity in populations distributed in such heterogeneous and dynamic habitats represents a challenge in evolutionary biology and species conservation (Avise *et al*., 1987). In the present study, we examined the phylogeography, population structure, and demographic history of *Inia* sp. To date, genetic comparisons among *Inia* subspecies have been reported by Banguera-Hinestroza *et al*. (2002), Vianna *et al*. (2010), and Ruiz-García (2010), who identified a strong phylogeographic pattern in a large spatial scale. We have performed a detailed study of two *Inia* sp. subpopulations from the Central Amazon in Brazil using the mitochondrial (mt)DNA control region (CR) and cytochrome *b* (cyt *b*) gene sequences and 14 autosomal microsatellites. We used the mtDNA CR to address the phylogenetic relationship between the Central Amazon population (Brazil) and other adjacent *Inia* sp. populations reported by Banguera-Hinestroza *et al*. (2002) aiming to understand the population structure at a macrogeographic scale. We also compared the two geographically close Brazilian populations using mtDNA CR, cyt *b*, and autosomal microsatellites to investigate the local pattern of population structure, gene flow and historical demography.

MATERIAL AND METHODS

Sample collection and DNA extraction

Tissue samples were collected over 14 years from a total of 43 dead animals from three neighbouring locations in the Central Amazon in Brazil: Tefé Lake ($N = 17$), Mamirauá Sustainable Development Reserve ($N = 16$), and Amanã Lake ($N = 10$) (Fig. 1; see also Supporting information, Table S1). Samples from Mamirauá and Amanã are herein treated as a single population (Mamirauá) because they originate from a continuous water environment at the left margin of the Amazon River, whereas Tefé is located at the right margin. Samples were obtained from animals that were found dead or were accidentally caught in nets. Muscle and skin tissues were preserved in 95% ethanol and stored at 4 °C. DNA was extracted in accordance with the standard phenol–chloroform protocol, *sensu* Sambrook & Russell (2001). DNA was quantified using a fluorescence-

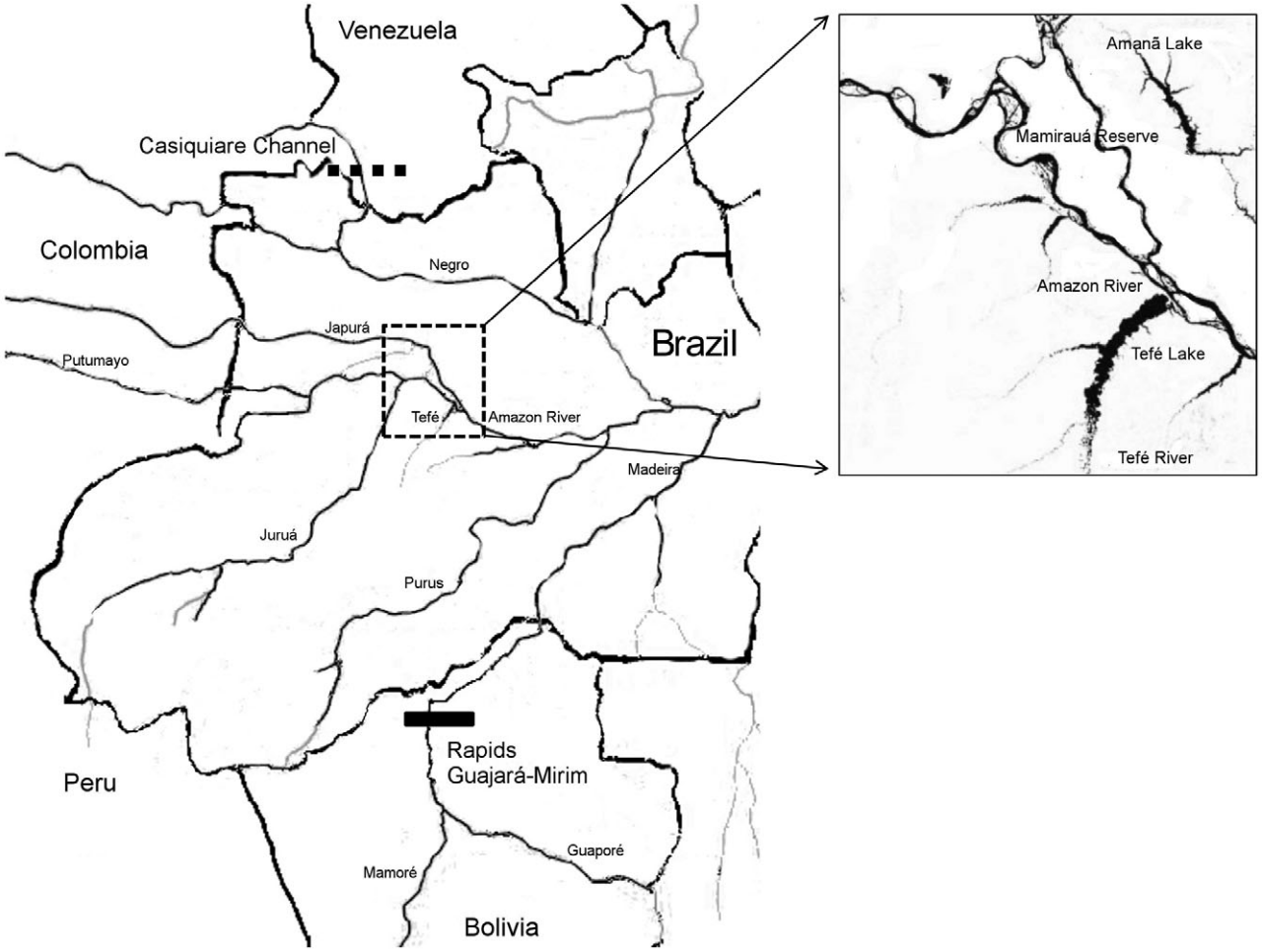

Figure 1. Map showing a zoom of the sampled areas in Brazil: the Tefé River and Lake, Mamirauá Reserve, and Amanã Lake in the central part of the Amazon basin. The large South American map shows a region of occurrence of *Inia* sp. with a solid bar indicating the barrier constraining *Inia* sp. contact in the Madeira River, and a dashed bar indicating the region where the Amazon and Orinoco basins are connected and may occasionally exchange populations in the rainy period.

based method on a Qubit Fluorometer, in accordance with the manufacturer's instructions (Invitrogen).

DNA AMPLIFICATION, SEQUENCING, AND SEX DETERMINATION

A 400-bp fragment of the mtDNA CR was amplified via the polymerase chain reaction (PCR) using primers CR-4 (Southern, Southern & Dizon, 1988) and CR-5 (Southern *et al*., 1988; Palumbi *et al*., 1991). Alternatively, for samples sequenced at Oregon State University, we used primers tPro-whale M13-Dlp-1.5 (Baker, Cipriano & Palumbi, 1996) and Dlp-8G (designed by G. Lento, *sensu* Dalebout *et al*., 2005) for the amplification reactions. The PCR amplification conditions were performed *sensu* Caballero *et al*. (2007).

The complete mtDNA cyt *b* gene (1140 bp) was amplified using primers L14121 and H15318 (Redondo *et al*., 2008). Each PCR mix contained 20–40 ng of genomic DNA, 1× Taq reaction buffer 1B (Phoneutria – 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.1% Triton X-100), 200 μM dNTPs, 0.5 μM of each primer, and 1 unit of Taq DNA polymerase (Phoneutria). The PCR cycling was performed in an Eppendorff Gradient thermocycler following the conditions described by Vianna *et al*. (2010).

PCR products were cleaned using 20% polyethylene glycol + 2.5 % NaCl, and both strands were sequenced using an ET dye terminator kit (GE Healthcare) in a MegaBACE automated capillary sequencer with the cycle sequencing temperature profile: 95 °C for 1 min, 35 cycles of 95 °C at 25 s, annealing at 50 °C for 15 s, and extension at 60 °C for 3 min. Two additional

primers were used to sequence the complete cyt *b* gene: XL14733 and MVZ4 (Kocher *et al*., 1989). For samples sequenced at Oregon State University, free nucleotides and primers were removed from the PCR products using shrimp alkaline phosphatase and *Exo*I and directly sequenced using the standard protocols of Big Dye terminator sequencing chemistry on an ABI 3730 automated capillary sequencer. To ensure sequence accuracy and generate high quality consensus sequences, we confirmed each sequence position from at least two independent primary PCRs with forward and reverse runs (Redondo *et al*., 2008; Morin *et al*., 2010). Individuals whose sex could not be determined by post-mortem examination were identified by PCR amplification of a fragment of the *SRY* gene multiplexed with fragments of the *ZFY*/*ZFX* genes as positive control, *sensu* Gilson *et al*. (1998).

Microsatellite genotyping

For the microsatellite studies, sample and data quality were checked in accordance with the recommendations described by Morin *et al*. (2010). Samples were genotyped at 14 dinucleotide microsatellite loci either developed directly for *Inia* sp. (Gravena *et al*., 2009) or using heterologous primers isolated from the other cetacean species (Table 2) and standardized for *Inia* sp. samples. Amplification reactions contained 20–40 ng DNA, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM dNTPs and 0.25 units of Platinum-Taq (Invitrogen). Each forward primer had a M13 sequence tail added to its 5′ end to allow fluorescent labelling (HEX, NED, FAM) following Schuelke (2000). The thermocycle profile for the microsatellites isolated from another species were carried out in 10 μL volumes, with 2.5 mM $MgCl_2$ and annealing temperature varying by locus (50 °C for MK5, MK6, and EV94; 55 °C for PPHO142, KWM12a, TtrGT48, and GT575). Microsatellite loci isolated from *Inia* were amplified in accordance with the procedure described by Gravena *et al*. (2009). PCR products were run in an ABI 3730 automated sequencer using GS500 LIZ size standard control and allelic peak sizes were analyzed using the GENEMAPPER, version 4.0 (Applied Biosystems).

Mitochondrial DNA analysis

For sequence quality control purposes, sequences of the mtDNA were 'base called' using the PHRED (Ewing *et al*., 1998). High quality consensus sequences (400 bp for CR and 1140 bp for cyt *b*) for each individual were produced from alignments of forward and reverse strand sequences with PHRAP (Green, 1994) and visualized/edited in CONSED, version 12.0 (Gordon, Abajian & Green, 1998). The consensus sequences from all individuals were aligned using CLUSTAL-X, version 1.83 (Thompson *et al*., 1997) to allow the identification of nucleotide variation and of the different haplotypes. In addition to the data obtained in the present study, we included all CR and cyt *b* sequences of *I. geoffrensis* specimens reported by Banguera-Hinestroza *et al*. (2002) in the analysis. To infer the relationship among the haplotypes for the populations in a geographic context, haplotype networks were constructed using the median-joining algorithm as implemented in NETWORK, version 4.5 (Bandelt, Forster & Röhl, 1999).

Standard indices such as haplotypic diversity (h) and nucleotide diversity (π) were estimated for each population using ARLEQUIN, version 3.1 software (Excoffier, Laval & Schneider, 2005). Analysis of molecular variance (AMOVA) and population pairwise distances were calculated as Φ_{ST}, with the Tamura–Nei (Tamura & Nei, 1993) substitution model of evolution ($\alpha = 0.078$). Exact test of population differentiation was performed with 10 000 Markov chain steps to test significance as implemented in ARLEQUIN. The number of migrants successfully entering a population per generation (N_m) was estimated using the formula of Slatkin (1991) as described in ARLEQUIN. Tajima's D-test (Tajima, 1989) and Fu's F_s test (Fu, 1997) were also calculated for each population to evaluate the possibility of recent population expansion using 1000 bootstrap replicates.

To check the reliability of results from phylogenetic analysis of mtDNA CR sequences, we estimated the substitution saturation using the DAMBE, version 5.1.5 (Xia & Xie, 2001). The phylogenetic relationships of the mtDNA CR haplotypes were reconstructed using BEAST, version 1.5.2 (Drummond & Rambaut, 2007) and the resulting trees were summarized using the TREEANNOTATOR and FIGTREE (Drummond & Rambaut, 2007). The time of origin for each lineage and time of divergence between lineages were estimated using the strict molecular clock with HKY + G model and substitution rate fixed in 0.078. A log-normal tree prior with a constant population size and log-normal calibration points were used to estimate the time to the most recent common ancestor. *Pontoporia* was used as a calibration reference and a mean date of 11.2 Mya with log-normal SD of 1.025 million years in accordance with the results obtained by Xiong *et al*. (2009). In addition, *Lipotes* was used as outgroup (GenBank accession number: NC_007629.1). The historical demography of each phylogeographic group was examined using Bayesian skyline plot (BSP) implemented in BEAST (Drummond *et al*., 2005). This method permits the analysis of population expansions or reductions and generates a plot of effective population sizes (N_e) over time. The

phylogenetic model chosen was HKY + G, as suggested by MODELTEST (Posada & Crandall, 1998), using a strict molecular clock to estimate BSPs for the populations from Bolivia, Colombian Orinoco, and Mamirauá + Tefé. All analyses in BEAST were run for 10 000 000 generations with a burn-in of 1 000 000. Results were then visualized in TRACER, version 1.4 (Drummond & Rambaut, 2007).

Alternatively, Bayesian analyses were conducted in MRBAYES, version 3.1 (Huelsenbeck & Ronquist, 2001). Bayesian analyses topology was also constructed under the HKY + G substitution model. Posterior probabilities > 0.95 were considered robust.

Microsatellite analysis

The software MICRO-CHECKER was used to check for likely problems as a result of scoring errors, allele dropout, and null alleles (Van Oosterhout *et al*., 2004). The potential frequency of null alleles was also estimated using CERVUS, version 2.0 (Marshall *et al*., 1998). The matching genotypes and the probability of identity, the probability of two individuals sharing the same genotype across all 14 loci were estimated using GENALEX, version 6.0 (Peakall & Smouse, 2006). Departures from Hardy–Weinberg equilibrium, tests of linkage disequilibrium for each locus, and genetic differentiation among populations (using F_{ST} and R_{ST}) were carried out using ARLEQUIN, version 3.11 (Excoffier *et al*., 2005). We calculated both F_{ST} and R_{ST}, although conventional F_{ST} was shown to be more reliable when the sample size is limited (Gaggiotti *et al*., 1999). Inbreeding coefficients (F_{IS}) and allelic richness (A), a measure of the number of alleles per locus independent of sample size (Petit, El Mousadik & Pons, 1998), were computed in FSTAT, version 2.9 (Goudet, Perrin & Waser, 2002).

Estimates of migration rates between the Mamirauá and Tefé populations were calculated using a maximum likelihood (ML) coalescent approach implemented in MIGRATE, version 3.0 (Beerli & Felsenstein, 1999). Initial runs were set estimating θ and M (immigration rate/mutation rate) based on F_{ST} calculations. Three replicate searches included ten short chains with a total of 10 000 genealogies samples and two long chains with 1 000 000 genealogies sampled, following a burn-in of 10 000 trees. For microsatellite data, a constant mutation rate of 10^{-4} was used (Whitaker *et al*., 2003; Hedrick, 2005) to transform estimates of θ into N_e based on formula $\theta = 4N_e\mu$ (where N_e is effective population size and μ is the mutation rate/locus/generation). To determine whether the migration is sex-biased, F_{ST} estimates were calculated for males and females separately. The statistical significance was estimated using permutation simulation with default settings implemented in ARLEQUIN, version 3.11. The potential for sex-bias was tested using the FSTAT, version 2.9.2.3 (Goudet, 2002) based on sex-specific expectations with respect to F_{ST}, variance of the assignment index (vAIc) and mean assignment index (mAIc).

The software BOTTLENECK, version 1.2 (Piry, Luikart & Cornuet, 1999) was used to evaluate deviations of mutation-drift equilibrium that could indicate recent bottlenecks in the Mamirauá and Tefé populations. Three different mutation models of microsatellite evolution were employed: (1) infinite allele model (IAM) (Ohta & Kimura, 1973); (2) stepwise mutation model (SMM) and (3) two-phased model (TPM), an intermediate between the IAM and SMM that better fits most microsatellite datasets (DiRienzo *et al*., 1994). The TPM parameters were set with 70% single-step mutations and 30% multiple-step mutations and a variance among multiple steps of 12. The significance was assessed with the Wilcoxon sign-rank test, a more powerful and robust test when used with few polymorphic loci (< 20). We also used a qualitative graphical descriptor of the shape of the allele frequency distribution that can differentiate between stable (L-shape indicator) and bottlenecked (mode-shift indicator) populations (Luikart *et al*., 1998).

RESULTS

Genetic diversity

mtDNA control region

We used 400 bp of the mtDNA CR to investigate the relationship among the three subspecies and different geographic locations. Our CR sequences from 30 Brazilian samples were aligned to 96 CR sequences obtained by Banguera-Hinestroza *et al*. (2002). We identified a total of 19 haplotypes and 49 variable sites in the aligned consensus fragments: six from Brazil, two from the Colombian Amazon (CA), six from the Bolivian Amazon (BA), and five from the Colombian Orinoco (CO) (Table 1). Among the four localities, Brazil displayed the highest haplotype diversity, followed by CO and BA. CA presented substantially lower haplotype diversity than that of all the other regions. The lowest nucleotide diversity was observed in the BA haplotypes, whereas the highest diversity was seen in the CO haplotypes (Table 1). Tajima's and Fu's tests revealed no significant results regarding population expansions for all geographic locations, except for the BA group, in which the Fs value indicated a recent expansion ($F_s = -2.678$, $P < 0.02$).

At a regional scale (within Brazil), we found a total of six CR mtDNA haplotypes from which five were restricted to the Central Amazon populations in

Table 1. Summary of mitochondrial DNA control data

Population	N	S	H_t	h	π
BrA	30	7	6	0.7943 ± 0.0277	0.0326 ± 0.0163
Mamirauá	20	2	3	0.4842 ± 0.1129	0.0013 ± 0.0012
Tefé	10	4	3	0.5111 ± 0.1643	0.0040 ± 0.0029
CO	17	27	5	0.6471 ± 0.1185	0.2238 ± 0.1198
CA	38	6	2	0.1494 ± 0.0739	0.0152 ± 0.0127
BA	41	5	6	0.5561 ± 0.0755	0.0129 ± 0.0115

N, sample size; S, polymorphic sites (parsimony informative); H_t, number of haplotypes; h, haplotype diversity; π, nucleotide diversity. For h and π, the standard deviations are also shown.

Brazil. All haplotypes found in Amanã grouped with Mamirauá haplotypes (both referred to as Mamirauá in the analyses) but separated from Tefé haplotypes, suggesting two different Brazilian populations: one located on the northern side (left margin) and another on the southern side (right margin) of the Amazon River. Three haplotypes were exclusively found in the Tefé Lake sample and two haplotypes were exclusively found in individuals from Mamirauá and Amanã. A similar genetic diversity was observed in the Tefé samples $h = 0.5111 \pm 0.1643$ compared to $h = 0.4842 \pm 0.1129$ found in the samples from Mamirauá (Table 1).

Microsatellites

The potential presence of null alleles was detected in five out of 14 microsatellites, thus, the loci: KWM12a, GT575, IG2B1, IG11B1, and IG8H1 were excluded from the *F*-statistics analysis. The probability of identity across all 14 loci was highest for Mamirauá (1×10^{-10}) and lowest for Tefé (7.4×10^{-11}). No matching genotypes were found among the samples. All loci were polymorphic, with a low to medium level of allelic diversity at each locus (2–7), and no alleles were found to be unique in any population (Table 2). Levels of microsatellite diversity were moderate with average heterozygosity 0.5464 ± 0.3059 in Mamirauá and 0.5697 ± 0.3611 in Tefé (results over nine loci). Negative values of F_{IS} indicated that there was no inbreeding in the Mamirauá or Tefé populations when considered separately. Five loci, described above, exhibited significant deviations from the Hardy–Weinberg equilibrium, possibly as a result of null alleles (Table 2). Significant linkage disequilibrium was also observed in five out of 45 pairs of loci, which may be a result of the relatively limited sample sizes.

The analysis of population subdivision using F_{ST} and R_{ST} showed no significant structure across the sampled areas. F_{ST} attributed only 1.2 % of the variation between populations and no significant positive values were obtained from the R_{ST} estimator.

Population differentiation

The differentiation of mtDNA CR sequences among the four regions was high ($F_{ST} = 0.53$; $\Phi_{ST} = 0.87$). Most of the variation occurred among populations, suggesting an extremely reduced maternal gene flow among localities (Table 3). Pairwise comparisons suggested that a significant part of the diversity is a result of the inclusion of the BA population whose Φ_{ST} values compared to those of Mamirauá, Tefé, CO and CA were close to 1, except for the latter where the value was slightly lower, 0.87 (Table 3). The AMOVA analysis also revealed a strong differentiation between the two Brazilian populations ($F_{ST} = 0.50$; $\Phi_{ST} = 0.70$, $P < 0.001$), which are located only 45 km apart.

The complete cyt *b* gene (1140 bp) was also sequenced in 20 individuals from the Brazilian Amazon. Two haplotypes were defined by a single transition. One haplotype (GenBank accession number EU554562) represented 13 individuals from Mamirauá and a second one was identified in seven individuals from Tefé (GenBank accession number EU554561). Thus, there was a specific cyt *b* haplotype for each one of the Brazilian populations, which reflects the remarkable maternal isolation suggested by the control region network (Fig. 2).

Phylogeographic structure and phylogenetics

The median-joining network (MJN) of the CR sequences showed two population clusters in Brazil (Fig. 2). Five of the six haplotypes found in Brazilian populations appeared to be unique, with only one haplotype shared between Mamirauá and the Colombian Amazon (MM3/CA1). The star-like tree found for the Mamirauá population showed an excess of rare haplotypes originating from the most frequent

Table 2. Summary of microsatellite data for *Inia* sp

	Mamirauá						Tefé						
Locus	*N*	*k*	*A*	H_O	H_E	Null allele frequencies	*n*	*k*	*A*	H_O	H_E	Null allele frequencies	Source
PPHO142	21	3	2.81	0.524	0.521	+0.0015	12	3	2.83	0.583	0.507	–0.0789	Rosel *et al.* (1999)
EV94Mn	21	6	4.22	0.857	0.741	–0.0874	12	5	4.50	0.667	0.750	+0.0443	Valsecchi & Amos (1996)
KWM12a	20	5	3.90	**0.450**	**0.665**	+0.1833	12	5	4.48	**0.500**	**0.707**	+0.1188	Hoelzel *et al.* (1998)
MK5	20	3	2.90	0.600	0.617	–0.0137	12	3	2.94	0.417	0.554	+0.0981	Krützen *et al.* (2001)
MK6	20	4	3.87	0.700	0.709	–0.0054	10	3	3.00	0.800	0.695	–0.0905	Krützen *et al.* (2001)
GT575	21	8	5.85	**0.619**	**0.820**	+0.1282	12	6	4.99	**0.500**	**0.761**	+0.1585	Bérubé *et al.* (2000)
TtruGT48	18	4	3.37	0.889	0.663	–0.1820	8	5	4.87	0.875	0.800	ND*	Caldwell *et al.* (2002)
IG10A1	16	6	5.27	0.750	0.808	+0.0130	7	7	7.00	0.857	0.890	ND*	Gravena *et al.* (2009)
IG2B1	21	2	1.82	**0.095**	**0.177**	+0.2857	10	3	2.90	**0.100**	**0.426**	+0.5984	Gravena *et al.* (2009)
IG11B1	20	6	5.02	**0.500**	**0.777**	+0.2147	7	4	4.00	**0.286**	**0.703**	ND*	Gravena *et al.* (2009)
IG1F1	19	5	4.22	0.737	0.690	–0.0440	11	4	3.51	0.455	0.558	+0.0938	Gravena *et al.* (2009)
IG8H1	19	6	4.26	**0.263**	**0.523**	+0.3706	10	6	5.00	**0.500**	**0.632**	+0.1487	Gravena *et al.* (2009)
IG11D2	20	4	2.67	0.300	0.276	–0.0733	8	2	2.00	0.375	0.325	ND*	Gravena *et al.* (2009)
IG10E	19	5	4.43	0.842	0.747	–0.0888	10	6	5.58	0.600	0.821	+0.1103	Gravena *et al.* (2009)
Probability of identity				1×10^{-10}						7.4×10^{-11}			
F_{IS}†				–0.177, $P < 0.05$						–0.131, $P < 0.05$			

N, sample size for each region; *k*, number of alleles at each locus; *A*, allelic richness; H_O, H_E, observed and expected heterozygosity, respectively. Significant deviations from Hardy–Weinberg equilibrium are shown in bold.

*Not done, at least ten individuals are required to perform null alleles frequencies estimates.

†Calculated over nine loci that did not exhibit signs of null alleles.

Table 3. Population pairwise distances using Φ_{ST} (above diagonal) and F_{ST} (below diagonal)

	BrA				
	Mamirauá	Tefé	CO	CA	BA
BrA					
Mamirauá	–	0.50	0.43	0.13	0.47
Tefé	0.70	–	0.41	0.75	0.45
CO	0.58	0.51	–	0.67	0.40
CA	0.56	0.76	0.63	–	0.64
BA	0.97	0.96	0.87	0.97	–

All Φ_{ST} values are significant at $P < 0.0001$. BrA, Brazilian Amazon; CO, Colombian Orinoco; CA, Colombian Amazon; BA, Bolivian Amazon.

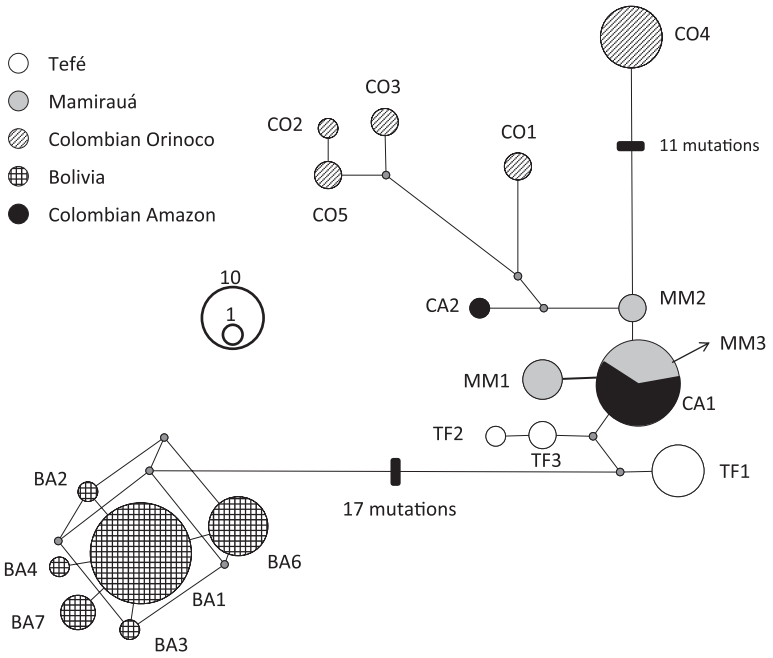

Figure 2. Median-joining network based on mitochondrial DNA control region haplotypes for *Inia geoffrensis*. Circle areas are proportional to the number of individuals representing that haplotype. Branch lengths are approximately proportional to mutation events (i.e. the shortest line length represents one substitution). Solid bars emphasize the high number of substitutions found separating haplotype clusters. Small grey dots indicate median vectors.

haplotype (MM3), suggesting population growth. Nevertheless, this pattern was not considered significant by previous expansion tests, possibly as a result of the small sample size. The haplotype MM2 connects with the remnant haplotypes from CA (CA2) and CO (CO1 to CO5). A remarkable separation between BA populations and all others is supported by 17 nucleotide substitutions (Fig. 2). This separation was further corroborated by phylogenetic reconstructions. The MJN also indicated some heterogeneity in the Colombian Orinoco population. Phylogenetic reconstructions using *Beast* (Fig. 3) and MRBAYES (see Supporting information, Fig. S1) based on mtDNA (CR), recovered almost identical topologies. The Colombian Orinoco, Colombian-Brazilian Amazon, and Bolivian populations illustrate three clear geographic clusters. The latter appears in both trees as a well supported monophyletic group. The Central Amazon haplotypes from Brazil fill a phylogenetic gap between CO and BA haplotypes, which is expected as a result of the intermediate geographic position between both localities, considering the connectivity of rivers and a likely correlation between genetic divergence and geographic distances.

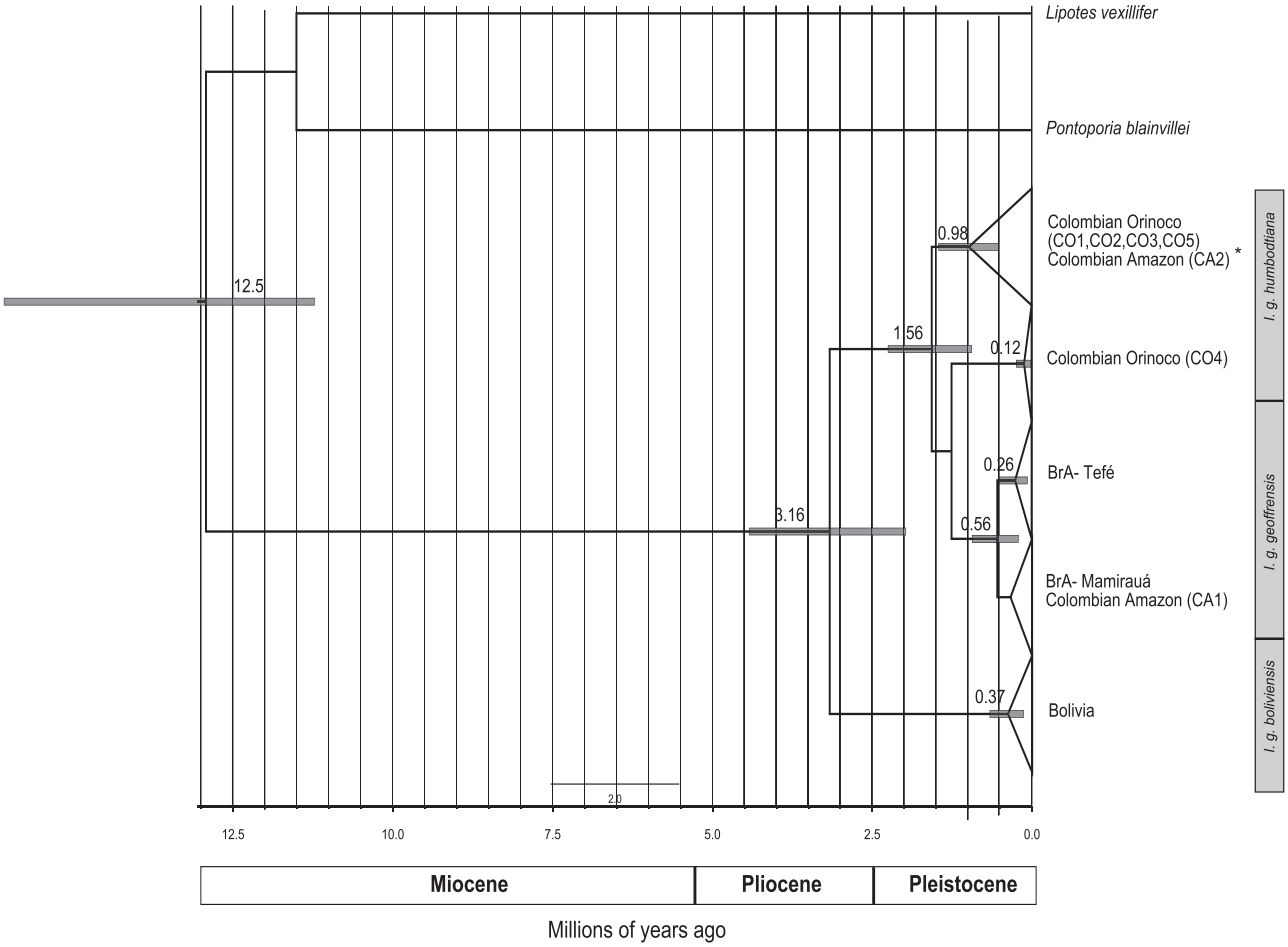

Figure 3. The Bayesian tree based on mitochondrial DNA control region. Numbers at each branch correspond to the mean divergence times derived from BEAST (Drummond & Rambaut, 2007). Node bars delimit the lower and upper 95% highest posterior density.

Sex-biased gene flow and effective population size estimation based on microsatellite data

We conducted an AMOVA analysis of the microsatellites, treating males and females of each population separately, aiming to assess the potential influence of sex-biased gene flow. The results revealed a significant but low structure for males ($N = 9$, MM and $N = 6$, TF) (F_{ST}: 0.014, $P < 0.05$) and no significant structure for females ($N = 9$, MM and $N = 5$, TF) (F_{ST}: 0.003, $P > 0.05$). In addition, *Fstat* tests showed no significant differences on sex-biased gene flow when using other estimators (R_{ST}, *v*AIc and *m*AIc scores, $P > 0.05$).

ML gene flow estimates based on microsatellite data were low, with less than two migrants per generation across both areas (Table 4). Reliable ML results could not be obtained for mtDNA data. According to Beerli (2006), single locus datasets with low variability (variable sites) do not allow estimating migration rates with great precision. Nevertheless, the exclusive occurrence of different mtDNA haplotypes in each area indicated little or no recent female gene flow.

Divergence time and historical demography

In accordance with the procedure implemented by Xia & Xie (2001) in DAMBE, no evidence of substantial saturation was detected in the mtDNA CR (Iss < Iss.c; $P < 0.05$) and therefore our sequence data set appears suitable for phylogenetic inference. The divergence between the Bolivian and the other *Inia* populations appears to have occurred in the late Pliocene, approximately 3.12 Mya. The next earliest split is observed between the Colombian Orinoco/Colombian Amazon (CA2) and the Colombian Orinoco (CO4)/ Brazilian Amazon haplotypes with an estimated mean divergence date value of 1.56 Mya. The

Table 4. Maximum likelihood estimates of gene flow between regions and effective population size (N_e) based on microsatellite data using Migrate

	N_e	95%CI	M_{1_2}	M_{2_1}	95%CI
Mamirauá	2375	2150–2650	–	1.31	1.17–1.46
Tefé	3050	2700–3475	1.20	–	1.07–1.34

M_{1_2}, migration from population 1 (Mamirauá) to population 2 (Tefé); M_{2_1}, migration from population 2 to population 1. The confidence interval (95% CI) is also shown.

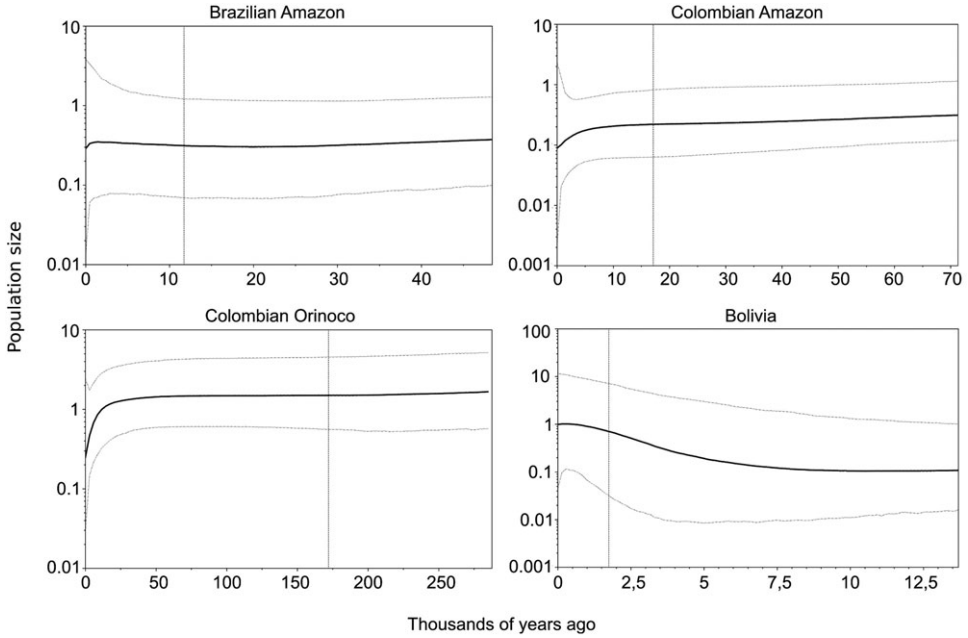

Figure 4. Bayesian skyline plots of each lineage of *Inia* sp., except for the Brazilian Amazon where the two populations (Mamirauá and Tefé) were combined. The central line represents the mean divergence time value for the log of population size (N_e*τ). The area between upper and lower lines corresponds to the 95% posterior density.

Colombian–Brazilian group (without CA2, which is closely related to the Orinoco haplotypes) shares a common ancestor at approximately 1.25 Mya and the youngest lineage is represented by the cluster of Tefé haplotypes formed in the Middle Pleistocene (approximately 0.26 Mya) (Fig. 3).

The Bayesian skyline plots (Fig. 4) detected stable population sizes through time for most populations analyzed, and a population decline for the Brazilian and Orinoco populations in recent years. By contrast, the Bolivian population underwent a surprisingly rapid population growth over the last 7000 years.

Bottleneck detection based on microsatellite data

Significant support ($P < 0.01$) for contemporary reductions in effective population size was found in the Mamirauá and Tefé populations under all three microsatellite mutational models: IAM, SMM, and TPM (results over nine loci). Additionally, the graphical method indicated bottleneck-induced distortions of allele distributions (Fig. 5).

DISCUSSION

Phylogeography and population structure

The results obtained in the present study provide further support for the remarkable macrogeographic pattern described by Banguera-Hinestroza *et al.* (2002). Both network (MJN) and tree reconstructions strongly support the occurrence of at least two Evolutionary Significant Units (Bolivia and others), thereby providing a basis to prioritize both areas as separate units for conservation efforts (Moritz, 1994).

Although the phylogeographic pattern revealed a strong geographic correlation for all *Inia* sp. groups, Mantel tests using pairwise F_{ST} distances previously

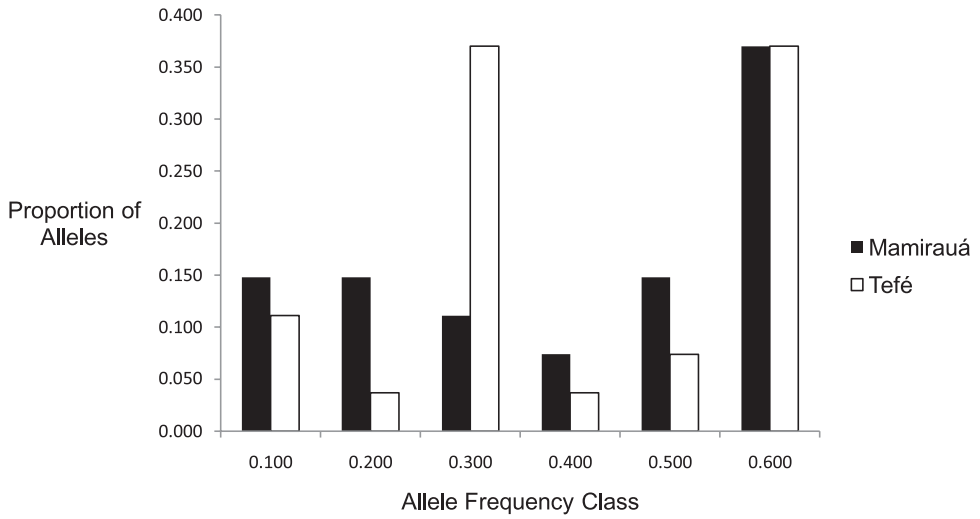

Figure 5. Mode-shift distortion distribution of allele frequencies for nine microsatellite loci in bottlenecked *Inia* sp. populations from Mamirauá and Tefé.

performed by Vianna *et al*. (2010) showed no significant correlation. If the populations have been separated for a long time, we would expect that genetic drift might have erased any isolation-by-distance pattern. In this case, considering the geographical range and diversity of habitats in the Orinoco and Amazon basins, it is difficult to predict the exact geographic paths that would facilitate the gene flow among the *Inia* sp. populations. On the other hand, genetic differentiation may be taking place between populations facing dissimilar environmental conditions and selective pressures, as well as increased geographic distances (Endler, 1982). The results obtained in the present study based on the mtDNA analysis showed a significant genetic differentiation between populations of Mamirauá and Tefé (within Brazil), indicating high female phylopatry, whereas male gene flow would be likely responsible for the low and nonsignificant differentiation observed with biparentally inherited microsatellites. Although mammalian females are generally more phylopatric than males (Greenwood, 1980), the degree of matrilineal phylogeography of *Inia* sp. is remarkable considering the short distance (45 km) between both Brazilian locations.

Sex-biased gene flow and effective population size

In a previous long-term study of *Inia* in the Mamirauá system, Martin and da Silva (2004b) identified a high degree of fidelity, with 90% of the *Inia* sighted in the system considered to be resident of the area. These same authors (Martin & da Silva, 2004a) observed a marked sexual segregation (except at low water season) in which males preferentially remained in the main river area and females tended to inhabit the more remote and protected portions of the habitat, such as lakes and várzeas (flooded forests). The shared haplotype between Mamirauá and the Colombian Amazon is consistent with the lack of geographic barriers between the Mamirauá (and Amanã) and the Colombian rivers that might allow occasional gene flow. Nonetheless, there was clear evidence of a strong differentiation between both areas ($\Phi_{ST} = 0.58$).

The lack of microsatellite differentiation suggests that long-term male-mediated gene flow may be maintaining a relative homogeneity between populations in Mamirauá and Tefé. This is in agreement with previous observations (Martin & da Silva, 2004a, b) that males are found in the main river systems, perhaps crossing over both margins, whereas females usually demonstrate fidelity to the adjacent flooded areas. However, the AMOVA and FSTAT analyses were non-informative with respect to male-biased gene flow. An extensive sampling and the use of more appropriate markers such as those in the Y chromosome is required to better evaluate this question.

Although no significant differentiation was detected with microsatellites, MIGRATE analysis with the same data showed some restricted gene flow between Mamirauá and Tefé with estimates of less than two individuals moving across both areas per generation. The low F_{ST} values may be also attributed to the increased effective population size in nuclear autosomal loci because mtDNA reflects a quarter of the effective population size of diploid markers and divergence tends to accumulate faster by drift (Palumbi, Cipriano & Hare, 2001). N_e estimates of *Inia* from the

Mamirauá Lake system (2 375) corresponds to approximately 18% of current census estimates for the Mamirauá Reserve (13 000), which covers an area of 11 240 km^2 (Martin & da Silva, 2004b). According to Frankham, Ballou & Briscoe (2010) approximately 10% of the census population size is a reasonable estimate of effective population size. Although Tefé Lake appears to harbour the highest densities of *Inia* in the Central Amazon (M. Marmontel, pers. comm.), no conclusive study to estimate actual numbers of *Inia* in this area performed so far.

Divergence date and historical demography

There is some controversy about the timing and cause of separation of Bolivian populations (*I. g. boliviensis*). Some studies suggest that the Bolivian *Inia* underwent a separation as a result of a recent barrier represented by 400 km of rapids between Guajará-Mirim and Porto Velho in Brazil (da Silva, 1994). The rapids are considered to have formed at the end of the Pleistocene, approximately 100 000 years ago (Grabert, 1967). By contrast, Banguera-Hinestroza *et al*. (2002) used molecular divergence to claim that the Bolivian *Inia* sp. has been isolated from the other subspecies for at least 2.6 million years (based on 400 bp of mtDNA CR, if a 2% divergence ratio is employed). Thus, allopatric separation of the Bolivian *Inia* would have resulted by the uplift of the Andes during the Cenozoic, leading to a disruption between upper Madeira River and the rest of the Amazon basin. The Bayesian trees presented here support monophyly for the Bolivian *Inia* (see Supporting information, Fig. S1) as already demonstrated in several other studies (Banguera-Hinestroza *et al*., 2002; Agnarsson & May-Collado, 2008; McGowen, Spaulding & Gatesy, 2009).

Ho *et al*. (2008) noted that the use of an external calibration point may lead to an overestimation of divergence times, and the data obtained in the present study may reflect this error. Although we presented mean time estimates for comparisons between the lineages, the goal of the study is to depict a historical trajectory of *Inia* sp. in South America without focusing on precise dates. The deep divergence time between the Bolivian *Inia* and the other lineages (3.12 Mya) indicates a very ancient species or species complex. At that time, the Amazon fluvial system had been reshaped by the rise of the Andes (Latrubesse *et al*., 2010), providing a new eastward dispersal pathway for *Inia*. Concurrently, an early dispersal might have occurred towards the Madeira drainage basin, which is considered by Westaway (2006) as the most ancient and stable river of the Amazon basin.

Our historical demographic analyses revealed that the Bolivian *Inia* underwent a recent demographic expansion, a sudden growth starting approximately 7000 years ago. It is tempting to consider that the formation of the rapids in the late Pleistocene happening some time between 11 000–126 000 years ago (Grabert, 1984), triggered demographic changes of the Bolivian *Inia*. Although single locus analysis does not appear to be ideal, our historical demographic analyses were consistent with other methods of demographic inference, including Fu's F_s and Tajima's D. In addition, small population size was demonstrated by Heled & Drummond (2008) not to be an issue in recovering past population dynamics.

The MJN network shows a strong genetic break with 17 substitutions separating Bolivian from Brazilian haplotypes. We could address the possibility that the unobserved intermediate haplotypes were lost as a result of a local extinction process. The subsequent demographic expansion observed in our analyses would have been led by the remnant individuals. However, future sampling and analysis of populations located downstream of the rapids in the Madeira River will provide a more complete picture of this scenario.

The divergence time observed between most of the Orinoco and Amazon populations (1.56 Mya) does not coincide with the estimates of the Orinoco–Amazon vicariant event approximately 10 Mya (Lundberg *et al*., 1998). This suggests that the formation of both lineages is likely a product of further dispersal events. The paraphyletic relationship between haplotypes of the two subspecies (*I. g. geoffrensis and I. g. humboldtiana*) suggests more recent exchanges across the basins (see Supporting information, Fig. S1).

Climatic changes in the Pleistocene were probably correlated with the diversification of all *Inia* lineages reported herein (Haffer, 1987). The BSP analysis of mtDNA data indicates recent population declines for the Orinoco and the Colombian Amazon but a stable population size over the past 40 000 years within Brazil (Mamirauá + Tefé). However, autosomal microsatellite data indicated that the resident *Inia* populations from Mamirauá and Tefé Lake have undergone an earlier decline during the Pleistocene, from 60–145 000 years ago (2–4 N_e generations).

Inia phylopatry and conservation implications

The species complex *Inia* sp. shows a significant population structure across large and small geographic scales, in addition to local female phylopatry. We suggest that, besides restrictions to long-range gene flow, heterogeneity of the water environment may also be leading to the significant differentiation observed between close neighbouring populations in both sides of the Amazon River.

Patterns of marked population differentiation were also observed in other cetacean species. Caballero *et al*. (2007) described a high differentiation in *Sotalia guianensis* populations distributed in a large geographic scale, and Möller *et al*. (2007) observed a significant differentiation in *Tursiops truncatus* populations that were located over 16 km apart, which is a smaller scale than that reported in the present study. The fine-scale genetic structure in *Tursiops* sp. has been attributed to habitat type and behavioural specializations (Natoli *et al*., 2005; Möller *et al*., 2007; Oremus *et al*., 2007; Wiszniewski *et al*., 2010). Altogether, it appears plausible that the range of environmental differences in riverine habitats associated with behavioural aspects of this species have played a role in promoting genetic subdivision in *Inia* sp. populations. In the case of the present study, the Amazon River system likely restricts female gene flow between neighbouring populations.

The mtDNA data of the present study allowed the resolution of the phylogenetic position of Brazilian populations related to the others with high support values. Moreover, the mtDNA results consistently reflected the complex population structure in *Inia* sp., displaying a highly marked spatial differentiation, even between neighbouring populations. This condition indicates that *Inia* sp. is more vulnerable to extinction because populations are relatively isolated by local female phylopatry. These data provide new important information that has implications for the conservation status of *Inia* sp. because this species is currently considered Data Deficient by IUCN (Reeves *et al*. 2010).

Finally, we suggest that conservation efforts should consider managing local populations of dolphins as semi-independent evolutionary units aiming to preserve *Inia* phylopatry in both macro- and microgeographic scales.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. The Bayesian tree based on the mitochondrial DNA control region. Numbers associate with nodes represent posterior probabilities from Bayesian Markov chain Monte Carlo searches conducted in MRBAYES. Haplotype frequencies are indicated to the right of the tree. CO, Colombian Orinoco; CA, Colombian Amazon; MM, Mamirauá-Brazil; TF, Tefé-Brazil; BA, Bolivian Amazon.

Table S1. *Inia* sp. individuals used in the present study. Sampling regions in Brazil, collection dates, and genetically and morphologically indentified sex. Positive signs indicates successful amplifications for each molecular marker. The GenBank accession numbers of haplotypes compared in the study are also shown.

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