GENETIC DIFERENTIATION IN POPULATIONS OF THE RIVER DOLPHINS GENUS *Inia* (Blainville, 1817) AND APPORTS TO THE TAXONOMIC STATUS WITH MOLECULAR ANALYSIS

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E. Banguera-Hinestroza[1,3*], H. Cárdenas[2], M. Ruiz-García[3], M. Marmontel[4], E. Gaitán[5], R. Vázquez[6] & F. García-Vallejo[1]

Resumen

Banguera-Hinestroza E., H. Cárdenas, M. Ruiz-García, M. Marmontel, E. Gaitán, R. Vázquez and F. García-Vallejo: Genetic diferentiation in populations of the river dolphins genus *inia* (blainville, 1817) and apports to the taxonomic status with molecular analysis. Rev. Acad. Colom. Cienc. **26**(101): 575-588. ISSN 0370-3908.

El delfín rosado del género *Inia* es endémico de las mayores cuencas hidrográficas del norte de Sudamérica. Este trabajo constituye el primero a nivel mundial que pretende dar aproximaciones acerca de la estructura genética de este género. Se colectaron 96 muestras de especímenes en las cuencas de los ríos Orinoco, Putumayo (400 km. de recorrido) y en los ríos Mamoré, Ipurupuru y Tijamuchi en la Amazonia boliviana y se incluyeron 5 muestras de la Amazonia brasileña. Se estudiaron 570pb de la región d-loop del ADN mitocondrial y 600pb del gen citocromo b. Los análisis filogenéticos y de subdivisión poblacional soportan la propuesta de dividir el género en dos especies filogenéticas, y revelan aspectos importantes acerca de la estructura genética de los grupos de *Inia* en estas regiones geográficamente separadas.

Palabras clave: *Inia*, mtDNA, D-loop region, Cyt-b gene, nucleotide diversity, Colombia y Bolivia, speciation, Iniidae.

1 Laboratorio de Biología Molecular y Patogénesis. Departamento de Ciencias Fisiológicas. Facultad de Salud. Universidad del Valle. A.A 25360, Cali, Colombia.

2 Laboratorio de Biología Molecular, Departamento de Biología. Sección de Genética. Facultad de Ciencias. Universidad del Valle. A.A 25360, Cali, Colombia.

3 Laboratorio de Inmunobiología, Biología Molecular y Genética de Poblaciones. Unidad de Genética. Departamento de Biología. Facultad de Ciencias. Pontificia Universidad Javeriana. Cra. 7ª No. 43-82. Bogotá, Colombia.

4 Instituto de Desenvolvimento Sustentable Mamirauá, Caixa Postal 0001 Tefe/AM, Brasil.

5 Centro Internacional de Agricultura Tropical (CIAT). A.A. 6713. Cali. Colombia. A.A. 6713, Cali, Colombia.

6 Universidad Técnica del Beni. Casilla 251. Trinidad. Bolivia.

Dirección actual: Universidad Javeriana, Cra 7 No. 43-82. Dpto. de Biología. Lab. de Genética de Poblaciones y Ecología Evolutiva.

Institución donde se realizó la investigación: Laboratorio de Biología Molecular y Patogénesis de la Universidad del Valle (Cali), Laboratorio de Biología Molecular Instituto von Humboldt - Centro Internacional de Agricultura Tropical (CIAT), Palmira (Valle).

Abstract

The pink dolphin genus *Inia* is endemic to the major river basins from northern South America. This work is the first one at woldwide level focused on the genetic structure of this genus. In this work, 96 DNA samples from skin of specimens of this genus were collected in the Orinoquian basin, the Putumayo River (in 400 km); and the Mamore, Tijamuchí and Ipurupuru rivers in the Bolivian Amazon and including five samples of the Brazilian amazon. These samples were used to sequence 570pb of the d-loop region and 600 bp of the mitochondrial Cyt-b gene. The phylogenetic analysis and the population subdivision, supporting the proposal to subdivide the *Inia* genus into two different allopatric species, and revealed important aspects about the genetic structure of *Inia* groups from these three geographically separated regions.

Key words: *Inia*, mtDNA, D-loop region, Cyt-b gene, nucleotide diversity, Colombia and Bolivia, speciation, Iniidae.

Introduction

The river dolphin of the genus *Inia* (Blainville, 1817), usually known as tonina, bufeo, boto or pink dolphin is classified within the Order Cetacea, Suborder Odontoceti, Superfamily Inioidea and Family Iniidae (**Heyning**, 1990; **Muizon**, 1988; **Fordyce** & **Barnes**, 1994; Fordyce et al., 1994; **Messenger** & **McGuire**, 1998). Populations of the river dolphin are distributed widely in many rivers from the Amazon and Orinoco basins. The range of distribution in South America includes many rivers from Bolivia, Brazil, Colombia, Ecuador, Peru, Venezuela and French Guyana, which cover an area equivalent to 7 million square kilometers (**Best** and da **Silva**, 1989a,b).

Distribution of *Inia* is constrained by a series of geographical barriers. Populations of Amazonas and Beni-Mamore (Bolivia and Brazil) are separated by rapids in the upper Madeira between Porto Velho and Guajara-Mirim (da Silva, 1994). The unique connection between populations of Amazonas and Orinoco is throughout the Casiquiari Channel, which reaches the Negro River, an affluent of the Amazonas. The Casiquiari waterway is considered as a barrier for *I. geoffrensis* because the water's pH is rather low, and there is low biomass productivity. Other potential barriers are the faults of the Negro River and the rapids of the Orinoco between Samariapo and Puerto Ayacucho. (**da Silva**, 1989a,b).

The first morphometric study of the genus *Inia* at a specific level was carried out by **Pilleri** and **Ghir** (1977). They considered this genus divided into two different species: *I. boliviensis* (D´Orbigny, 1834), which is distributed in the Beni-Mamore rivers, and *I. Geoffrensis*, which was subdivided into two subspecies *I. g. geoffrensis* (**Van Bree** and **Robineau**, 1973) in the Amazon basin, and *I. g. humboldtiana* (**Pilleri** and **Gihr**, 1977) in the Orinoquian basin. Despite this, **Casinos** and **Ocaña** (1979) claimed that the clinal variation in the populations of *Inia geoffrensis,* based on craniometrical studies were not in agreement with the existence of two species and in fact supported the hypothesis of the existence of only one species with three different subspecies. However, more recently, **da Silva** (1994) again proposed the subdivision of the genus into the two aforementioned species, based on morphometric and meristic analyses.

Nevertheless, the most used and recognized taxonomical status of this genus still includes three subspecies: *I. geoffrensis boliviensis* for the system of Bolivian rivers, *I. geoffrensis geoffrensis* for the Amazonian basin rivers and *I. geoffrensis humboldtiana* for the Orinoco basin (**Van Bree** and **Robineau**, 1973; **Trebbau** and **Van Bree**, 1974; **Best** and **da Silva**, 1989a,b; **Rice** 1998)

In order to analyze the genetic variation of these three supposed subspecies of *I. geoffrensis*, a fragment of 570 bp of the mitochondrial D-loop DNA (96 individuals) and 600 bp of the Cyt-b mitochondrial gene from *Inia* specimens (38 individuals) were sequenced. In addition, the most underlying analyses carried out were the determination of the different haplotypes found; the nucleotide diversity within and between the different populations was analyzed; and the phylogenetic relationships among these three feasible taxa was recovered by using genetic distance matrices and maximum parsimony procedures. A striking and noteworthy differentiation (for both genes) was clearly revealed among the *Inia* sp. populations from the Orinoco and Amazonas rivers, with the dolphins from the Ipurupuru and Mamore rivers in the Bolivian Amazon. Therefore, this result agrees quite well with the existence of two genetically different clades, corresponding to the presence of two different allopatric species within the genus *Inia*.

Materials and methods

Collection of Samples

Skin samples were taken by making a biopsy of 1cm^2 of caudal fin. All specimens were captured in fishing nests, taking special care to ensure the physical integrity of each dolphin. After the biopsy, the wound was covered with antibiotic cream, and the specimen was released under safe conditions. The geographical points sampled were as follows. Several lagoons were explored in a transect of 443 km from Puerto Leguizamo to Puerto Encanto in the Colombian Amazonia, and a total of 38 specimens were collected. In the Orinoco basins, a total of 11 stations along of Orinoco, Guaviare and Inirida rivers were covered. Only 17 specimens were captured, including two specimens from the Arauca River, which is an affluent to the Orinoco River. In the Bolivian Amazonas, several lagoons were explored in the Mamore River, as well as two locations along the Ipurupuru (affluent of the Guapore /Itenez River) and Tijamuchi rivers. A total of 41 specimens were surveyed.

In total, 96 river dolphins were sampled for the mitochondrial D-loop region; and a subarray of these samples, together with other samples from Brazil (Jarua Grande and Anagua lagoons near to the Tefe River), were also analyzed for the mitochondrial cytochrome-b gene.

The sample sites and their respective coordinates are shown in Table 1 and Figure 1. All skin samples were stored in a solution of 20% DMSO (dimethyl sulfoxide), hvpersaturated with NaCl, according to **Hoelzel** (1991) for periods of 4-8 weeks at room temperature until their processing in the laboratory.

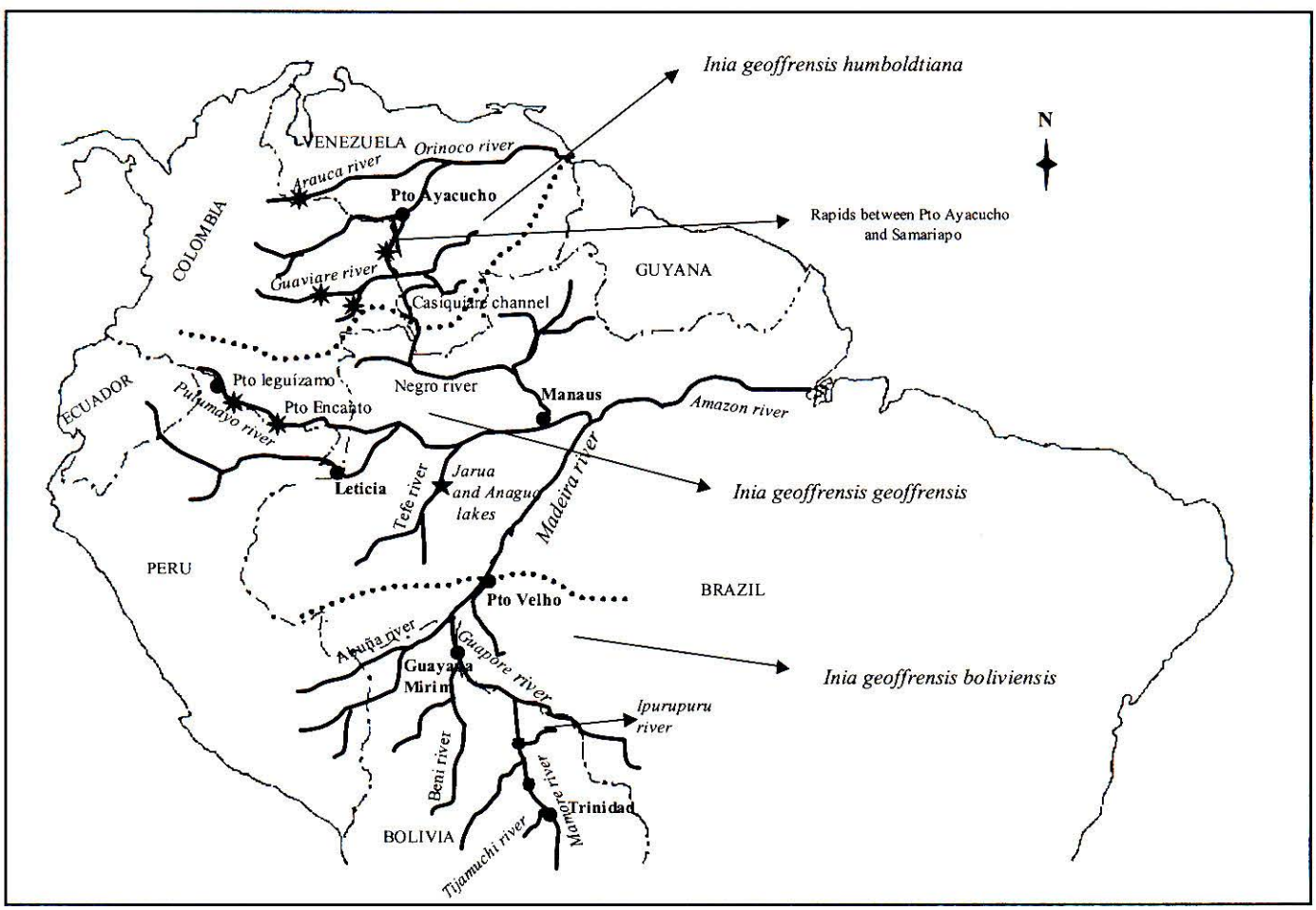

Figure 1. Map representing the geographical areas surveyed in the Orinoco Basin, in the Colombian, in the Brazilian, and in the Bolivian Amazon studied. Stars point out the places which were sampled and dolphins were captured. Dotted lines indicate the rapids and other obstacles for the migration of *Inia* from one basin to other.

Table 1. Sample localities of the *Inia* individuals presented in this study, geographical coordinates and sample sizes.

Locality	Number of sites sampled	Coordinates	Number of individuals
Orinoco			
Inirida River	3	68° 11´ 8.28" W – 3°57´26.32" N 68° 1´ 4.69" W – 4° 1´54.46" N 68° 1´43.51" W – 3°19´47.70" N	4 2 5
Orinoco River	1	67° 53´ 14.99" W - 4° 5´ 45.92" N	2
Guaviare River	1	67° 58´ 1.46" W - 3°56´28.65" N	2
Arauca River	1	71° 13.´W - 6° 50´N	2
Amazon			
Putumayo River	3	74° 25´ W - 0° 32´ S 73° 51´ W - 1° 8´ S 73° 39´ W - 1° 15 S	15 13 10
Jarua Grande Lake	1	----------	4
Anagua Lake	1	----------	1
Bolivian Amazon			
Ipurupuru River	2	65°3´W - 14°18´S	12
Tijamuchi River	1	----------	1
Mamoré River	5	65° 3´ W – 14° 33´S 65° 00´ W – 14° 46´S	28
Total	**19**		**101**

DNA amplification and sequencing

Extraction of DNA from skin samples was performed by using the QIAamp tissue kit (Qiagen Inc.) following the manufacturer's instructions. DNA quality was monitored by electrophoresis in 0.8% agarose gels.

The D-loop region was amplified by PCR, using specific combinations of light-strand TRO (5´CCTCCCT AAGACTCAAGGAAG-3´) and heavy-strand DH6 (5´AAATACAYACAGGYCCAGCTA-3´) oligonucleotide primers, donated by the Southwest Fisheries Science Center (La Jolla, CA). From 20 to 100 ng of DNA were employed in a reaction buffer 1X (1.5 mM MgCl2, 0.8 mM of dNTP mix), 1 mM of a pair of oligonucleotide primers and 2 units of Taq DNA polymerase in a total volume of 50 µl, which were submitted to PCR reaction

by using a thermal cycler (MJ Research PT-100). Cycling conditions were performed as follows: 2 min. at 95°C, 30 sec. at 94°C, 1 min. at 50°C and 1.5 min. at 72°C. Then 35 cycles were done with a final extension step of 3 min. at 72°C. Amplified mtDNA was cleaned throughout sephadex columns, and in several cases was also used polyethylene glycol/NaCl, both with good results. Cleaned amplicons were resolved by electrophoresis in 1% agarose gels. The cytochrome b gene was amplified by using the primers Tglu (5´TGACTTGAARAACCAYCGTTG-3´) and MHB2 (5´CTGGTTTGATGTGTGYTGGAGT-3´). A similar cycling profile for this gene was used, the only difference being the 52°C annealing temperature.

The fragments of 570 bp from the mitochondrial D–loop and 600 bp from the Cyt-b gene of 96 *Inia* sp. specimens were sequenced using an ABI PRISM™ 377 DNA Sequencer (Perkin Elmer However, not all 96 samples were employed to sequence the cyt-b gene (n = 38). In addition, 5 Brazilian Amazon samples were sequenced, as well as 2 additional genebank accession sequences for one *Inia* individual (Accession no. AF304068) and the outgroup (Accession no. AF229170). Sequencing reaction was performed in a final volume of 10 □l, which contained 20 to 100 ng of mitochondrial D-loop DNA; 1 □l of oligonucleotide primers (3.2 mM), which corresponded to TRO for the heavy chain and DH6 for the light chain; and 4 □l of Dye dideoxy terminator premix. The reaction was carried out by means of a thermal cycler (MJ Research PT-100) at 80°C, followed by 25 denaturation cycles at 95°C for 10 sec., at 52°C for 10 sec. and a final extension step at 60°C for 4 min. All DNA fragments were sequenced twice and in those cases of mismatching a new PCR reaction was done and sequenced again. The sequences were run in a 4.5% polyacrylamide denaturant gel for 7 hours.

Sequence Analysis

Sequence alignments and editing were performed by using the sequencer 3.0 and the Clustal X (1.62 b) programs.

Mitochondrial genetic structure within and between populations, and several population genetic parameters such as nucleotide diversity □), average number of nucleotide differences (k), average number of nucleotide substitutions per site between populations (Dxy), and the number of net nucleotide substitutions per site between populations were calculated by means of the DNAsp (**Rozas** and **Rozas**, 1995) and **Arlequin** (**Schneider** et al., 2000) programs. Haplotypic diversity was calculated according to **Nei´s** (1978) and **Tajima** and **Nei´s** (1984) equations.

Concordance between geographical variation and genetic heterogeneity was analyzed by using a molecular variance analysis (AMOVA), assuming an overall *Inia* population subdivided into three geographical subpopulations. The AMOVA procedure is included within the Arlequin program (**Schneider** et al., 2000).

The haplotype relationships, obtained from the D-loop and cyt-b mitochondrial genes among all populations analyzed, were determined by applying the MEGA program (version 1.2) (**Kumar** et al., 1993) with Tajima's and Kimura´s two parameters (**Kimura**, 1980) and **Jukes Cantor's** (1969) genetic distances. Several transitions to transversion ratios were used. All yielded identical results. Trees were constructed using the Neighbor-Joining (**Saitou** and **Nei**, 1987) and the UPGMA algorithm procedures. Maximum parsimony trees were searched by using the PAUP program (4.0b) (**Swofford**, 1998), with the heuristic and the branch and bound procedures, with unordered character-state data. The consensus tree was obtained with the 50% rule. All phylogenetic trees obtained were statistically tested by using a bootstrap with 1000 repetitions. The control-region sequence and the cyt-b gene sequence of *Pontoporia blainvillei* were employed as outgroup elements to root the trees obtained given that the Pontoporiidae, today represented by only this species, have been considered sister taxa of the Iniidae (**Fordyce & Barnes**, 1994; **Fordyce** et al., 1994; **Messenger & McGuire**, 1998).

For estimating divergence times, the percent divergence estimates proposed by **Wilson** et al., (1985) for the D-loop region and by **Irwin** et al., (1991) for the Cyt-b gene were used and calibrated by using different geomorphological accounts and several fossil records of Pontoporidae.

Results

Nucleotide Variation

Of the 52 nucleotide substitutions found, 40 were informative from a phylogenetic standpoint for the 570 bp D-loop mitochondrial region from the 96 sequences obtained (Fig. 2a). When the outgroup was included, 142 polymorphic sites were determined, 42 of them phylogenetically informative. For the different Cyt-b haplotypes found (600 bp), 35 sites were polymorphic; but only 27 sites (2 variants) were informative from a phylogenetic standpoint, and one site was informative (3 variants). Including the outgroup, 119 sites were polymorphic although only 30 informative sites (2 variants), and 4 sites (3 variant) were recorded (Fig. 2b).

Haplotypes	N	6	1 6	2 3	2 4	2 7	2 8	4 4	4 5	4 6	5 4	6 2	6 3	6 6	7 8	9 1	9 9	1 0 4	1 0 5	1 1 5	1 2 7	1 2 8	1 3 0	1 3 5	1 3 6	1 3 7	1 3 9	1 4 1
Colombian Orinoquía 1	2	C	A	A	G	G	T	C	C	C	A	G	A	C	T	A	A	T	G	T	C	C	C	A	T	T	A	T
Colombian Orinoquía 2	1	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	C	.	C
Colombian Orinoquía 3	2	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	.	.	C
Colombian Orinoquía 4	10	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	C	.	C
Colombian Orinoquía 5	2	.	.	.	.	.	.	.	.	.	G	.	G	.	.	.	.	.	A	.	.	.	.	G	C	.	G	.
Bolivian Amazon 1	14	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 2	1	T	G	G	A	A	C	T	T	A	.	A	.	.	C	.	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 3	3	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 4	1	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 5	12	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 6	9	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Bolivian Amazon 7	1	T	G	G	A	A	C	T	T	A	.	A	.	.	C	G	G	C	.	C	T	T	T	.	C	.	.	.
Colombian Amazon 1	35	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colombian Amazon 2	3	.	G	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Haplotypes	N	1 7 2	1 8 1	1 9 9	2 1 1	2 2 7	2 2 9	2 4 4	2 4 6	2 6 4	2 6 9	2 7 0	2 7 1	3 1 1	3 1 9	3 2 1	3 2 2	3 2 4	3 5 2	3 8 5	3 8 8	4 1 1	4 4 7	4 5 2	5 3 5	5 3 8
Colombian Orinoquía 1	2	C	A	C	T	G	T	G	T	G	C	A	A	A	A	C	C	G	T	C	T	C	C	C	A	T
Colombian Orinoquía 2	1	.	.	.	.	A	C	.	.	A	T	.	.	.	G	T	.	C	.	T	.	.	T	.	.	C
Colombian Orinoquía 3	2	.	.	.	.	A	C	.	C	.	T	.	.	.	.	.	.	C	.	T	.	.	T	.	.	C
Colombian Orinoquía 4	10	.	.	.	.	A	C	.	.	A	T	.	.	.	G	.	.	C	.	T	.	.	T	.	.	C
Colombian Orinoquía 5	2	T	C	T	C	.	.	A	.	.	T	G	.	G	.	.	.	C	.	T	.	.	T	.	G	.
Bolivian Amazon 1	14	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C	.	T	.	G	.
Bolivian Amazon 2	1	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C	.	T	.	G	.
Bolivian Amazon 3	3	T	.	T	.	.	.	A	.	.	.	.	G	.	.	.	.	C	C	G	C	.	T	.	G	.
Bolivian Amazon 4	1	T	.	T	.	.	.	A	.	A	T	.	G	.	.	.	.	C	C	G	C	.	T	T	G	.
Bolivian Amazon 5	12	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C	T	T	.	G	.
Bolivian Amazon 6	9	T	.	T	.	.	.	A	.	.	T	.	.	.	.	.	.	C	C	G	C	.	T	.	G	.
Bolivian Amazon 7	1	T	.	T	.	.	.	A	.	.	T	G	G	.	.	.	.	C	C	G	C	.	T	T	G	.
Colombian Amazon 1	35	T	.	T	C	.	.	A	.	A	T	.	.	.	.	.	.	C	C	T	C	.	.	.	.	.
Colombian Amazon 2	3	C	.	T	.	.	.	.	.	A	T	.	.	.	.	.	T	C	T	T	C	.	.	.	G	.

Figure 2. A/ Variable sites in the mitochondrial control region for the genus *Inia*, which defined 14 different haplotypes for the three populations of *Inia sp.* analyzed. The points reveal those sequences which are identical to the Colombian Orinoquia 1 haplotype (line 1). The haplotypes are named in agreement of the geographical region which they belong. The nucleotide positions are pointed out in the first file in a vertical mode. B/ Variable sites for the Cytochrome b gene for the genus *Inia*, which defined 9 different haploypes for the three *Inia* sp. Populations analyzed. The points reveal those sequences identical to the Brazilian Amazon 1 haplotype (line 2, Accesion No X922534. GENEBANK). The nucleotide positions are pointed out in the first file in a vertical mode. N = number of individuals.

Colombian Orinoquía 1	2	C	A	C	T	G	T	G	T	G	C	A	A	A	A	C	C	G	T	C	T
Colombian Orinoquía 2	1	.	.	.	.	A	C	.	.	A	T	.	.	.	G	T	.	C	.	T	.
Colombian Orinoquía 3	2	.	.	.	.	A	C	.	C	.	T	.	.	.	.	.	.	C	.	T	.
Colombian Orinoquía 4	10	.		.	.	A	C	.	.	A	T	.	.	.	G	.	.	C	.	T	.
Colombian Orinoquía 5	2	T	C	T	C	.	.	A	.	.	T	G	.	G	.	.	.	C	.	T	.
Bolivian Amazon 1	14	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C
Bolivian Amazon 2	1	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C
Bolivian Amazon 3	3	T	.	T	.	.	.	A	.	.	.	.	G	.	.	.	.	C	C	G	C
Bolivian Amazon 4	1	T	.	T	.	.	.	A	.	A	T	.	G	.	.	.	.	C	C	G	C
Bolivian Amazon 5	12	T	.	T	.	.	.	A	.	.	T	.	G	.	.	.	.	C	C	G	C
Bolivian Amazon 6	9	T	.	T	.	.	.	A	.	.	T	.	.	.	.	.	.	C	C	G	C
Bolivian Amazon 7	1	T	.	T	.	.	.	A	.	.	T	G	G	.	.	.	.	C	C	G	C
Colombian Amazon 1	35	T	.	T	C	.	.	A	.	A	T	.	.	.	.	.	.	C	C	T	C
Colombian Amazon 2	3	C	.	T	.	.	.	.	.	A	T	.	.	.	.	.	T	C	T	T	C

Figure 2A

Haplotipos	*N*	*8*	*4*	*5*	*6*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*1*	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*2*	*3*	*3*	*3*	*3*	*3*	*3*	*4*	*4*	*5*	*5*	*5*	*5*	*5*
			5	*1*	*6*	*0*	*2*	*2*	*2*	*3*	*4*	*6*	*7*	*9*	*9*	*0*	*4*	*4*	*4*	*5*	*8*	*9*	*9*	*0*	*0*	*2*	*6*	*6*	*7*	*4*	*7*	*6*	*7*	*7*	*8*	*8*
						2	*0*	*8*	*9*	*8*	*4*	*9*	*7*	*5*	*8*	*1*	*0*	*3*	*4*	*8*	*8*	*1*	*4*	*1*	*6*	*3*	*1*	*5*	*5*	*1*	*1*	*9*	*4*	*6*	*5*	*9*
Brazilian Amazon 1	*1*	*A*	*T*	*A*	*C*	*T*	*C*	*T*	*T*	*G*	*T*	*C*	*T*	*T*	*T*	*T*	*G*	*T*	*T*	*A*	*T*	*C*	*C*	*G*	*G*	*C*	*C*	*T*	*T*	*C*	*T*	*C*	*C*	*A*	*A*	*C*
Colombian Amazonian 1	*3*	.	.	.	*T*	*C*	.	.	*C*	*A*	*C*	.	.	*C*	.	.	*A*	*C*	.	.	*C*	.	*T*	.	.	.	*T*	.	*C*	*T*	*C*	.	*T*	.	*G*	.
Colombian Amazonian 2	*12*	.	.	.	*T*	*C*	*T*	.	*C*	*A*	*C*	.	.	.	.	.	*A*	*C*	.	.	*C*	.	*T*	.	.	.	*T*	.	*C*	.	*C*	.	*T*	.	*G*	.
Colombian Orinoquía 1	*2*	.	.	.	.	.	.	.	.	.	.	.	*C*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colombian Orinoquía 2	*1*	.	.	.	*T*	*C*	*T*	*C*	*C*	*A*	*C*	.	.	.	.	*C*	*A*	.	.	.	.	.	*T*	.	.	.	*T*	.	*C*	.	*C*	.	*T*	.	*G*	.
Colombian Orinoquía 3	*8*	*G*	.	.	*T*	*C*	.	.	*C*	.	*C*	*T*	.	.	.	.	*A*	.	.	.	.	.	*T*	.	.	.	*T*	.	*C*	.	*C*	.	*T*	.	*G*	.
Colombian Orinoquía 4	*1*		.	.	*T*	*C*	*T*	*C*	*C*	*A*	*C*	.	.	.	.	*C*	*A*	.	.	.	.	.	*T*	*A*	.	.	*T*	.	*C*	.	*C*	.	*T*	.	*G*	.
Bolivian Amazon 1	*1*		*C*	*T*	*T*	*C*	.	*T*	*C*	.	*C*	.	.	.	*C*	*C*	*A*	.	*C*	*G*	.	*T*	*T*	.	*A*	*T*	*T*	*C*	.	.	*C*	*T*	.	*G*	*C*	*T*
Bolivian Amazon 2	*9*		*C*	*T*	*T*	*C*	.	*T*	*C*	.	*C*	.	.	.	*C*	*C*	*A*	.	*C*	*G*	.	*T*	*T*	.	*A*	.	*T*	*C*	.	.	*C*	*T*	.	*G*	*C*	*T*

Figure 2B

Characterization and Geographical Distribution of the D-loop and Cyt-b Haplotypes Found in the Amazon and Orinoquia Basin Inia sp. Populations

A total of 14 different haplotypes, having one or more noticeable nucleotide substitutions, were found among the *Inia* mitochondrial D-loop DNA region analyzed. Figure 2a,b shows all haplotypes characterized and includes the different nucleotide substitutions, which defined them. Five haplotypes were exclusively distributed in specimens surveyed from the Orinoco region rivers (N=17) (Colombian Orinoquia 1, 2, 3, 4, 5) with 30 variable nucleotide positions. Only two haplotypes were recorded in specimens from the Putumayo River (N=38) (Colombian Amazon 1, 2), with only 7 polymorphic nucleotide positions. In the Bolivian rivers it was possible to discriminate an overall number of 7 haplotypes (N=41) (Bolivian Amazon, 1, 2, 3, 4, 5, 6, 7), which were differentiated by one or two nucleotide substitutions.

For the Cyt-b mitochondrial gene, 9 different haplotypes were determined. In the Bolivian Amazon 2 different haplotypes (Bolivian Amazon 1, 2) in the 10 Bolivian individuals sequenced were recorded, with only one transition polymorphic site present. On the other hand, the 12 Orinoquian specimens surveyed showed 4 different haplotypes (Colombian Orinoquia 1, 2, 3, 4), which differentiated among them by 19 transition polymorphic sites. Finally, the 10 individuals analyzed from the Colombian Amazon showed two different haplotypes, which presented 13 transition polymorphic sites (Colombian Amazon 1, 2). In addition, several Brazilian specimens were studied (N=6), five showed the most frequent haplotype discovered in the Colombian Amazon and one (obtained in the GENEBANK) presented a different haplotype.

An analysis of geographical distribution of the D-loop and Cyt-b haploytpes clearly revealed that each of the three geographical regions under study had its own haplotypes. Nevertheless, in the case of the Cyt-b gene there is a haplotype in the Brazilian Amazon (Brazilian-Amazon 1, Accession no. AF304068), which was differentiated from only one Colombian Orinoquia haplotype in one bp. In addition of this study, a minutely spatial autocorrelation analysis, together with a variogram and an isolation-by-distance analyses in each geographical region, showed clear spatial structure, but the results will be presented elsewhere.

Geographical Subdivision

The AMOVA analysis for the D-loop mitochondrial region showed that, by means of 1000 random permutations and by using the Kimura two-parameters distance, the major fraction of the overall molecular variance was due to the heterogeneity among the three population areas studied (F_{st} = 0.90, P < 0.0001). Several comparisons carried out using population pairs revealed that the three populations were highly differentiated among them from a statistical standpoint. The pairwise difference of the genetic population between the Bolivian Amazon and the Colombian Orinoquia was F_{ST} = 0.89 (P < 0.00001) and between Bolivia and the Colombian Amazon, F_{ST} = 0.95 (P < 0.00001). The genetic differentiation between both Colombian areas was F_{ST} = 0.76 (P < 0.00001). The AMOVA result for the Cyt-b gene agrees quite well with that found with the D-loop region (F_{ST} = 0.78 (P < 0.0001). The genetic heterogeneity between Bolivia and the Colombian Orinoquia (F_{ST} = 0.81; P < 0.00001) and between Bolivia and Amazonian Colombia (F_{ST} = 0.91; P < 0.00001) was substantially higher than the genetic differentiation between both Colombian areas (F_{ST} = 0.47; P < 0.0001).

Haplotype Phylogenetic Relationships

Mitochondrial D-loop region

Table 2 shows the estimates for the nucleotide □) and haplotypic (h) diversities found within and among the three geographical areas studied in the Colombian Orinoco, the Colombian Amazon and the Bolivian Amazon for the D-loop region. The nucleotide diversity for all 96 sequences analyzed was 2.8%. Obviously, this estimate was higher than those found for each individual geographical region (1.48% for the Orinoquian area, 0.18% for the Colombian Amazon and 0.20% for the Bolivian Amazon). This in turn will allow us to put forward the straightforward differences among the *Inia sp.* populations studied. Although the nucleotide diversity was higher in the Orinoco population, the haplotypic diversity was higher in the Bolivian population. Table 3 presents the number of net nucleotide substitutions per site between pairs of these three populations (Da). As can be seen, the molecular divergence between the Orinoquian and the Bolivian populations was 6.53% as compared to 5.32% between the Colombian Amazon and the Bolivian population (for the hypervariable region of 400 bp), which suggests a noticeable genetic differentiation between the Colombian and the Bolivian populations.

Phylogenetic relationships among the *Inia* sp. populations by using the 570 bp D-loop haplotypes studied, recovered by two different procedures, offered similar results. The genetic distance tree showed here was based on the Jukes-Cantor genetic distance and the neighbor-joining algorithm. The use of the Tamura-Nei, and the Kimura two-parameter genetic distances offered the same results. Clearly, the topology of all trees obtained differentiated two clades with high bootstrap levels. One of them included all the haplotypes belonging to the Bolivian populations. The other clade included the populations of the Colombian Amazon and the Orinoco basin. (Fig. 3). In the same sense, the maximum parsimony trees with the branch and bound procedure and with a transitions-transversions ratio equals 1 gave the same results as the tree shown here. The consistency index was 0.71; whereas the retention index was 0.86, with a minimal length tree of 78 steps.

Cyt-b gene

Table 2 shows the nucleotide (□) and the haplotypic (h) diversity obtained with the Cyt-b gene. The overall nucleotide diversity was 1.78%. In the case of the Bolivian sample the nucleotide diversity was only 0.0003%; whereas the nucleotide diversity in the Colombian Orinoquia reached 0.99%, clearly showing that this area has the higher levels of nucleotide diversity for the Cyt-b as well as for the D-loop region. The Colombian-Brazilian Amazon showed a nucleotide diversity of 0.47%. The haplotype diversity was also higher in the Colombian Orinoquia (h = 0.56) than in the Colombian-Brazilian Amazon (h = 0.42) and Bolivia (h = 0.20). Table 3 shows

Table 2. Estimations of the Haplotypic diversity and percentage of nucleotide diversity (π) for both the mitochondrial control region (570pb) and the Cytochrome b gene (600pb) for the three *Inia* populations studied

POPULATIONS	Haplotypic diversity in the control region	Haplotypic diversity in the cytochrome b gene	Nucleotide diversity (?) in the control region	Nucleotide diversity (?) in the cytochrome b gene
Colombian Orinoquia	0.647±0.118	0.561±0.154	1.48%	1.16%
Amazon	0.149±0.074	0.425±0.138	0.20%	0.47%
Bolivian Amazon	0.761±0.038	0.200±0.154	0.18%	0.03%

Table 3. Net genetic divergence (D_A) among the populations of Inia studied. In the upper main diagonal the genetic divergence for the Cyt-b gene (600 bp). Whereas in the lower main diagonal the genetic divergence for the mitochondrial control region (only hypervariable region, (400 bp)).

	Colombian Orinoco	*Amazon*	*Bolivian Amazon*
Colombian Orinoco		0.59%	2.49%
Amazon	2.50%		2.98%
Bolivian Amazon	6.53%	5.32%	

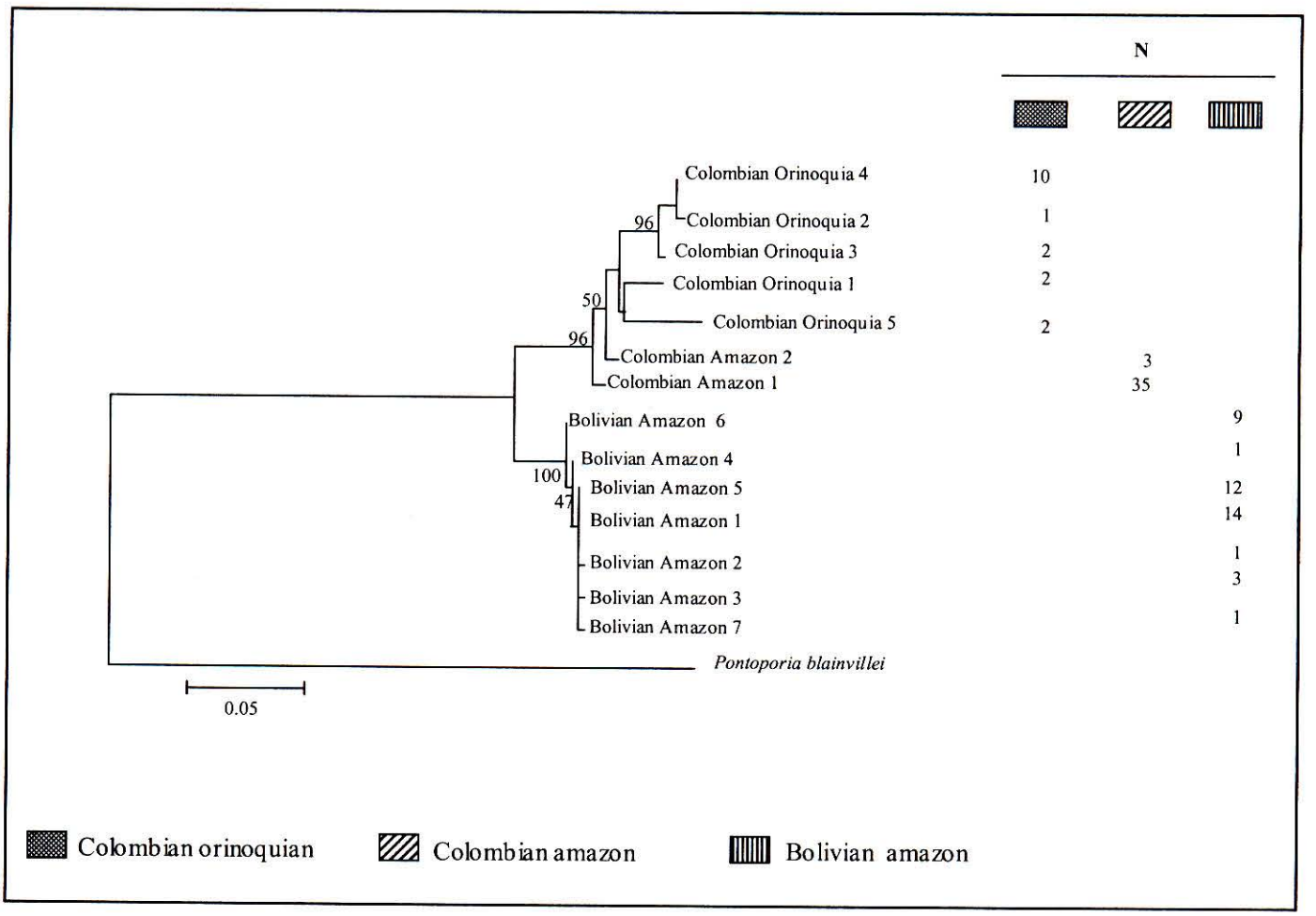

Figure 3. Dendrogram with the neighbor-joining algorithm and the Tamura-Nei genetic distance (1993) with a = 0.5 among the different *Inia* haplotypes found for the mitochondrial control region. The number on the tree nodes are the bootstrap percentages.

the Da statistic among the three areas studied for the Cyt-b gene. The highest value was discovered between the Colombian-Brazilian Amazon and the Bolivian Amazon (2.98%), while the value between the Colombian Orinoquia and the Bolivian Amazon was 2.49%. On the other hand, the genetic differentiation between the Colombian Orinoquia and the Colombian-Brazilian Amazon was very small (0.59%).

The phylogenetic relationships recovered among the cyt-b haplotypes using the different distances and the neighbor-joining algorithm, were substantially similar to those obtained throughout the D-loop mitochondrial analysis (Fig. 4). The Bolivian haplotypes conformed a clade with high bootstrap values, separate from that array established by the Orinoquian and the Colombian-Brazilian Amazon haplotypes (Fig. 4). The striking relationship between the Orinoquian and the Colombian-Brazilian Amazon haplotypes for the Cyt-b gene is remarkable, which is not in agreement with the possibility of two different subspecies of *Inia* in these two different basins, *I. geoffrensis geoffrensis* and *I. geoffrensis humboldtiana*.

Discussion

For the first time, data were obtained about the molecular intra- and interpopulational variation for the mitochondrial D-loop region and for the Cyt-b gene of the river dolphin *Inia sp.* from different populations throughout its geographical range. As there were no previous data for individuals of the same species among other South American rivers, our data should be compared with those recorded from other species of marine cetaceans and a few other terrestrial mammal species.

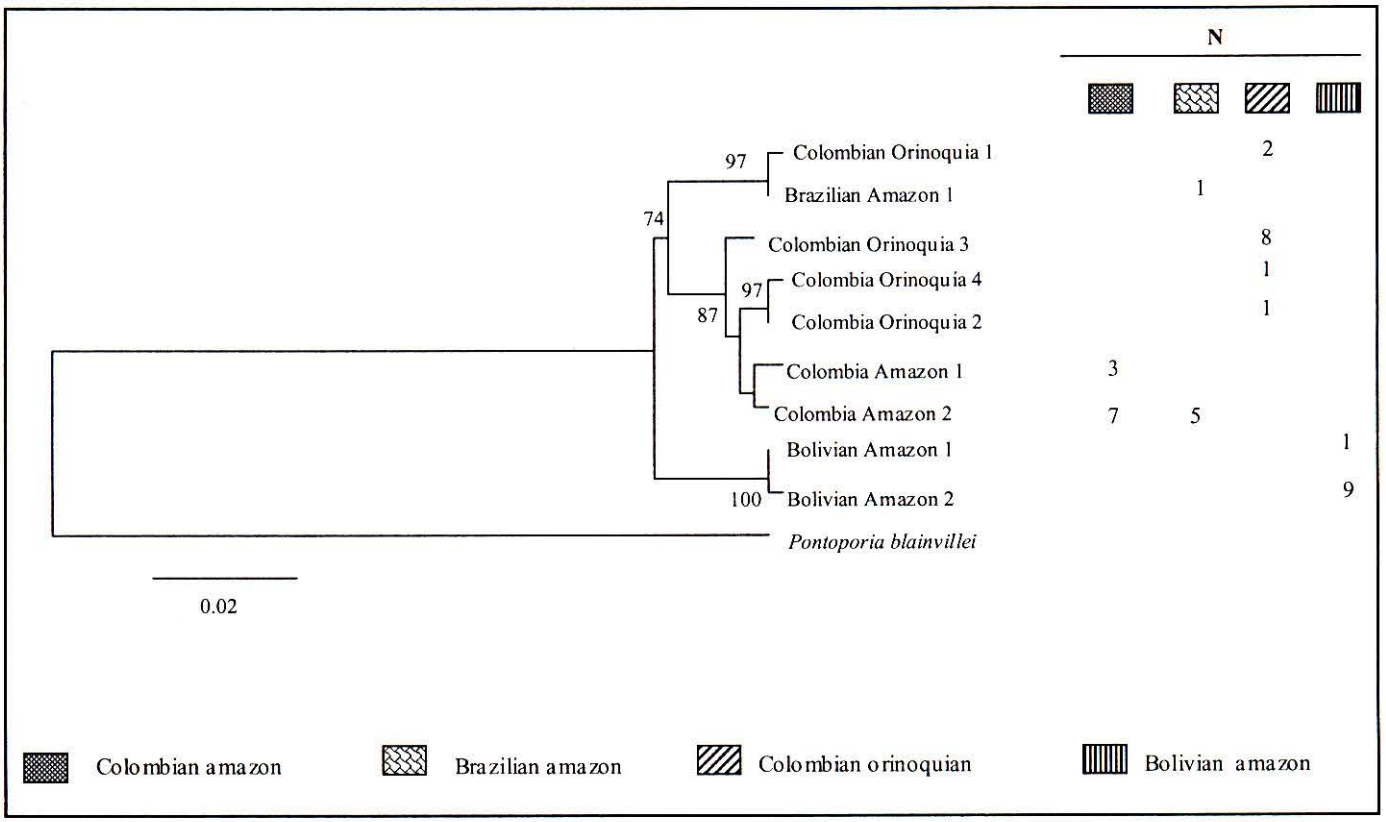

Figure 4. Dendrogram with the neighbor-joining algorithm and the Tamura-Nei genetic distance (1993) with a = 0.5 among the different *Inia* haplotypes found for the mitochondrial Cytochrome b gene. The number on the tree nodes are the bootstrap percentages.

Variation of the Mitochondrial D- loop and Cyt-b Sequences in Inia

The estimated nucleotide diversity for the D-loop region (1.48%) and for the Cyt-b (1.16%) for the Orinoquian population puts forward a considerable level of genetic diversity in this population. These values were fourfold for the D-loop region and from 2 to 390-fold for the Cyt-b gene of those found in the Bolivian and the Colombian-Brazilian Amazon rivers. The nucleotide diversity for the D-loop reported for the Bolivian and Colombian Amazon (0.2% and 0.18%, respectively) and for the Cyt-b (0.003% and 0.68%) proved to be similar to those values found in other regional Cetacean populations (**Hoelzel**, 1991; **Baker** et al., 1993; **Rosel** et al., 1994; **Pastene** et al., 1996; **Sechi** et al., 1998). Therefore, the most baseline result found in the Orinoquia, under the neutrality model of **Tajima** (1989), is the elevated nucleotide diversity found in their *Inia* sp. populations. This fact could be interpreted in several ways: (a) Several authors claimed that the geographical areas with the highest genetic diversity are the central range in the distribution of a given species (e.g., **Dobzhansky**, 1971). If this were the case, then the Orinoquia could be the original area of distribution of *Inia*, which means and adds credence to the argument that the introduction of its ancestry inside South-America could have been via the Atlantic Ocean. (b) Another interpretation is that in the last 1.5-2 million years, different maternal lineages from different geographical origins could have been migrated inside the Orinoquian river systems. (c) This difference could also be due to the higher population size in the Orinoquian rivers than in the Colombian or Bolivian Amazon. Differential mutation rates among these areas could be a less parsimonious explanation. In whatever event, this geographical area has considerable genetic diversity, which has important biological conservation implications.

On the other hand, the overall nucleotide diversity found in *Inia* (2.8% D-loop) proved to be similar to humpbacks worldwide, but generally superior to other nucleotide diversity values found in other Cetacean species,

such as the case reported by **Rosel** et al., (1994) for several *Delphinius* spp. (2.1%), for *Delphinapterus leucas* (0.51%; **O´Corry-crowe** et al., 1997), for the Chinese *Tursiops truncates* (1.9%; **Wang** et al., 1999), for certain local *Megaptera novaeangliae* populations (0.89% for the Mexican population and 0.63% for the Hawaii population; **Medrano-González** et al., 1995) and considerably higher than three species of *Phocoena* (0.39%) (**Rosel** et al, 1995).

The absence of common haplotypes among the *Inia* sp. populations suggests the occurrence of multiple events of genetic isolation as a mechanism for generating the haplotype diversity observed. According to **Avise** et al., (1989), when a population has been recently divided into two new ones, some haplotypes could be more closely related between them than with other haplotypes within its own population. A compelling addition to this inference is the case of the haplotype Colombian Orinoquia 1 for the D-loop region, which was genetically closer to the two Colombian Amazonian haplotypes than to the Colombian Orinoquia haplotypes. With the Cyt-b gene, the haplotype Colombian Orinoquia 1 is also genetically more related to the Colombian-Brazilian Amazon haplotypes than the remaining Colombian Orinoquia haplotypes. This result could be evidence that a relatively recent gene flow between Amazon and Orinoquian populations could have occurred in Colombia. Therefore the claims of **Van Bree** and **Robineau** (1973), **Pilleri** and **Gihr** (1977), and **Casinos** and **Ocaña** (1979) that there are different subspecies of *I. geoffrensis* in the Orinoco Basin (*I. g. humboldtiana*) and in the Amazon (*I. g. geoffrensis*), which is based on morphological variables, is not supported by the molecular data. Moreover, several authors (**Best** and **Da Silva**, 1984, 1989; **Meade** and **Koehnken**, 1991) have registered individuals of this species in the Negro River and in the Casiquiari Channel, which have been considered by other authors to be a potential isolation factor between the Amazon and the Orinoco basins. As commented earlier, our molecular data agree quite well with the fact that an important gene flow is connecting the Amazon and the Orinoco *I. geoffrensis* populations.

It should be noted that, on the contrary, only two haplotypes for the D-loop region and three for the Cyt-b gene were detected in the Colombian Amazon (and for the Colombian-Brazilian Amazon in the case of the last gene). For the D-loop region, one of them, the Colombian Amazon 2 haplotype, had a frequency of 92%. Clearly, the most prominent genetic mechanisms invoked to generate the high prevalence of this haplotype is the gene drift and/or the founder effect. Perhaps, this geographical area was recently colonized by *Inia*. Therefore, this haplotype would be considered as a founder introduced within this Western Amazonian area by female colonizers.

Genetic Divergence among Inia Populations

The values of genetic divergence (Da) calculated among the different *Inia sp.* populations were compared with other species of Cetaceans by using both mitochondrial control region and Cyt-b sequences. The *Inia* values ranged from 6.53% (Bolivian Amazon vs Colombian Orinoquia) to 2.50% (Colombian Amazon vs Colombian Orinoquia) for the D-loop region. In the case of the Cyt-b gene, these values ranged from 2.98% (Bolivian Amazon vs. Colombian-Brazilian Amazon) to 0.59% (Colombian-Brazilian Amazon vs. Colombian Orinoquia). Needless to say, these *Inia* values were very high for the existence of a unique *Inia* species. They are even higher than those previously calculated between *Eubalena glacialis* and *E. australis* (1.8%) (**Schaeff** et al, 1991), between *Stenella attenuata* and *S. longirostris* (4%) (**Dizon** et al, 1991), between *Mesoplodon*, *Berardius* and *Hyperoodon* (4.7%) (**Dalebout** et al., 1998), between *Tursiops truncatus* and *T. aduncus* (4.4%) (**Wang** et al., 1999), between several *Delphinus* spp, (1.11%) (**Rosel** et al., 1994), between *Balaenoptera musculus* and *B. physalus* (3.4%) and between *B. borealis* and *B. acutorostrata*) (Arnason et al., 1993) for the D-loop region. They were also much higher than those calculated in populations of a same species. In the case of the minke whales (*Balenoptera acutorostrata*), divergence values between two northern Pacific populations ranged from 0.22 to 3.99%, between Antarctic and northern Pacific populations (**Baker** et al, 1993). For instance, we have data for terrestrial mammals that agree quite well in the sense of the high levels of genetic heterogeneity discovered between the Bolivian and the Colombian *Inia* sp. populations. This is the case between two Cervidae species of the genus *Mazama* for this same mitochondrial region (*M. americana* and *M. gouzaoubira*, divergence of 2.1%; **Ruiz-García** et al., 2001).

Another valuable insight into understanding the high degree of genetic heterogeneity between the Colombian and Bolivian *Inia* sp. population relays on the average F_{st} (=0.90) value for the D-loop region and on the average F_{ST} (= 0.86) value for the Cyt-b gene, which indicated that the genus *Inia* is really divided into two different species, the Colombian and the Bolivian ones.

Given the foregoing, our results agree quite well with those obtained at a morphometric level, reported by **Pilleri** and **Ghir** (1977) and **da Silva** (1994). Together, all these data have led us to support the hypothesis of the exist-

ence of one species in Bolivia, *I. boliviensis* and only one species in Colombia and Brazil, *I. geoffrensis*.

The existence of 400 km of rapids in the upper Madeira-Mamoré River suggest that a process of allopatric separation of this genus could be a reasonable explanation that supports the results obtained in this study. In agreement with **Pilleri** and **Pilleri** (1982), the formation of these two species could be at the final Pliocene and in the beginning of the Pleistocene as it is known that the Beni lands in Bolivia were once a lake completely isolated from other water sources during the Neogene. In the Holocene-Pleistocene, this lake was tapped and the 400 km of the Madeira-Mamoré rapids were conformed It is possible that *Inia* reached this area via the Abuña pass, which is present today, previous to the formation of the rapids between Guayara-Mirim and Porto Velho (**Grabert** 1967; **Pilleri** et al, 1984). Therefore the isolation of *I. boliviensis* could have occurred during the Pleistocene, approximately 5 million years ago, when the Andes mountains were formed (**Grabert**, 1984), which is sustained by the fact that in this period the current geomorphologic aspect of the Andes was reached (**Lundberg** et al, 1998). This moment could be decisive for the allopatric isolation of the Bolivian *Inia* population with regard to the Amazon and the Orinoquia populations.

The current controversy with respect to the divergence times within the genus *Inia*, and of this genus with other related taxa could be resolved partially with the results presented here. Whether we accept a ratio of 1-2% divergence per one million years for the D-loop region (**Wilson** et al., 1985), the evolutionary time of separation between populations can be calculated. The first estimate has been tested in marine mammals, whereas the second is typically of terrestrial mammals. We obtained an estimate of 4.7-4.1 million years of divergence between both taxa for all the 570 bp studied, assuming a 1% divergence ratio. If the 2% divergence ratio is employed, the upper estimate is 3.3-2.7 million years and the lower estimate is 2.4-2.1 million years. For the Cyt-b gene, **Irwin** et al. (1991) showed that the divergence per million of years is about 0.5%. Taking this value, the evolutionary divergence between *I. boliviensis* and *I geoffrensis* could be about 5.96-4.98 million years. The average value for both mitochondrial regions (taking 1 and 0.5% of divergence, respectively) is about 5.33-4.54 million years, which is quite similar to the 5 million years of separation between *I. geoffrensis* and *I. Boliviensis*, claimed by **Grabert** (1984) and **Da Silva** (1994).

The most ancient fossil, which was recovered from the Iniidae family and dated at 7-8 million years ago, was proposed to belong, however, to a subfamily different from the Iniinae (**Cozzuol**, 1996). In addition, the most ancient fossil of Pontoporidae came from the late Miocene (*Brachydelphis mazeasi*, from Peru, with an antiquity of 11-13 million years ago, (**Muizon**, 1988; **Cozzuol**, 1996). This family is proposed to be the sister taxa of the Iniidae. Assuming this fact, and taking into account that the nucleotide divergence between *Inia* and *Pontoporia* was 17.8% for the D-loop region, it is possible to obtain several other time-divergence estimates. If a ratio ranging from 1 to 2% per million years is assumed, then the separation time between *Inia* and *Pontoporia* could range from 17.8-8.9 million years, respectively, which agrees quite well with the 11-13 million years established for *Brachydelphis*. Nevertheless, the rates of mitochondrial evolution could be different among different taxa. Although, the molecular clock could be accelerated by gene drift in small and isolated populations, we believe that these estimates could be highly realistic. Based on the foregoing considerations, we propose to review the taxonomy of *Inia* in order to modify its taxonomic species status with two allopatric species: *I. boliviensis* in Bolivia and one unique *I. geoffrensis* species in the Orinoco and Amazon rivers.

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