Molecular Identification of Evolutionarily Significant Units in the Amazon River Dolphin *Inia* sp. (Cetacea: Iniidae)

E. Banguera-Hinestroza, H. Cárdenas, M. Ruiz-García, M. Marmontel, E. Gaitán, R. Vázquez, and F. García-Vallejo

The Amazon river dolphin, genus *Inia*, is endemic to the major river basins of northern South America. No previous studies have focused on the genetic structure of this genus. In this work, 96 DNA samples from specimens of this genus were collected in the Orinoco basin (four rivers), the Putumayo River, a tributary of the Colombian Amazon and the Mamoré, and the Tijamuchí and Ipurupuru rivers in the Bolivian Amazon. These samples were used to amplify a fragment of 400 bp of the mitochondrial DNA (mtDNA) control region. In addition, 38 of these samples were also used to sequence 600 bp of the mitochondrial cytochrome *b* gene. The analysis of the population structure subdivision with an analysis of molecular variance (AMOVA) revealed important aspects about the genetic structure of *Inia* groups from these three geographically separate regions. By comparing the control region DNA and cytochrome *b* sequences, distinct types of nonshared haplotypes were observed. The net genetic divergence of control region sequences was 6.53% between the Orinoco and Bolivian rivers, 5.32% between the Putumayo and Bolivian rivers, and 2.50% between the Orinoco and Putumayo rivers. For the cytochrome *b* gene, these values were 2.48%, 2.98%, and 0.06%, respectively. The nucleotide sequences were analyzed phylogenetically using several genetic distance matrices and applying neighbor-joining, maximum likelihood, and maximum parsimony procedures. The results support the proposal to subdivide the *Inia* genus into at least two evolutionarily significant units: one confined to the Bolivian river basin and the other widely distributed across the Amazon and Orinoco basins.

From the Laboratorio de Biología Molecular y Patogénesis, Departamento de Ciencias Fisiológicas, Facultad de Salud, Universidad del Valle, A.A 25360, Cali, Colombia (Banguera-Hinestroza and García-Vallejo); Laboratorio de Biología Molecular, Departamento de Biología, Sección de Genética, Facultad de Ciencias, Universidad del Valle, A.A 25360, Cali, Colombia (Cárdenas); Laboratorio de Inmunobiología y Genética de Poblaciones Molecular, Unidad de Genética, Departamento de Biología, Facultad de Ciencias, Pontificia Universidad Javeriana, Cra 7ª No. 43-82, Bogotá, Colombia (Ruiz-García); Instituto de Desenvolvimento Sustentable Mamirauá, Caixa Postal 0001 Tefe/AM, Brazil (Marmontel); Centro Internacional de Agricultura Tropical (CIAT), A.A. 6713, Cali, Colombia (Gaitán); and Universidad Técnica del Beni, Casilla 251, Trinidad, Bolivia (Vázquez). Funding for this study was provided by Fondo FEN Colombia and the Laboratorio de Biología Molecular y Patogénesis (Universidad del Valle). Partial support was also provided by Laboratorio de Biología Molecular Instituto von Humboldt, the Health Secretary from Departamento de Guainia, the Corporación para el Desarrollo del Nororiente Amazónico (C.D.A.), and the Pto. Leguízamo (Departamento de Putumayo) and Pto. Inirida (Departamento de Guainía) majors. We are also grateful to Bernardo Ortiz (TRAFFIC office for South America) and Drs. Joe Tohme (Biotechnology Unit at CIAT Cali, Colombia) and Luis Pastene (Cetacean Research Institute, Japan) for their respective collaborations. Thanks to Marila Lazaro (Laboratorio de Evolución, Facultad de Ciencias, Montevideo, Uruguay) for providing the control region consensus sequence of *Pontoporia.* We also appreciate the help offered by Angela Garcia (Department of Fisheries and Aquatic Sciences, University of Florida) and Drs. Mario Cozzuol (Universidad Federal de Rondônia, Porto Velho, Brazil), Bill Perrin, and Richard LeDuc (Southwest Fisheries Science Center, La Jolla, CA) for their critical review of the manuscript. In addition, our thanks go to the Bolivian Ministry of the Environment for facilitating the obtaining of CITES permissions, to the Colombian Environment Ministry, to the Regional Development Corporation (Corpoamazonía), and to the Colombian National Parks. Many thanks to the referees, the associate editor (Dr. C. Scott Baker), and Stephnie Ploen for their corrections and comments. Address correspondence to Manuel Ruiz-García at the address above, or e-mail: mruiz@javeriana.edu.co.

© 2002 The American Genetic Association 93:312–322

The Amazon river dolphin of the genus *Inia* (Blainville 1817), also known as tonina, bufeo, boto, or pink dolphin, is classified within the order Cetacea, suborder Odontoceti, superfamily Inioidea, and family Iniidae (de Muizon 1988; Fordyce and Barnes 1994; Fordyce et al. 1994; Messenger and McGuire 1998; Rice 1998). Populations of the Amazon river dolphin are distributed widely in many rivers in the Amazon and Orinoco basins. The range of distribution in South America includes rivers in Bolivia, Brazil, Colombia, Ecuador, Peru, Venezuela, and French Guyana, covering an area equivalent to 7 million km^2 (Best and da Silva 1989a,b).

The distribution of *Inia* is constrained by a series of geographical barriers. Populations from the Amazon and Beni-Mamoré (Bolivia and Brazil) are separated by rapids in the upper Madeira River between Porto Velho and Guajara-Mirim (da Silva 1994). The unique connection between populations from the Amazon and Orinoco is facilitated by the Casiquiari Channel, which reaches the Negro River, a tributary of the Amazon. The Casiquiari Channel is considered a barrier for *I. geoffrensis* because the water's pH is rather low and biomass productivity is low. Other potential barriers are the rapids of the Negro River and the rapids of the Orinoco between Samariapo and Puerto Ayacucho (Figure 1) (Best and da Silva 1989a,b).

The first morphometric study of the genus *Inia* at the species level was carried out by Pilleri and Ghir (1977). They divided the genus into two different species: *I. boliviensis* (D'Orbigny 1834), which is distributed in the Beni-Mamoré River, and *I. geoffrensis.* The latter was further 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 Orinoco basin. Despite this, Casinos and Ocaña (1979) claimed that the clinal variation in the populations of *I. geoffrensis,* based on craniometric studies,

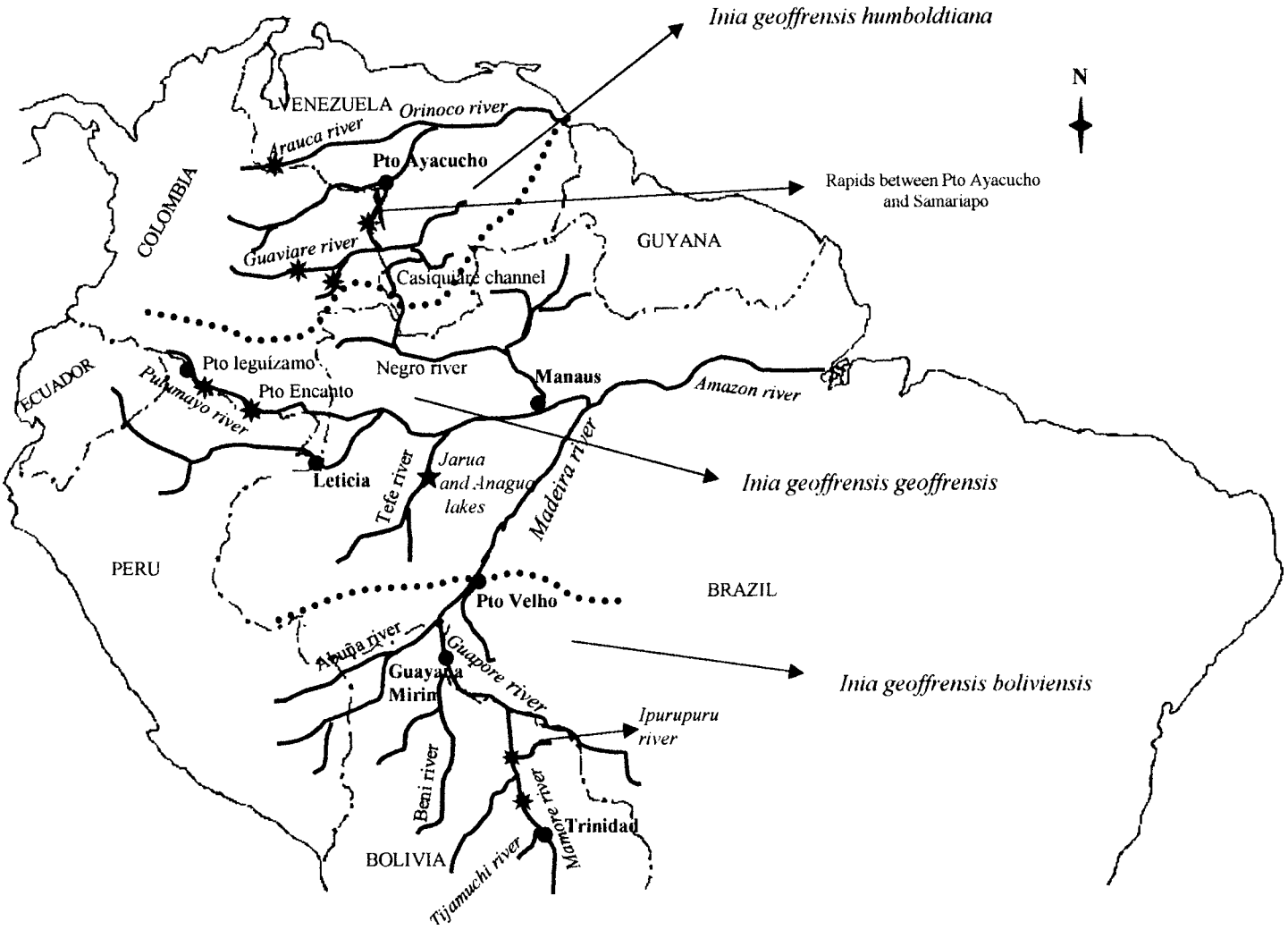


Figure 1. Map representing the geographical areas studied in the Orinoco Basin and in the Colombian, Brazilian, and Bolivian Amazon. Stars indicate the places where dolphins were captured and sampled. Dotted lines indicate rapids and other obstacles to the migration of *Inia* from one basin to another.

was 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. Nevertheless, since 1989 the most widely used and recognized subdivision 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 (Best and da Silva 1989a,b). More recently, da Silva (1994) again proposed subdividing the genus into the two aforementioned species, based on morphometric analyses. This study (da Silva 1994) augmented the geographic range and the number of samples in comparison to previous studies. However, the most recognized classification considers only one species with three subspecies (Rice 1998).

In order to analyze the genetic variation of these three proposed subspecies of *I. geoffrensis*, a fragment of 400 bp of the mitochondrial DNA (mtDNA) control region (96 individuals) and 600 bp of the cytochrome *b* mitochondrial gene (38 individuals) from *Inia* specimens were analyzed. The control region of mtDNA is a portion that evolves particularly rapidly and thus allows fine-scale resolution of population structure and microevolutionary divisions (Rosel et al. 1995; Wang et al. 1996). The statistics calculated from the sequences obtained were the mean number of pairwise differences, the nucleotide diversity and net genetic divergence within and between the different populations, the ϕ_{ST} and F_{ST} statistics among population pairs, the Tamura and Nei (1993) genetic distances among the same pairs, and an analysis of molecular variance (AMOVA). The phylogenetic relationships among these three feasible taxa were 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 Amazon rivers compared with the dolphins from the rivers in the Bolivian Amazon. These results are therefore in good agreement 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 means of a biopsy of 1 cm^2 from the caudal fin. All dolphins were captured in fishing nests, taking special care to ensure the safety of

each individual. After the biopsy, the wound was covered with antibiotic cream and the animal was released under safe conditions. The number of individuals and the geographic regions sampled are shown in Table 1 and Figure 1. In the Colombian Orinoco, a total of 17 individuals were sampled in 11 locations along the Orinoco, Guaviare, Inirida, and Arauca rivers. In the Colombian Amazon, several lagoons were explored in a transect of 443 km from Puerto Leguizamo to Puerto Encanto in the Putumayo River. In these areas, a total of 38 individuals were caught. In the Bolivian Amazon, several lagoons were explored in the Mamoré, Ipurupuru, and Tijamuchí rivers. A total of 41 specimens were surveyed in this area. A total of 96 dolphins were captured for this study. All these animals were analyzed for the mtDNA control region. A subset of these samples (n = 32), representing all the different haplotypes found for the mtDNA control region, were also analyzed for the mitochondrial cytochrome *b* gene. In addition, cytochrome *b* sequences were obtained from five Amazon river dolphins from the Jarua Grande and Anagua lagoons near the Tefé River in the Brazilian Amazon, and one additional sequence was obtained from GenBank (accession number AF304068). In total, 38 samples were analyzed for the cytochrome *b* gene.

Two sequences of the Franciscana (*Pontoporia blainvillei*) were used as an outgroup. The sequence used in the analysis of the mtDNA control region was donated by Marila Lazaro (Uruguay), while the sequence for the cytochrome *b* analysis was obtained from GenBank (AF229170). The Pontoporiidae, today represented by only this one species, the Franciscana, have been considered sister taxa of the Iniidae (Fordyce and Barnes 1994; Fordyce et al. 1994; Messenger and McGuire 1998).

All skin samples obtained in the field were stored in a solution of 20% dimethyl sulfoxide (DMSO), supersaturated with NaCl, according to Hoelzel (1991), for periods of 4–8 weeks at room temperature until processed in the laboratory.

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
Colombian Orinoco			
Inirida River	3	68°11′8.28″ W–3°57′26.32″ N	4
		68°1′4.69″ W–4°1′54.46″ N	2
		68°1′43.51″ W–3°19′47.70″ N	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	15
		73°51′ W–1°8′ S	13
		73°39′ W–1°15′ S	10
Jarua Grande Lake	1	—	4
Anagua Lake	1	—	1
Bolivian Amazon			
Ipurupuru River	2	65°3′ W–14°18′S	12
Tijamuchí River	1	—	1
Mamoré River	5	65°3′ W–14°33′ S	28
		65°00′ W–14°46′ S	
Total	19		101

DNA Amplification and Sequencing

Extraction of DNA from skin samples was performed using the QIAamp tissue kit (Qiagen Inc.) following the manufacturer's instructions. DNA quality was visualized by electrophoresis in 0.8% agarose gels. The control region was amplified by polymerase chain reaction (PCR) using specific combinations of light-strand TRO (5′-CCTCCCTAAGACTCAAGGAAG-3′) and-heavy-strand DH6 (5′AAATACAYACAGGYCCAGC TA 3′) oligonucleotide primers donated by the Southwest Fisheries Science Center (La Jolla, CA). Twenty to 100 ng of DNA were employed in a reaction with 10 mM Tris-HCL, pH 8.3, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 mM of each oligonucleotide primer, and two units of *Taq* DNA polymerase in a total volume of 50 μl. The amplified fragments were submitted for amplification using a thermal cycler (MJ Research PT-100). Cycling conditions were performed as follows: 2 min at 95°C, 30 s at 94°C, 1 min at 50°C, and 1.5 min at 72°C. Thirty-five cycles were performed, with a final extension step of 3 min at 72°C. Amplified mtDNA was cleaned using sephadex columns, and in several cases polyethylene glycol/NaCl was also used, both with good results. Cleaned PCR products were visualized 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′) (Palumbi et al. 1991). A similar cycling profile as for the control region was used for this gene, with 52°C as the annealing temperature. All amplified fragments were sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer).

The sequencing reaction was performed in a final volume of 10 μl containing 20–100 ng of PCR-amplified product, 1 μl of oligonucleotide primers (3.2 mM), and 4 μl of dye dideoxy terminator premix. The cycling profile was as follows: 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 s, at 52°C for 10 s, and a final extension step at 60°C for 4 min. Sequenced products were purified by ethanol precipitation. All DNA fragments were sequenced in both directions, and in cases of mismatching, the PCR reaction was repeated and the product sequenced again. Sequence alignments and editing were performed using Sequencer 3.0 (Gene Codes Corp.) and Clustal X (version 1.62 b) (Thompson et al. 1997).

The GenBank accession numbers for the different mitochondrial control region haplotypes and for the diverse cytochrome *b* gene haplotypes found are as follows:

AF521113, AF521114, AF521115, AF521116, and AF521117 for the five mitochondrial control region haplotypes for the Orinoco,

AF521118, AF521119, AF521120, AF521121, AF521122, AF521123, and AF521124 for the seven mitochondrial control haplotypes for the Bolivian Amazon,

AF521125 and AF521126 for the Colombian Amazon,

AF521105 and AF521106 for the Bolivian cytochrome *b* haplotypes,

AF521107, AF521108, AF521109, and AF521110 for the Orinoco cytochrome *b* haplotypes, and

AF521111 and AF521112 for the Colombian Amazon haplotypes.

Sequence Analysis

Mitochondrial genetic structure within and between populations, and several population genetic parameters such as the mean number of pairwise differences, the nucleotide diversity (π), and the net number of nucleotide substitutions per site (D_A) among populations, were calculated using DNAsp (Rozas and Rozas 1995) and Arlequin (Schneider et al. 2000). The haplotypic diversity according to Nei's (1978) and Tajima and Nei's (1984) equations was calculated as well. The ϕ_{ST} incorporates information on the degree of genetic distance between haplotypes, in this case the gamma distance with the Tamura and Nei (1993) model of evolution, where $\alpha = 0.5$ as recommended for the control region sequences (Kumar et al. 1993) as well as the frequencies of haplotypes in each population. The F_{ST} statistic considers only differences in haplotype frequencies. From these genetic heterogeneity statistics, gene flow estimates among the three geographic areas were calculated using the Arlequin program.

Concordance between geographic and genetic diversity was analyzed using an AMOVA, assuming an overall *Inia* population subdivided into three geographical subpopulations. The AMOVA procedure is included in the Arlequin program (Excoffier et al. 1992, Schneider et al. 2000) and was used to calculate both the ϕ_{ST} and F_{ST} statistics. O'Corry-Crowe et al. (1997) suggested that the AMOVA with ϕ_{ST} is more appropriate than F_{ST} when the haplotypes are divergent and phylogeographical structure is large, as in the current data. Thus ϕ_{ST} rather than F_{ST} values are presented in this study, although both were calculated and found to be in agreement.

The phylogenetic relationships of the control region and cytochrome *b* haplotypes were reconstructed using the MEGA program (version 1.2) (Kumar et al. 1993) with Tamura and Nei (1993), Tajima and Nei (1984), Kimura's two parameters (Kimura 1980), and Jukes and Cantor (1969) genetic distances. All genetic distances gave identical reconstructions. However, only the results for the Tamura and Nei (1993) analysis are shown here, because its properties are considered more appropriate for the mtDNA control region. Several transition:transversion ratios were also used, with identical results. Trees were constructed using the neighbor-joining (Saitou and Nei 1987) and unweighted pair group method with arithmetic mean (UPGMA) algorithm procedures in MEGA. Maximum parsimony trees were searched by using the PAUP* program (version 4.0b) (Swofford 1998), with the heuristic and the branch and bound procedures, using simple sequence addition of sequences and tree bisection-reconnection branch swapping 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 1,000 repetitions. The maximum likelihood procedures (among them, the F84 model of substitution, the Hasegawa et al. [1985] model of sequence evolution, and a maximum-likelihood estimate of the transition to transversion rate) were also employed and the Kishino–Hasegawa maximum likelihood test was performed to determine whether different trees were significantly different (Kishino and Hasegawa 1989). Maximum-likelihood distances were calculated with and without an allowance for invariant sites (*I*). The value of *I* was fixed at 0.666 based on a maximum-likelihood evaluation of the minimum evolution tree derived from the original data. The starting tree was obtained from the neighbor-joining analysis, and the shape parameter (α) for the gamma distribution of rate heterogeneity among sites was empirically derived. Nevertheless, the most accurate trees were identical to those obtained with the Tamura and Nei (1993) genetic distances with the UPGMA, neighbor-joining, and maximum parsimony trees. The control region and the cytochrome *b* gene sequences of *P. blainvillei* were employed as an outgroup.

Results

A total of 400 bp for the mtDNA control region for 96 individuals and 600 bp for the cytochrome *b* gene for 38 specimens were sequenced. The number of variable sites, the number of haplotypes found, the mean number of pairwise differences within each geographic region, and the nucleotide and haplotypic diversity levels are shown in Figure 2 and Table 2.

mtDNA Control Region

A total of 14 different haplotypes were found among the *Inia* mtDNA control region sequences. Five haplotypes were found exclusively in specimens from the Orinoco region rivers ($n = 17$) (Colombian Orinoco 1, 2, 3, 4, 5) (Figure 2A), with 27 variable nucleotide positions (25 transitions and 2 transversions). Only two haplotypes were recorded in specimens from the Putumayo River ($n = 38$) (Colombian Amazon 1, 2) (Figure 2A), with only six polymorphic nucleotide positions (all transitions). In the Bolivian rivers it was possible to discriminate seven haplotypes ($n = 41$) (Bolivian Amazon, 1, 2, 3, 4, 5, 6, 7) (Figure 2A) based on five different transitions. The mean number of pairwise differences in the Orinoco basin was 7.984 ± 3.904. This value was significantly greater than the values found for the Colombian Amazon (0.912 ± 0.646) or the Bolivian rivers (0.641 ± 0.509). Haplotypic diversity was greatest in the Bolivian rivers ($h = 0.761 \pm 0.038$) and the Orinoco basin ($h = 0.647 \pm 0.118$), whereas the haplotypic diversity in the Colombian Amazon was significantly lower ($h = 0.149 \pm 0.074$) (Table 2). The nucleotide diversity of the Orinoco basin was considerably higher ($\pi = 1.996\%$), while the Colombian Amazon ($\pi = 0.228\%$) and the Bolivian rivers ($\pi = 0.160\%$) presented a more restricted nucleotide diversity (Table 2). The greatest net genetic divergence (D_A) was found between the Colombian Orinoco and the Bolivian rivers ($D_A = 6.53\%$) and between the Colombian Amazon and the Bolivian rivers ($D_A = 5.32\%$) (Table 3). The net genetic divergence between the Colombian Orinoco basin and the Colombian Amazon was much smaller ($D_A = 2.50\%$) (Table 3). The ϕ_{ST} values agree closely with this last point. The highest pair values were between the Colombian Orinoco and the Bolivian Amazon ($\phi_{ST} = 0.917$) and between the Colombian Amazon and the Bolivian Amazon ($\phi_{ST} = 0.969$). This value between both Colombian populations (Orinoco and Amazon) was slightly lower ($\phi_{ST} = 0.764$) (Table 4). Estimates of female gene flow (Nm_f) based on the ϕ_{ST} were extremely low among the three regions, especially between the Colombian and Bolivian regions (Table 4).

Cytochrome *b* Gene

For the cytochrome *b* mitochondrial gene, nine different haplotypes were distinguished. In the Bolivian Amazon, two different haplotypes (Bolivian Amazon 1, 2) were found, differentiated by one transition (Figure 2B). In the 12 specimens from the Orinoco basin, four different haplotypes were found (Colombian Orinoquia 1, 2, 3, 4), differentiated

A

Haplotypes	N	6	1	2	2	2	2	4	4	4	5	6	6	6	7	9	9	1	1	1	1	1	1	1	1	1	1
			6	3	4	7	8	4	5	6	4	2	3	6	8	1	9	0	0	1	2	2	3	3	3	3	3	4
																		4	5	5	7	8	0	5	6	7	9	1
Colombian Orinoquia 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 Orinoquia 2	1	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	C	.	C
Colombian Orinoquia 3	2	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	.	.	C
Colombian Orinoquia 4	10	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	C	C	.	C
Colombian Orinoquia 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	1	1	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
		7	8	9	1	2	2	4	4	6	6	7	7	1	1	2	2	2	5	8	8
		2	1	9	1	7	9	4	6	4	9	0	1	1	9	1	2	4	2	5	8
Colombian Orinoquia 1	2	C	A	C	T	G	T	G	T	G	C	A	A	A	A	C	C	G	T	C	T
Colombian Orinoquia 2	1	.	.	.	.	A	C	.	.	A	T	.	.	.	G	T	.	C	.	T	.
Colombian Orinoquia 3	2	.	.	.	.	A	C	.	C	.	T	.	.	.	.	.	.	C	.	T	.
Colombian Orinoquia 4	10	.	.	.	.	A	C	.	.	A	T	.	.	.	G	.	.	C	.	T	.
Colombian Orinoquia 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

B

Haplotypes	N	8	4	5	6	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	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(Genbank AF304068)	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 Orinoquia 1	2	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Colombian Orinoquia 2	1	.	.	.	T	C	T	C	C	A	C	.	.	.	.	C	A	.	.	.	.	.	T	.	.	.	T	.	C	.	C	.	T	.	G	.
Colombian Orinoquia 3	8	G	.	.	T	C	.	.	C	.	C	T	.	.	.	.	A	.	.	.	.	.	T	.	.	.	T	.	C	.	C	.	T	.	G	.
Colombian Orinoquia 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 2. **(A)** Variable sites in the mitochondrial control region for the genus *Inia*, defining 14 different haplotypes for the three populations of *Inia* sp. analyzed. The points indicate those sequences that are identical to the Colombian Orinoco basin 1 haplotype (line 1). The haplotypes are named according to the geographical region to which they belong. The nucleotide positions are given in the first file in a vertical mode. **(B)** Variable sites for the cytochrome *b* gene for the genus *Inia*, defining nine different haplotypes for the three *Inia* populations analyzed. The points show the sequences identical to the Brazilian Amazon 1 haplotype (line 2, GenBank accession no. X922534). The nucleotide positions are given in the first file in a vertical mode. *N* = number of individuals.

by 19 transitions (Figure 2B). Finally, in the 10 individuals analyzed from the Colombian Amazon, two haplotypes were found, differentiated by 13 transition polymorphic sites (Colombian Amazon 1, 2) (Figure 2B). In addition, several Brazilian specimens were sequenced (n = 6). Five represent the most frequent haplotype discovered in the Colombian Amazon (CA2) and one (obtained from GenBank) represented a unique haplotype (BA1) (Figure 2B).

The mean number of pairwise differences among cytochrome *b* haplotypes in the Colombian Orinoco was 7.133 ± 3.657 (Table 2). This value was significantly greater than the values found for the Colombian Amazon (2.747 ± 0.155) and for the Bolivian Amazon (0.201 ± 0.269) (Table 2). Nucleotide diversity shared the same geographic pattern (Table 2). The greatest haplotypic diversity was that from the Colombian Orinoco basin (h = 0.561 ± 0.154),

Table 2. Estimations of the mean number of pairwise differences, haplotypic diversity, and percentage of nucleotide diversity for both the mitochondrial control region and the cytochrome *b* gene for the three *Inia* populations studied

	Mean no. pairwise differences		Haplotypic diversity		Nucleotide diversity	
Populations	Control region	Cytochrome *b* gene	Control region	Cytochrome *b* gene	Control region	Cytochrome *b* gene
Colombian Orinoco Basin (n = 17)	7.984 ± 3.904	7.133± 3.657	0.647 ± 0.118	0.561 ± 0.154	1.996%	1.16%
Amazon (n = 43)	0.912 ± 0.646	2.747 ± 0.155	0.149 ± 0.074	0.425 ± 0.138	0.228%	0.46%
Bolivian Amazon (n = 41)	0.641 ± 0.509	0.201 ± 0.269	0.761 ± 0.038	0.200 ± 0.154	0.160%	0.03%
Total	12.786 ± 4.312	11.816 ± 4.110	0.931 ± 0.072	0.778 ± 0.109	5.669%	3.376%

h = haplotypic diversity; π = percentage of nucleotide diversity.

whereas the haplotypic diversity in the Colombian Amazon is intermediate (h = 0.425 ± 0.133). In contrast to the mtDNA control region analysis, the dolphins from the Bolivian rivers showed the lowest haplotypic diversity (h = 0.200 ± 0.154) (Table 2).

Net genetic divergence (D_A) of cytochrome *b* among the *Inia* populations was greatest between the Colombian Orinoco and Bolivian Amazon rivers (D_A = 2.49%) and between the Colombian Amazon and Bolivian Amazon rivers (D_A = 2.99%) (Table 3). The net genetic divergence between the Colombian Orinoco basin and the Colombian Amazon was considerably smaller (D_A = 0.59%) (Table 3). Thus this trend is identical to that found in the control region, although the net genetic divergence is only about half of that determined in the control region. This suggests that the rate of evolution of the mtDNA control region is twofold greater than that of the cytochrome *b* gene. Population differentiation of cytochrome *b* haplotypes was similar to that found in the control region haplotypes (Table 4). The differences between the Bolivian Amazon rivers and Colombian Orinoco (ϕ_{ST} = 0.81; $P <$ .00001) and between the Bolivian Amazon rivers and Colombian Amazon (ϕ_{ST} = 0.91; $P <$ .00001) were substantially greater than between the two Colombian areas (ϕ_{ST} = 0.47; $P <$ .0001), although all values were highly significant (Table 4). The gene flow estimates confirmed a high degree of isolation of the three river dolphin populations (M = 0.28) (Table 4).

Phylogenetic Relationships of Haplotypes

Mitochondrial control region. The phylogenetic reconstructions of *Inia* sp. control region haplotypes recovered by the three different procedures showed identical topologies. The tree presented here is based on the Tamura and Nei (1993) genetic distance and the neighbor-joining algorithm (Figure 3). The topology of all obtained trees differentiated two clades with high bootstrap levels. One of the clades included all the haplotypes belonging to the Bolivian populations. The other clade included the populations of the Colombian Amazon and the Orinoco basin (Figure 3). The maximum parsimony trees with the branch and bound procedure and with a transition:transversion ratio of 1 until 20 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.

Cytochrome b gene. The phylogenetic relationships recovered among the cytochrome *b* haplotypes using different distances, maximum likelihood, and maximum parsimony were substantially similar to those obtained throughout the mitochondrial control region analysis. The Bolivian haplotypes formed a clade with high bootstrap values separated from the array established by the Orinoco and the Colombian–Brazilian Amazon haplotypes. The maximum parsimony tree yielded a tree length of 38 steps, with a consistency index of 0.95 and a retention index of 0.95. The striking relationship between the haplotypes from the Orinoco basin and the Colombian–Brazilian Amazon haplotypes for the cytochrome *b* gene is remarkable, and not in agreement with the possibility of two genetically divergent subspecies of *Inia* in these two different basins. If *I. g. geoffrensis* and *I. g. humboldtiana* are really two well-developed subspecies, then their divergence is relatively recent. Nevertheless, the sample sizes are too small to draw definite conclusions.

The geographical distribution of both the control region and cytochrome *b* haploytpes thus clearly revealed that each of the three geographical regions under study has its own distinct haplotypes. Nevertheless, in the case of the cytochrome *b* gene, there is a haplotype in the Brazilian Amazon (Brazilian-Amazon 1, accession no. AF304068) that was differentiated from only one Colombian Orinoco basin haplotype by 1 bp. The phylogenetic reconstruction for cytochrome *b* indicates that at least two events of genetic contact between the Orinoco and Amazon populations occurred. In addition, an autocorrelation index for DNA analysis (AIDA) spatial autocorrelation analysis, together with a variogram and an isolation-by-distance analysis within each geographic region, showed a clear significant spatial structure (results are presented elsewhere).

Discussion

For the first time, data were obtained about the molecular intra- and interpopulational variation for the mitochondrial control region and for the cytochrome *b* gene of the Amazon 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.

Variation of the Mitochondrial Control Region and Cytochrome *b* Sequences in *Inia* Among the Colombian Amazon and Orinoco Basin Dolphins

The estimated nucleotide diversity for the control region (1.99%) and for the cytochrome *b* gene (1.16%) for the population from the Orinoco basin indicates a considerable level of genetic diversity in this population. These values were from 9 to 12 times higher for the control region and from 2.5- to 39-fold higher for the cytochrome *b* gene compared to those found in the Bolivian and Colombian–Brazilian Amazon rivers. The nucleotide diversity for the control region reported for the Bolivian and Colombian Amazon (0.16% and 0.228%, respectively) and for cytochrome *b* (0.03% and 0.46%) proved to be similar to those values found in other regional cetacean populations (Baker et al. 1993; Hoelzel 1991; Pastene et al. 1996; Rosel et

al. 1994; Sechi et al. 1998). Therefore, the most baseline result found in the population from the Orinoco basin, under the neutrality model of Tajima (1989), is the elevated nucleotide diversity found in the *Inia* sp. populations of this river. This fact can be interpreted in several ways: First, several authors claim 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 population from the Orinoco basin could be the original area of distribution of *Inia*, which adds credence to the argument that the introduction of its ancestry inside South America could have been via the Atlantic Ocean. Second, another interpretation is that in the last 1.5–2 million years, different maternal lineages from different geographical origins could have migrated within the Orinoco river system. Third, this difference could also be due to the larger population size in the Orinoco than in the Colombian or Bolivian Amazon. In whichever event, this finding has important biological conservation implications. Among other things, it could mean that if the nuclear gene diversity in the dolphins of the Orinoco is in agreement with that found at the mitochondrial level, then this population could have a greater capacity for adaptation in front of feasible environmental changes than the other Amazon river dolphin populations studied.

There are no mtDNA haplotypes shared between the Orinoco and Amazon *Inia* in our sample, but the phylogenetic relationships of these haplotypes is paraphyletic with respect to these two regions. The haplotypes of the Bolivian *Inia*, however, are reciprocally monophyletic with respect to both the Orinoco and Amazon *Inia.*

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 (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 Orinoco 1 for the control region, which was genetically closer to the two Colombian Amazonian haplotypes than to the Colombian Orinoco haplotypes. With the cytochrome *b* gene, the haplotype Colombian Orinoco 1 is also genetically more related to the Colombian–Brazilian Amazon haplotypes than the remaining Colombian Orinoco haplotypes. This result could be evidence that a relatively recent gene flow between populations from the Amazon and the Orinoco 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 were based on morphological variables, are not fully supported by the molecular data. Moreover, several authors (Best and da Silva 1983, 1989a; Meade and Koehnken 1991) have reported individuals of this species in the Negro River and in the Casiquiari Channel. These regions have been considered by other authors to be a potential isolation factor between the Amazon and Orinoco basins. As commented earlier, our molecular data show that low gene flow connected the Amazon and Orinoco *I. geoffrensis* populations at some point in the past. Nevertheless, another alternative interpretation is possible. However, the absence of shared haplotypes suggests this gene flow is not recent or ongoing.

Therefore the molecular evidence of relatively recent contact among these two *Inia* populations is not strong counterevidence of different *I. geoffrensis* subspecies in the Orinoco and Amazon basins. Indeed, in the case of the D-loop tree, which has better resolution, the difference between the Orinoco basin and the Colombian Amazon is relatively clear. It also could suggest that some of the dolphins from the Orinoco basin are derived from an Amazon lineage. Nevertheless, a recent quantitative morphologic analysis of the same animals captured for the molecular genetics analysis (Ruiz-García et al. 2002) showed that there was no significant difference between males and females of both the Orinoco and Amazon basins according to a canonical population analysis. On the contrary, the males from the Colombian Amazon and Orinoco in particular were significantly different from the animals captured in the Bolivian rivers; this agrees quite well with the molecular data reported here. Therefore, the question about the existence of two *I. geoffrensis* subspecies in the Orinoco and Amazon basins remains open to additional studies with molecular nuclear markers and larger sample sizes from throughout the river basins.

It should be noted that only two haplotypes for the control region and three for the cytochrome *b* gene were detected in the Colombian Amazon (and for the Colombian–Brazilian Amazon in the case of the last gene). For the control region, one of these, the Colombian Amazon 2 haplotype, had a frequency of 92%. Clearly the most prominent genetic mechanism invoked to generate the high prevalence of this haplotype is a bottleneck, gene drift, and/or a founder effect. Perhaps this geographical area was recently colonized by *Inia*, and this haplotype would be considered as a founder introduced within this western Amazonian area by female colonizers. However, alternative scenarios are consistent with the molecular data. It is possible that *Inia* originated in the Amazon and that the Orinoco River was later colonized by the Lower Amazon populations, although at least two events of contact between these two populations happened, one in the Colombian Amazon and another in the Brazilian Amazon. Perhaps the high gene diversity in the Orinoco is not because this is the original distribution area of *Inia*, but rather because this population has expanded recently (cladogenesis), while the Amazon population remained a relatively constant size or had bottlenecks more recently.

Table 3. Net genetic divergence (D_A) among the populations of *Inia* studied

	Colombian Orinoco	Amazon	Bolivian Amazon
Colombian Orinoco		0.59%	2.49%
Amazon	2.50%		2.99%
Bolivian Amazon	6.53%	5.32%	

Above the diagonal is the genetic divergence for the cytochrome *b* gene (600 bp); below the diagonal is the genetic divergence for the mitochondrial control region (400 bp).

Genetic Diversity in the Overall *Inia* Population and Genetic Divergence Among *Inia* Populations

The overall nucleotide diversity found in *Inia* (2.8% control region) proved to be similar to humpbacks worldwide (2.4%), but was generally higher than values found in other cetacean species, such as the case reported by Rosel et al.

Table 4. Genetic heterogeneity and estimated female migrants per generation among the three *Inia* populations studied

	Colombian Orinoco	Amazon	Bolivian Amazon
ϕ_{ST}			
Colombian Orinoco	—	0.764*	0.917*
Amazon	0.430*	—	0.969*
Bolivian Amazon	0.810*	0.910*	—
Nm_f			
Colombian Orinoco	—	0.15397	0.04488
Amazon	0.28191	—	0.01568
Bolivian Amazon	0.05864	0.02472	—

Above the diagonal are the results for the mitochondrial control region; below the diagonal are the results for the cytochrome *b* gene. ϕ_{ST} = genetic heterogenity; Nm_f = female migrants per generation.
*$P < .0001$.

(1994) for several *Delphinus* sp. (2.1%), for *Delphinapterus leucas* (0.51%; O'Corry-Crowe et al. 1997), for the Chinese *Tursiops truncatus* (1.9%; Wang et al. 1999), for Ziphiidae such as *Hyperoodon* and *Berardius* (<2%; Dalebout et al. 1998), and 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 values for three species of *Phocoena* (0.39%; Rosel et al. 1995), for Pacific Ocean populations of *Phocoenoides dalli* (1.42%; Escorza-Treviño and Dizon 2000), or for the northwest Atlantic *Phocoena phocoena* populations (1.1%; Rosel et al. 1999). The mtDNA diversity of *Inia* sp. is strongly partitioned between the Colombian and Bolivian populations. The overall ϕ_{ST} value for the control region (ϕ_{ST} = 0.917) and the overall ϕ_{ST} value for the cytochrome *b* gene (ϕ_{ST} = 0.810) are elevated. These values are substantially higher than those found for other cetaceans, such as the cases of the *D. leucas* in North America (ϕ_{ST} = 0.409, control region; Brown Gladden et al. 1997), *P. dalli* in the north Pacific (ϕ_{ST} = 0.119, control region; Escorza-Treviño and Dizon 2000), *P. phocoena* in the northwest Atlantic Ocean (ϕ_{ST} = 0.034, control region; Rosel et al. 1999), or *Pontoporia blainvelli* in Rio Grande do Sul and Rio de Janeiro (ϕ_{ST} = 0.403, control region; Secchi et al. 1998), and in the Hector's dolphin (Pichler et al. 1998).

More striking are the values of control region divergence (D_A) calculated among the different *Inia* sp. populations compared with other species of cetaceans. The *Inia* values ranged from 6.53% (Bolivian Amazon versus Colombian Orinoco basin) to 2.50% (Colombian Amazon versus Colombian Orinoco basin). Needless to say, these *Inia* values were very high for the existence of a unique *Inia* species. The divergence of Bolivia from the Colombian Amazon and Orinoco basin (6.63% and 5.32%) was greater than previously calculated between several well accepted species of cetaceans (e.g., *Stenella attenuata* and *Stenella longirostris* [4%]; Dizon et al. 1991), some *Mesoplodon, Berardius* sp., and *Hyperoodon* sp. (4.7%; Dalebout et al. 1998), *Tursiops truncatus* and *Tursiops aduncus* (4.4%; Wang et al. 1999), and several *Delphinus* sp. (1.11%; Rosel et al. 1994). In addition, we have data for neotropical terrestrial mammals that agree quite well in the sense of the high levels of genetic heterogeneity discovered between the Bolivian and Colombian *Inia* sp. populations. This is the case between two Cervidae species of the genus *Mazama* for the same mitochondrial region (*M. americana* and *M. gouzaoubira*, divergence 2.1%; Ruiz-García et al. in press).

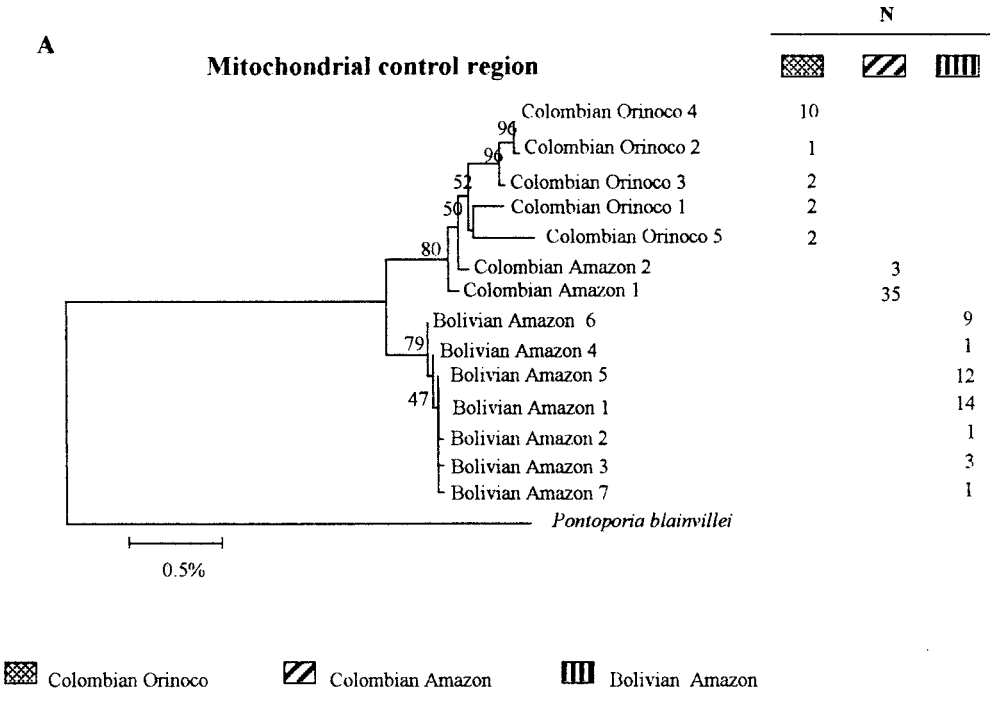
The marked divergence of mtDNA between the Colombian and Bolivian populations is consistent with previous morphological investigations. Da Silva (1994), analyzing specimens of the three main river systems, including a large number of specimens from the Brazilian Amazon, revealed that the populations of *Inia* were clearly separable into two different species, each well defined by cranial morphometrics. Our own biometric study with 12 morphological variables from the entire body of the captured animals revealed striking differences among the Bolivian dolphin set and the Colombian Amazon and Orinoco animal sets (Ruiz-García et al. 2002), particularly for the males. These morphological differences possibly justify species status for *I. geoffrensis* and *I. boliviensis*. Nevertheless, analyses of nuclear genetic markers are required until we have enough evidence that the Bolivian population is a fully developed, different species from the Amazon and Orinoco basin populations.

Therefore we propose to subdivide *Inia* into two evolutionarily significant units (ESUs), as defined by Moritz (1994). According to this definition, ESUs must be substantially reproductively isolated from other conspecific population units and must represent an important component in the evolutionary legacy of a given taxon. The mitochondrial data examined here seem to satisfy both conditions for the Bolivian river dolphin population in relation to the ones from the Amazon and Orinoco basins.

The Molecular Evolution of *Inia*

A stretch of 400 km of rapids in the upper Madeira-Mamoré River almost certainly forms a barrier to the movement of Bolivian *Inia*. As a result, allopatric separation could be a reasonable explanation that supports the results obtained in this study. In agreement with Pilleri et al. (1984), the formation of these two species could be at the end of the Pliocene and the beginning of the Pleistocene, since the Beni lands in Bolivia were once a lake completely isolated from other water sources during the Neogene (Grabert 1984). In the Holocene–Pleistocene, this lake was tapped and the 400 km of the Madeira-Mamoré rapids were formed. It is possible that *Inia* reached this area via the Abuña pass, which is present today, before the formation of the rapids between Guayara-Mirim and Porto Velho (Grabert 1967; Pilleri et al. 1984). Therefore, the isolation of Bolivian *Inia* could have occurred during the Pleistocene, approximately 5 million years ago, when the Andes mountains were formed (Grabert 1984). This is supported by the fact that in this period the current geomorphology of the Andes was attained (Lundberg et al. 1998). This moment could be decisive for the allopatric isolation of Bolivian *Inia* with regard to the Amazon and Orinoco basin populations.

The current controversy with respect to divergence times within the genus *Inia*, and of this genus with other related taxa, can be resolved partially with the results presented here. The rate of control region divergence in cetaceans has been estimated to be approximately 1%/million years for both Odonotocetes and Mysticetes (Baker et al. 1993; Hoelzel and Dover 1991). These estimates are consistent with several more recent studies. Escorza-Treviño and Dizon (2000), based on the fact that Barnes (1985) estimated a separation of 5–8 million



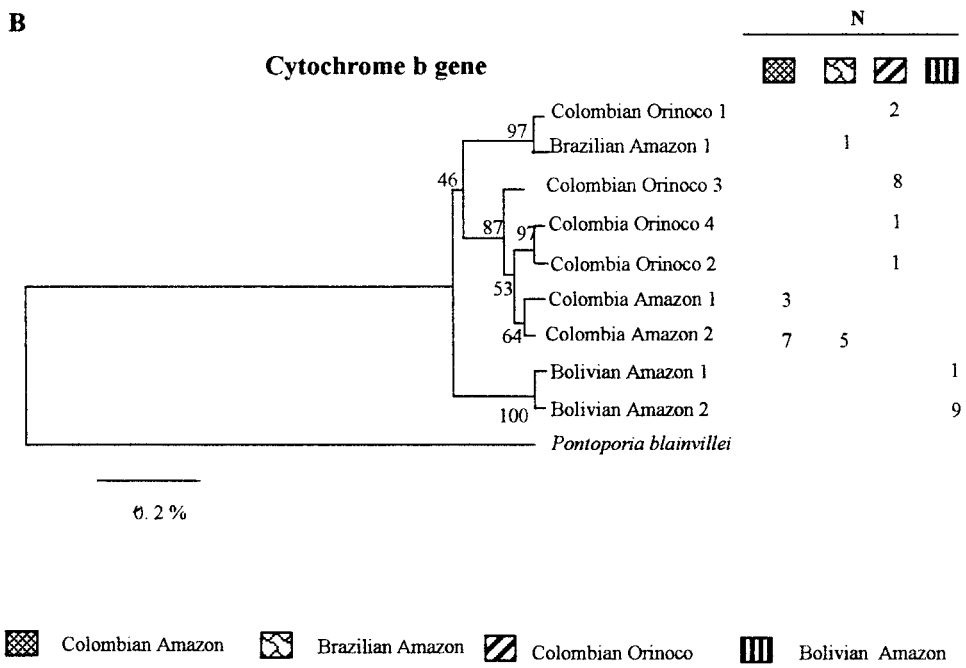


Figure 3. **(A)** Phylogeny of the mtDNA control region using the neighbor-joining algorithm and the Tamura and Nei (1993) genetic distance with $\alpha = 0.5$ among the different *Inia* haplotypes. The numbers on the tree nodes are the bootstrap percentages based on 1,000 simulations. **(B)** Phylogeny of the cytochrome *b* gene using the neighbor-joining algorithm and the Tamura and Nei (1993) genetic distance with $\alpha = 0.5$ among the different *Inia* haplotypes. The numbers on the tree nodes are the bootstrap percentages greater than 50% based on 1000 simulations. The scale shows the inferred proportion of nucleotide changes along each branch based on Tamura and Nei genetic distances.

years among *Phocoenoides* and *Phocoena* using the fossil record, estimated a ratio of 0.71–1.14%/million years for the mitochondrial control region for the first genus quoted. Similarly, McMillan and Bermingham (1996) estimated a rate of evolution of 0.94%/million years for the control region in that genus. Tikel (1997) estimated 2%/million years based on fossil evidence for the dugong and Garcia-Rodriguez et al. (1998) showed that the most feasible molecular clock for the manatee (*Trichechus manatus*) was 1–2%/million years. Even feline-specific mtDNA divergence rates vary from 0.97% to 1.39%/million years (Lopez et al. 1997). Assuming a 1% divergence rate for the control region, we obtained an estimate of

5.3–6.5 million years of divergence between both *Inia* populations for the 400 bp studied. If the 2% divergence ratio is employed, the estimate is 2.6–3.2 million years. For the cytochrome *b* gene, Irwin et al. (1991) showed that the divergence per million years is about 0.5%. Taking this value, the evolutionary divergence between Bolivian *Inia* and Colombian and Amazonian *Inia* could be about 5–6 million years. The average value for both mitochondrial regions (taking 1% and 0.5% of divergence, respectively) is about 5.1–6.2 million years. This is quite similar to the 5 million years of separation between Bolivian *Inia* and Colombian and Amazonian *Inia* claimed by Grabert (1984) and da Silva (1994). These temporal estimates are of the same magnitude as those calculated for the three mtDNA lineages discovered in *T. manatus* by García-Rodriguez et al. (1998) (3.5–7 million years).

The most ancient fossil, which was recovered from the Iniidae family and dated at 7–8 million years old, was proposed to belong 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 estimate of 11–13 million years old; Cozzuol 1996; de Muizon 1988). This family is proposed to be the sister taxon of the Iniidae. Assuming this, and taking into account that the nucleotide divergence with the Tajima and Nei distance between *Inia* and *Pontoporia* is 8.8% for cytochrome *b*, it is possible to obtain several other time-divergence estimates. If a ratio ranging from 0.5% to 1%/million years is assumed, then the separation time between *Inia* and *Pontoporia* could range from 8.8 to 17.6 million years, which agrees with the 11–13 million years established for *Brachydelphis* (de Muizon 1988). Nevertheless, the rates of mitochondrial evolution could be different among different taxa. Based on the previous considerations, we suggest an urgent need to review the taxonomy of *Inia* in order to confirm the proposal of at least two extant evolutionarily units, which could reach species status: *I. boliviensis* in Bolivia and a unique *I. geoffrensis* species in the Orinoco and Amazon rivers. This will require some nuclear gene markers and a more exhaustive geographic mtDNA sample from the Amazon.

Within the Amazon and Orinoco basins, future study is also needed to investigate allele frequency differences between males and females. In other cetacean species, comparisons of nuclear and mtDNA diversity suggest different patterns of gene flow for males and females. In the North American beluga whales, for example, the ϕ_{ST} values (0.047–0.072) for microsatellites were significantly lower than the ϕ_{ST} value obtained for the mitochondrial control region (0.409) (Brown Gladden et al. 1999). Even the geographical break in microsatellite allele frequency did not coincide with the break in mtDNA haplotype distribution of this species in the Canadian Arctic islands. Similarly Wang et al. (1996) found that female porpoises were more phylopatric than males and Escorza-Treviño and Dizon (2000) showed a highly male-biased dispersal among *P. dalli* populations. Such studies will aid in delineating further genetic units within the extensive range of the Amazon and Colombian *Inia*.

References

Avise JC, 1989. A role for molecular genetics in the recognition and conservation of endangered species. Trends Ecol Evol 4:279–281.

Baker CS, Perry A, Bannister JL, Weinrich MT, Abernethy RB, Calambokidis J, Lien J, Lambertsen RH, Urbán Ramírez J, Vasquez O, Clapham PJ, Alling A, O'Brien SJ, and Palumbi SR, 1993. Abundant mitochondrial DNA variation and world-wide population structure in humpback whales. Proc Natl Acad Sci USA 90:8239–8243.

Barnes LG, 1985. Evolution, taxonomy and antitropical distribution of the porpoises (Phocoenidae, Mammalia). Mar Mamm Sci 1:149–165.

Best RC and da Silva V, 1983. Distribution of freshwater dolphins and manatees in the upper Rios Negro and Orinoco [abstract]. Presented at the 5th Biennial Conference on the Biology of Marine Mammals, Boston; 17–21.

Best RC and da Silva VMF, 1989a. Amazon river dolphin, Boto. *Inia geoffrensis*, de Blainville, 1817. In: Handbook of marine mammals. London: Academic Press; 1–23.

Best RC and da Silva VMF, 1989b. Biology, status and conservation of *Inia geoffrensis* in the Amazon and Orinoco river basins. Occasional paper SSC 3. Geneva, Switzerland: IUCN Species Survival Commission; 23–33.

Brown Gladden JG, Ferguson MM, and Clayton JW, 1997. Matriarchal genetic population structure of North American beluga whales *Delphinapterus leucas* (Cetacea: Monodontidae). Mol Ecol 6:1033–1046.

Brown Gladden JG, Ferguson MM, Friesen MK, and Clayton JW, 1999. Population structure of North American beluga whales (*Delphinapterus leucas*) based on nuclear DNA microsatellite variation and contrasted with the population structure revealed by mitochondrial DNA variation. Mol Ecol 8:347–363.

Casinos A and Ocaña J, 1979. A craniometrical study of the genus *Inia* D'Orbigny, 1834, Cetacea, Platanistoidea. Saugetierkunde Mitteilung 27:194–206.

Cozzuol MA, 1996. Contributions of southern South America to vertebrate paleontology. Munchber Geowissenschaftliche Abhandlungen 30:321–342.

Dalebout ML, Van Helden A, Van Waerebeek K, and Baker CS, 1998. Molecular genetic identification of southern hemisphere beaked whales, Cetacea:Ziphiidae. Mol Ecol 7:687–694.

da Silva V, 1994. Aspects of the biology of the Amazonian dolphin genus *Inia* and *Sotalia fluviatilis* (PhD dissertation). Cambridge: Cambridge University.

De Muizon CH, 1988. Les vértebrés fossiles de la Formation Pisco, Pérou. Troisième partie: les Odontocètes, Cetacea, Mammalia) du Miocène. Travaux Institut Francais D'Etudes Andines, Paris, Éditions Recherche sur les Civilisations, Memoire 78:1–244.

Dizon AE, Southern SO, and Perrin WF, 1991. Molecular analysis of mtDNA types in exploited populations of spinner dolphins *Stenella longirostris*. Final Report to the International Whaling Commission 3:83–202.

Dobzhansky TH, 1971. Evolutionary oscillations in *Drosophila pseudoobscura*. In: Ecological genetics and evolution. Oxford: Blackwell Scientific; 109–133.

Escorza-Treviño S and Dizon AE, 2000. Phylogeography, intraspecific structure and sex-biased dispersal of Dall's porpoise, *Phocoenoides dalli*, revealed by mitochondrial and microsatellite DNA analyses. Mol Ecol 9:1049–1060.

Excoffier L, Smouse PE, and Quattro JM, 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Fordyce RE and Barnes LG, 1994. The evolutionary history of whales and dolphins. Annu Rev Earth Planet Sci 22:419–455.

Fordyce RE, Barnes LG, and Miyazaki N, 1994. General aspects of the evolutionary history of whales and dolphins. Island Arc 3:373–391.

García-Rodriguez AI, Bowen BW, Domming D, Mignucci-Giannoni A, Marmontel M, Montoya-Ospina RA, Morales-Vela B, Rudin M, Bonde RK, and McGuires PM, 1998. Phylogeography of the West Indian manatee (*Trichechus manatus*): how many populations and how many taxa? Mol Ecol 7:1137–1149.

Grabert H, 1967. Sobre o desaguamento natural do sistema fluvial do Rio Madeira desde a construção dos Andes. Biota Amazon 1:209–214.

Grabert H, 1984. Migration and speciation of the South American Iniidae, Cetacea-mammalia. Zeitschrift Saugetierkunde 49:334–341.

Hasegawa M, Kishino H, and Yano T, 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 21:160–174.

Hoelzel AR, 1991. Analysis of regional mitochondrial DNA variation in the killer whale: implications for cetacean conservation. Report to the International Whaling Commission, special issue 13:81–120.

Hoelzel AR and Dover GA, 1991. Evolution of the cetacean mitochondrial D-loop region. Mol Biol Evol 8:475–493.

Irwin DM, Kocher TD, and Wilson AC, 1991. Evolution of the cytochrome *b* gene of mammals. J Mol Evol 32:128–144.

Jukes TH and Cantor CR, 1969. Evolution of protein molecules. In: Mammalian protein metabolism (Munro HN, ed). New York: Academic Press.

Kimura M, 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:11–120.

Kishino H and Hasegawa M, 1989. Evolution of the maximum likelihood estimate of the evolutionary tree topology from DNA sequence data and the branching order in Hominidae. J Mol Evol 29:170–179.

Kumar S, Tamura K, and Nei M, 1993. MEGA: molecular evolutionary genetics analysis, version 1.01. University Park: Pennsylvania State University.

Lopez JV, Culver M, Stephens JC, Jonson WE, and O'Brien SJ, 1997. Rates of nuclear and cytoplasmatic

mitochondrial DNA sequence divergence in mammals. Mol Biol Evol 14:277–286.

Lundberg J, Marshall L, Guerrero J, Horton B, Malabarba C, and Wesselingh F, 1998. The stage for neotropical fish diversification: history of a tropical South American river. In: Phylogeny and classification of neotropical fishes (Malabarba LR, Reis R, Vari R, Lucena Z, and Lucena CA, eds). Porto Alegre, Brasil: Pontificia Universidade Católica Do Rio Grande Do Sul; 13–48.

McMillan WO and Bermingham E, 1996. The phylogeographic pattern of mitochondrial DNA variation in the Dall's porpoise *Phocoenoides dalli.* Mol Ecol 5: 47–61.

Meade RH and Koehnken L, 1991. Distribution of the river dolphin, tonina *Inia geoffrensis* in the Orinoco River basin of Venezuela and Colombia. Interciencia 16:300–312.

Medrano-Gonzalez L, Aguayo-Lobo A, Urban Ramirez J, and Baker CS, 1995. Diversity and distribution of mitochondrial DNA lineages among humpback whales, *Megaptera novaeangliae*, en the Mexican Pacific Ocean. Can J Zool 73:1735–1743.

Messenger SL and McGuire JA, 1998. Morphology, molecules and the phylogeny of cetaceans. Syst Biol 47:90–124.

Moritz C, 1994. Defining ''evolutionarily significant units'' for conservation. Trends Ecol Evol 9:373–375.

Nei M, 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590.

O'Corry-Crowe GM, Sudam RS, Rosenberg A, Frost KJ, and Dizon AE, 1997. Phylogeography, population structure and dispersal patterns of the beluga whale *Delphinapterus leucas* in the western Nearctic revealed by mitochondrial DNA. Mol Ecol 6:955–970.

Palumbi S, Martin A, Romano S, and McMillan WO, 1991. The simple fool's guide to PCR. Honolulu: University of Hawaii.

Pastene LA, Hiroshi H, Kazuo W, Yoshitaka B, and Mutsuo G, 1996. Phylogenetic relationship in the minke whale world-wide as revealed by two independent analyses of mitochondrial DNA. Resúmenes 7ma Reunión de Especialistas en Mamíferos Marinos Acuáticos de América del Sur y primer congreso de la sociedad latinoamericana de especialistas en mamíferos acúaticos, Viña del Mar, Chile; 53.

Pichler F, Dawson S, Slooten E, and Baker CS, 1998. Geographic isolation of Hector's dolphin populations described by mitochondrial DNA sequences. Conserv Biol 12:676–682.

Pilleri G and Gihr M, 1977. Observations on the Bolivian, *Inia boliviensis,* (D'Orbigny, 1834) and the Amazonian bufeo, *Inia geoffrensis* (Blainville 1817), with a description of a new subspecies (*Inia geoffrensis humboldtiana*). Invest Cetacea 8:11–76.

Pilleri G, Marcuzzi G, and Pilleri O, 1984. Speciation in the Platanistoidea: systematic, zoogeographical and ecological observations on recent species. Invest Cetacea 14:15–46.

Rice DW, 1998. Marine mammals of the world. Systematics and distribution. Special publication no 4. Lawrence, KS: Society for Marine Mammalogy.

Rosel PE, Dizon AE, and Haygood M, 1995. Variability of the mitochondrial control region in population of the harbour porpoise, *Phocoena phocoena,* on interoceanic and regional scales. Can J Fish Aquat Sci 52:1210–1219.

Rosel PE, Dizon AE, and Heyning JE, 1994. Genetic analysis of sympatric morphotypes of common dolphins, genus *Delphinus.* Mar Biol 119:159–167.

Rosel PE, France SC, Wang JY, and Kocher TD, 1999. Genetic structure of harbour porpoise *Phocoena phocoena* populations in the northwest Atlantic based on mitochondrial and nuclear markers. Mol Ecol 8:S41–S54.

Rozas J and Rozas R, 1995. DnaSP: DNA sequence polymorphism an interactive program for simulating population genetics parameters from DNA sequence data. Comput Applic Biosci 11:621–625.

Ruiz-García M, Banguera-Hinestroza E, Cárdenas H, and García F, 2002. Morphological analysis of three *Inia* (Cetacea: Iniidae) populations from Colombia and Bolivia. J Mammal (submitted).

Ruiz-García M, Randi E, Martinez-Agüero M, and Alvarez D, in press. Relaciones filogenéticas entre diversos géneros de ciervos neotropicales (Familia Cervidae, Orden Artiodactyla) mediante secuenciación de la región mitocondrial D-loop y marcadores microsatélites Revista de Biología Tropical. Int J Trop Conserv Biol.

Saitou N and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Schneider S, Roessli D, and Excoffier L, 2000. ARLEQUIN version 2000: a software for population genetic data analysis. Geneva, Switzerland: University of Geneva.

Sechi E, Wang J, Murray B, Rocha-Campos C, and White B, 1998. Population differentiation in the Franciscana, (*Pontoporia blainvillei*), from two geographic locations in Brazil as determined from mitochondrial DNA control region sequences. Can J Zool 76:1622–1627.

Swofford DL, 1998. PAUP* version 4b. Phylogenetic analysis using parsimony computer program. Champaign, IL: Illinois Natural Survey.

Tajima F, 1989. Statistical methods for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tajima F and Nei M, 1984. Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol 1:269–285.

Tamura K and Nei M, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG, 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882.

Tikel D, 1997. Using a genetic approach to optimise Dugong (*Dugong dugon*) conservation management (PhD dissertation). North Queensland, Australia: James Cook University.

Van Bree PJ and Robineau D, 1973. Notes sur les holotypes de *Inia geoffrensis geoffrensis* (Blainville, 1817) et *Inia geoffrensis boliviensis* (Dórbigny 1834), Cetacea, Platanistidae. Mammalia 37:659–663.

Wang JY, Chou LS, and White BN, 1999. Mitochondrial DNA analysis of sympatric morphotypes of bottlenose dolphins, genus: *Tursiops* in Chinese waters. Mol Ecol 8:1593–1602.

Wang JY, Gaskin DE, and White BN, 1996. Mitochondrial DNA analysis of harbour porpoise, *Phocaena phocaena*, subpopulations in North American waters. Can J Fish Aquat Sci 53:1632–1645.

Received June 1, 2001
Accepted August 8, 2002

Corresponding Editor: C. Scott Baker