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Chapter 6

Molecular Differentiation among *Inia geoffrensis* and *Inia boliviensis* (Iniidae, Cetacea) by Means of Nuclear Intron Sequences

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We analyzed autosomal (Actine, CAT, CHRNA, GBA, IFN and Lactoalbumine) and Y-chromosome (DY8S, SmyS7, DY7S, and UBEY7) intron sequences from *Inia boliviensis* (Bolivia) and *Inia geoffrenis* (Peru and Colombia) captured from the Amazon and Orinoco watersheds of South America. The low level of genetic diversity we report, considering the number of base pairs sequenced and supported by as few as six nucleotide substitutions in some samples, is in disagreement with mitochondrial (control region and cyt-b) studies of these same populations. Although there are several possible explanations for this low diversity we suggest it may be due a homogenous aquatic habitat and the affect of constrictive natural selection on the *Inia*'s nuclear genome or, more probably, to a more recent temporal divergence (Pleistocene) between the pink river dolphin populations than had been considered previously. Nevertheless, relative genetic differentiation statistics combined with phylogenetic procedures with the polymorphic sequence introns revealed almost complete isolation of the Bolivian and Amazon-Orinoco populations. Furthermore, most of the G_{ST}, F_{ST}

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and N_{ST} values were equal to 1 and respective gene flow estimates were equal to 0, supporting complete genetic isolation. Therefore, these molecular data provide additional evidence that the Bolivian *Inia* population is a different and significant evolutionary unit.

Keywords: *Nuclear intron sequences, molecular phylogenetics, natural selection, recent temporal divergence, Inia boliviensis, Inia geoffrensis.*

Introduction

The taxonomic classification of the pink river dolphin (*Inia*), an endemic species of the Orinoco and Amazon river basins, has been problematic at the inter and intragenetic levels (Pilleri and Ghir, 1977; Casinos and Ocaña, 1979; da Silva, 1994; Hamilton et al., 2001; Banguera et al., 2002) ever since the formal inception of the taxon's name by Geoffroy St. Hilarie. The traditional classification (Best and da Silva, 1993) considers the genus to be monospecific (*Inia geoffrensis*) with three subspecies (*I. g. boliviensis*, *I. g. geoffrensis* and *I. g. humboldtiana*) that are distributed in the Bolivian Amazon, non-Bolivian Amazon and Orinoco basins respectively. However, Pilleri and Gihr's (1977) morphological analysis led them to suggest that the Bolivian form was a different species (*I. boliviensis*). Alternatively, Casinos and Ocaña (1979) claimed that the morphological differentiation among the forms of this species was typical of a clinal distribution of one species. Additional morphometric and morphological evidence in support of the existence of two species was presented by Da Silva (1994) who noted that unlike in the Amazon, Bolivian dolphins did not possess a third occipital condyle. Unfortunately, her conclusions were biased due to the small number of exemplars analyzed from Bolivia, some of which were largely incomplete. Hamilton et al., (2001) analyzed the complete cytochrome b and partial 12S and 16S rRNA mitochondrial sequences as an attempt to resolve the phylogeny question regarding the different river dolphin species. Their attempt was marginally successful as they provided some evidence that supported specific differences between *I. geoffrensis* and *I. boliviensis*.

Nevertheless, Banguera et al.'s (2002) molecular genetics paper provided additional and stronger evidence that the Bolivian taxon was a different species. For the two mitochondrial genes studied (control region and Cyt-b), the Bolivian form did not share any haplotype with the other populations (one from the Orinoco and other from the western upper Amazon in Colombia) and presented a significant and extremely high genetic heterogeneity in comparison to the other two populations. The Bolivian form was monophyletic and separate from the other *Inia* clusters within all of the phylogenetic trees obtained. In this paper we provide new molecular evidence (sequences of ten autosomic and Y chromosome introns) that tests if the Bolivian population is a different evolutionary clade and determine if the molecular evolution of nuclear and Y chromosome sequences are as substantial as that of the two mitochondrial genes reported by Banguera et al.'s (2002).

Materials and Methods

Samples

The river dolphins sequenced within this study comprised only a small fraction of those captured during four expeditions (2002-2005) within the Orinoco (Colombia: Orinoco, Guaviare, Inirida, and Arauca rivers) and Amazon river basins (Bolivian Amazon: Mamoré, Iruyañez, Guaporé, Ipurupuru and Tijamuchí rivers; other Amazon areas in Peru, Ecuador, Brazil and Colombia: Amazon, Ucayali, Tapiche, Puinahuva channel, Marañón, Samiria, Napo, Curaray, Putumayo, Javarí and Negro rivers). We performed a caudal fin biopsy for each of the 220 dolphins captured. Several of these animals were sequenced for four Y chromosome introns (UBEIY7, SMY7, DBY7 and DBY8; Hellborg and Ellegren, 2003) and for six autosomic introns (Lactalbumin gene, Actin, CAT, IFN, GBA and CHRNA1; Palumbi and Baker, 1994, Milinkovitch et al., 1998, Lyons et al., 1997). The Y chromosome intron samples were analyzed as follows. For UBEIY7, 8 exemplars were sequenced; one from the Orinoco river, four from Perú (one from Marañón river and three from the Curaray River), two from Colombia (Putumayo river), and one from Bolivia (Mamoré river). For SMY7, 13 individuals were sequenced, two from the Orinoco River, six from Perú (Ucayali, Marañón and Curaray rivers) and five from Bolivia (Mamoré and Iruyañez rivers). For DBY8, 7 exemplars were sequenced, two Peruvian individuals (Marañón and Curaray rivers) and five Bolivian ones (Mamoré and Iruyañez rivers). Only four animals were sequenced for each nuclear autosome intron, two from Peru (Curaray river) and two from Bolivia (Mamoré river).

Molecular Markers

DNA was extracted by means of the Phenol-Chloroform procedure (Sambrock et al., 1989) and modified for small samples (Baker et al. 1993). All the samples showed high DNA quality. For the first Lactalbumin intron (602 bp), the total PCR volume was 30 µl, with 19.6 µl of H_2O, 3 µl of Buffer, 2 µl of BSA, 3 µl of $MgCl_2$, 0.3 µl of dNTPs, 1 µl of both primers (Forward: Lac2F: 5'-CCAAAATGATGTCCTTTGTC-3', Reverse Lac1: 5'-CTCACTGTCACAGGAGATGT-3') and 0.1 µl AmpliTaq. The PCR profile for this marker consisted of 2 minutes at 94ºC (first step), 31 cycles at 94ºC for 15 seconds (second step), 15 seconds at 54 ºC, 30 seconds at 72 ºC, and a final extension for 10 minutes at 72ºC. The total PCR volume for the first Actin intron (1039 bp) was 30 µl, with 19. 6 µl of H_2O, 3 µl of Buffer, 2 µl of BSA, 3 µl of $MgCl_2$, 0.3 µl of dNTPs, 1 µl of both primers (Act 1385: 5'-CTTGTGAACTGATTACAGTCC-3'; Act 3: 5'-GGTTATCTGATGTATTCC-3') and 0.1 µl AmpliTaq. The PCR profile for this marker consisted of 2 minutes at 94ºC (first step), 34 cycles at 94ºC for 30 seconds, 40 seconds at 55ºC, 1 minute at 72ºC and a final extension of 10 minutes at 72ºC. The total PCR volumes for the CAT (Catalasa; 580 bp), IFN (Interferon; 372 bp), GBA (Glucocerebrosidasa; 245 bp) introns were each 30 µl, with 14. 6 µl of H2O, 2.5 µl of Buffer, 2 µl of BSA, 3.5 µl of MgCl2, 0.3 µl of dNTPs, 1 µl of both primers 10 µM (Forward: CAT-F: 5'-AAAGACTGACCAGGGCATCA-3', Reverse: CAT-R:5'-AGGGGTAGTCCTTGTGAGGCC-3';Forward: IFN-F: 5'-TTCTCCTGCCTGAAGGACAG-

3', Reverse: IFN-R: 5'-GGATCTCATGATTTCTGCTCTGAC-3'; Forward: GBA-F :5'-AAAAGCTTCGGCTACAGCTC-3',Reverse: GBA-R: 5'-TCCCTTCACTTTC TGGAA CTTC-3', respectively) and 0.1 µl AmpliTaq. The PCR profile for these markers consisted of a first step of 12 minutes at 94ºC, 35 cycles at 94ºC for 30 seconds, 45 seconds at 55ºC and 30 seconds at 72ºC, and a final extension of 10 minutes at 72ºC. For the CHRNA1 (cholinergic receptor Nicotine alpha polypeptide; 361 bp) intron, the total PCR volume was 30 µl, with 14. 6 µl of H2O, 2.5 µl of Buffer, 2 µl of BSA, 3.5 µl of MgCl2, 0.3 µl of dNTPs, 1 µl of both primers 10 µM (Forward: CHRNA1-F: 5'-GACCATGAAGTCAGACCAGGAG-3', Reverse: CHRNA1-R: 5'-GGAGTATGTGGTCCATCACCAT-3') and 0.1 µl GoldTaq. The PCR profile for CHRNA1 began with 12 minutes at 94ºC, followed by 10 cycles for 20 seconds at 94ºC, 20 seconds at 64-55ºC and decreasing the temperature from 72ºC over 40 seconds and 10 cycles. This marker required a third step of 30 cycles at 94ºC for 20 seconds, 55ºC for 20 seconds, 72ºC for 40 seconds and a final extension of 72ºC for 10 minutes. For the four Y chromosome introns (UBEIY7 (457 bp), SMY7 (465 bp), DBY7 (272 bp) and DBY8 (151 bp)), the total PCR volume was 30 µl, with 14.6 µl of H2O, 2.5 µl of Buffer, 2 µl of BSA, 3.5 µl of MgCl2, 0.3 µl of dNTPs, 1 µl of both primers 10 µM (Forward: UBE1Y7-F: 5'-tggctctgcctttsttyagc-3', Reverse: UBE1Y7-R: 5'-aggttgtatgccytgyaca-3'; Forward: SMY7-F: 5'-tggaggtgcccraartgta-3', Reverse: SMY7-R: 5'-aactctgcaaastrtactcct-3'; Forward: DBY7-F: 5'-ggtccaggagargctttgaa-3', Reverse: DBY7-R: 5'-cagccaattctcttgttggg-3'; Forward: DBY8-F: 5'-ccccaacaagagaattggct-3', Reverse: DBY8-R: 5'-cagcaccaccatakactaca-3', respectively) and 0.1 µl GoldTaq. The PCR profiles for the SMY7, DBY7 and DBY8 introns were the same and each consisted of a first step of 95ºC for 12 min, a second step of 20 cycles at 94ºC for 30 seconds, 60-50ºC for 1 minute decreasing temperature during 20 cycles and a 0.5 ºC per cycle decrease over 1.5 minutes from a start temperature of 72ºC. A third step was required of 20 cycles at 95ºC for 30 seconds, 55ºC for 1 minute and 72ºC for 1.5 minutes. The last step was a final extension of 72ºC for 10 minutes. The first step of the UBE1Y7's PCR profile was 95ºC for 12 min, followed by a second step of 20 cycles at 94ºC for 30 seconds, 55-45ºC for 1 minute decreasing 0.5 ºC per cycles for 20 cycles and 72ºC for 1.5 minutes. A third step was required of 20 cycles at 95ºC for 30 seconds, 45ºC for 1 minute and 72ºC for 1.5 minutes. The last step was an extension of 72ºC for 10 minutes. Therefore, a total of 4,544 bp of the ten introns were analyzed for two Peruvian and two Bolivian dolphins. The excess primers and nucleotides were removed from the PCR product using SAP (shrimp alkaline phosphatase) and ExoI (exonuclease I) (USB). The PCR products were sequenced in both directions using the Big Dye™ kit in an ABI 3100 sequencer.

Molecular Population Statistic Analysis

The sequence alignment was carried out with the ClustalX, and Phred v.020425 programs (Ewing and Green, 1998, Ewing et al., 1998) and was visually inspected. Several indels (insertions-deletions) of some of the analyzed introns (DBY8, UBEIY7, CAT and Actin) had informative phylogenetic signals. When it was possible, these insertions-deletions were included in the statistical analyses. The first analyses performed were those that

described the genetic variability of the sequences analyzed globally and within each population studied as well as for possible demographic changes. These statistics were the number of haplotypes, haplotype diversity (h, with standard deviation, SD), nucleotide diversity (π; with SD), π with Jukes and Cantor correction, average number of nucleotide differences (k, with stochastic, sampling and total variance assuming free and no recombination), raggedness (Harpending, 1994), Ramos-Onsins and Rozas R2 (2002), τ and different estimates of θ (= $4Ne\mu$) per site from π, from S (number of polymorphisms), from Eta (total number of mutations with SD for free and no recombination) and per sequence from S. A second set of analyses was performed to calculate genetic differentiation and gene flow estimates among the diverse populations analyzed. The statistics within this group were D_{xy} (average number of nucleotide differences per site between populations with and without the Jukes and Cantor correction), D_a (number of net substitutions per site between populations with and without the Jukes and Cantor correction), a chi-square contingency analysis, H_s, H_{st}, K_s, K_{st}, K^*_s, K^*_{st}, Z_s, Z^*_s , Snn (Hudson, 2000), (with the probability obtained by a permutation test with 1000 replicates), G_{ST} (and its respective gene flow estimate, Nm from haplotype information; Nei, 1973), Δ_{ST} and γ_{ST} (and its respective gene flow estimate, Nm from sequence information; Nei, 1982), N_{ST} with the Jukes and Cantor correction (and its respective gene flow estimate, Nm from sequence information; Lynch and Crease, 1990), F_{ST} (and its respective gene flow estimate, Nm from sequence information; Hudson et al., 1992a, b) and ϕ_{ST} (AMOVA; Excoffier et al., 1992). A third set of analyses was applied to determine possible linkage disequilibrium and recombination parameters in the polymorphic sequences. The Fisher´s exact and the chi-square tests as well as the D, D', r, ZnS (Kelly, 1997), Za, ZZ (Rozas et al., 2001) and the Wall's B and Q statistics were carried out to detect possible linkage disequilibrium, while Rm (minimum number of recombination events, Hudson and Kaplan, 1985) and R (estimation of the recombination parameter; Hudson, 1987) were obtained to measure the importance of the recombination. A fourth analysis was applied to detect some evidence of natural selection affecting the sequences analyzed. For this task, different versions of the Fu-Li (1993)'s test and the Tajima (1989)´s D test were carried out. Finally, we determined the phylogenetic relationships among the dolphins from Bolivia, Amazon and Orinoco, by using the following genetic distances: p-distance (Nei and Kumar, 2000), Kimura 2 parameters (Kimura, 1980), Jukes- Cantor (Jukes and Cantor, 1969), Tajima and Nei (Tajima and Nei, 1984), Tamura 3 parameters (Tamura, 1992), Tamura and Nei (Tamura and Nei, 1993), Log det (Nei and Kumar, 2000). When it was possible, we included homogeneous and heterogeneous evolutionary rate patterns among lineages. We also utilized different strategies in regards to the rates among sites (uniform or different -gamma distributed-). The phylogenetic trees were obtained through the algorithms UPGMA (Sokal and Michener, 1958), neighbor-joining (Saitou and Nei, 1987), and minimum-evolution (Rzhetsky and Nei, 1992, 1993) with 500 bootstraps. These analyses were completed with the DNASP 4.1, MEGA 3.1 and ARLEQUIN 2.0 programs.

Results

The sequences obtained for the Peruvian and Bolivian animals for 10 nuclear introns are shown in Figure 1.

DY8S

Inia boliviensis

GAGACCGGTATGAAAACTACAAGCAATAATTTATTGTTTATAAAAACATCACTA
GAATCAATGAAAAAGGTAATAATCAGTAAAAATGCATATTTACTTTTCTGGCTT
CTTCATA-GATCTGTACAGCCAATTCTCTTGTTGGGG-AAAAA

Inia geoffrensis (Peru)

TAGAGGGGTATGAAAACTACAAGCAATAATTTATTGTTTATAAAAACATCACTA
GAATCAATGAAAAAGGTAATAATCAGTAAAAATGCATATTTACTTTTCTGGCTT
CTTCATA-GATCTGTACAGCCAATTCTCTTGTTGGGG-AAAAA

SMY7S

Inia boliviensis

TACTCCTGGGTAGCCTGCTCAAAGCCAAAGGCTTCCGGGGGTCTCTTACACTCCT
GAAATAGAAAATATTTCAGTAAGACAGAGGCAATAGACAACAAACCCCTTCAA
AACACCAGCTAGAGATATATCTTATGCTATTCCTCCACTTTAATCTTCCAGGTAC
AATAGTTTCTTTAATATCTACTAAACTTATTTCCTTTTGGTTCCTTTGGTTCACAT
CATCTGGATTACAGTCCTTCCTGTCTGTCTTAAATTTCGACCATCCTACAGCTACT
CTACCCAACCATTCTCTCATTTACTGTGTCACTGATAGCTAATTATAACCTAACA
AAAGTTATTAACTTAGAAAAATGGAGGGACCTACTTTACTTTTCCCTAAAACCA
GGAGTCTGTATTAAAAACAATTACCAAGAACTAAATTTAAAACATCTATGGTAG
ACAGCCCTTACCGCCATGATACACTTC

Inia geoffrensis (Peru)

TACTCCTGGGTAGCCTGCTCAAAGCCAAAGGCTTCCGGGGGTCTCTTACACTCCT
GAAATAGAAAATATTTCAGTAAGACAGAGGCAATAGACAACAAACCCCTTCAA
AACACCAGCTAGAGATATATCTTATGCTATTCCTCCACTTTAATCTTCCAGGTAC
AATAGTTTCTTTAATATCTACTAAACTTATTTCCTTTTGGTTCCTTTGGTTCACAT
CATCTGGATTACAGTCCTTCCTGTCTGTCTTAAATTTCGACCATCCTACAGCTACT
CTACCCAACCATTCTCTCATTTACTGTGTCACTGATAGCTAATTATAACCTAACA

AAAGTTATTAACTTAGAAAAATGGAGGAACCTACTTTACTTTTCCCTAAAACCA
GGAGTCTGTATTAAAAACAATTACCAAGAACTAAATTTAAAACATCTATGGTAG
ACAGCCCTTACCGCCATGATACACTTT

DY7S

Inia boliviensis

TTCCTTTATAAAATAAGTAGTTTTTGCTTAGAAAAATTTTTGTAGATATCTGTTG
GCACCGTTCTTTCTAATTGAAGCCAAATATACTTTATTTCCTTTTCAATCTATTTA
CCCAATGAAAACAGCTGTTTGGGTGTAATCCTTAAATTACAGAGGGGATTTTGC
TGTCATGAACTTCAGAAATGAATAAAAAAGTTTTGAGTCTTTGAGCTTAATGTAT
AAATTGTCCTTACTTCTTAGGAAAATGGAAGGTATGGACGCCGTAAACAATA

Inia geoffrensis (Peru)

TTCCTTTATAAAATAAGTAGTTTTTGCTTAGAAAAATTTTTGTAGATATCTGTTG
GCACCGTTCTTTCTAATTGAAGCCAAATATACTTTATTTCCTTTTCAATCTATTTA
CCCAATGAAAACAGCTGTTTGGGTGTAATCCTTAAATTACAGAGGGGATTTTGC
TGTCATGAACTTCAGAAATGAATAAAAAAGTTTTGAGTCTTTGAGCTTAATGTAT
AAATTGTCCTTACTTCTTAGGAAAATGGAAGGTATGGACGCCGTAAACAATA

UBEY7

Inia boliviensis

CACA__GTAAGCTCTCAGTAATGACCGTCTTTTTGAGTGAGTGAATAAATTGGGT
GGAGAGCTAGCTGCAGGACTAAGAGAGGGGGAACCACAGTGTCTTAAAGCTGG
AGAACCATTAGAGGTCACTTGGCTGTGCATCAGAGTCACCTTTAATGTTCTGCTT
TCACTAGTTCCAGGATAGGATCTCAGAATCTGAATTCAAAACAGAGGCTAACTC
CAGGTGGTCCTGATGTGGCGCCTTGAGTGTGGTCACATCGCCTCAGTGTATAGG
AGGGGAAAGCTGGGTCCAGGGAATGGAGGCTAGTTTGTTACGGCTCAGATTAGC
CTTGAGCCCCCTGGACTCAGAGGACTTTCCCAGACCTACCCTGTCCAGTGGGTG
AGGAACAAGTCCTTGTGCTCTGTAAGAAGCAGGGATGGTGGGTTTTCTGTTTT

Inia geoffrensis (Peru)

CACACAGTAAGCTCTCAGTAATGACCGTCTTTTTGAGTGAGTGAATAAATTGGG
TGGAGAGCTAGCTGCAGGACTAAGAGAGGGGGAACCACAGTGTCTTAAAGCTG
GAGAACCATTAGAGGTCACTTGGCTGTGCATCAGAGTCACCTTTAATGTTCTGCT
TTCACTAGTTCCAGGATAGGATCTCAGAATCTGAATTCAAAACAGAGGCTAACT

CCAGGTGGTCCTGATGTGGCGCCTTGAGTGTGGTCACATCGCCTCAGTGTATAG
GAGGGGAAAGCTGGGTCCAGGGAATGGAGGCTAGTTTGTTACGGCTCAGATTA
GCCTTGAGCCCCCTGGACTCAGAGGACTTTCCCAGACCTACCCTGTCCAGTGGGT
GAGGAACAAGTCCTTGTGCTCTGTAAGAAGCAGGGATGGTGGGTTTTCTGTTTT

Actin

Inia boliviensis

AAAGAAACTAAAATAAAAGCCCAGGTCACTCACTCTCACAGAACCTAATGTTAG
ATGAAAATCAGCATTGTTACCAAATGTAATTTTACATCTGTTAGCTGTCAACAAA
ATACATGTGGACAGTCACATGGTAATTTTACATAGCATTATGTAGAAACAAAAT
GTTGATGGCAGCCACAGGGTAAAAACTGAGAGTTTCATCTCAGAAATTCTCCTT
CACAGCAGAAATGTATTTTGCACATTGAGCCTATCTGATTGATATTTAAGCTTTC
AATTTGGCATAGATTCACAAGAACAAATAATAGAAGCCTCTCCCCATGAATGAA
CTCTGTCCCTTTGTTGAACTTCTGGGATGGAGATATTTTGCATAAATCACTACTT
ATGGAACTATAGTAGCCACAGCTGTTTTGCAATTTTAAGTCACTCCAGTGCAATT
TTAAATTTTAGTAATGAGAATTCTCTCTTTTTTAAAGCTTCACCTTAATGATTAAA
AATACATCCCACTACTTTAGGCAGACTG----------
TCAGTGTTGATTGGTATGAATTTATGAGAAATCAAGCACAGAGTGATCATTTGT
AGAAATAATGTTTTTGCTTATAACGGTTAGATATAATCTTGGGTAGGTGACAACT
TCGAAAGTACAGTTTAAAGGATTTATAAGAGGCAAAATGCCATTTAACTAATAT
GTAAAATTTTACATATCCATTCAACAAATAATGATTGAGTGCCGAATATGTGCC
AGGCAATTTGTCATAGTGGCGAACAAGACAGACACAATCTTTGCCCTAAGAGAG
CTCATCAAGGGATGCAGAAGATAAATGACACAAAGTTGCCTTACCCGCAGTGTT
CTCTGCTATTTGAGAGAACTATTCAGGTCTGTTTCCAATCAACTAAGGCTGTTTC
TTAAGAACTCAGTGACAGTAGGATTCAGCATATCATTGTTGGTACACGAAGGAA
CTTTCTATCGCAATGATTAACCCTTTGTGTTTGGACTGTATCC

Inia geoffrensis (Peru)

AAAGAAACTAAAATAAAAGCC---
GTCACTCACTCTCACAGAACCTAATGTTAGATGAAAATCAGCATTGTTACCAAA
TGTAATTTTACATCTGTTAGCTGTCAACAAAATACATGTGGACAGTCACATGGTA
ATTTTACATAGCATTATGTAGAAACAAAATGTTGATGGCAGCCACAGGGTAAAA
ACTGAGAGTTTCATCTCAGAAATTCTCCTTCACAGCAGAAATGTATTTTGCACAT
TGAGCCTATCTGATTGATATTTAAGCTTTCAATTTGGCATAGATTCACAAGAACA
AATAATAGAAGCCTCTCCCCATGAATGAACTCTGTCCCTTTGTTGAACTTCTGGG
ATGGAGATATTTTGCATAAATCACTACTTATGGAACTATAGTAGCCACAGCTGTT
TTGCAATTTTAAGTCACTCCAGTGCAATTTTAAATTTTAGTAATGAGAATTCTCT
CTTTTTTAAAGCTTCACCTTAATGATTAAAAATACATCCCACTACTTTAGGCAGA
CTGGCGTCGTTTAAAAGACGGCCAGTCCACTACTTTAGGCAGATGTCAGTGTTG

ATTGGTATGAATTTATGAGAAATCAAGCACAGAGTGATCATTTGTAGAAATAAT
GTTTTTGCTTATAACGGTTAGATATAATCTTGGGTAGGTGACAACTTCGAAAGTA
CAGTTTAAAGGATTTATAAGAGGCAAAATGCCATTTAACTAATATGTAAAATTT
TACATATCCATTCAACAAATAATGATTGAGTGCCGAATATGTGCCAGGCAATTT
GTCATAGTGGCGAACAAGACAGACACAATCTTTGCCCTAAGAGAGCTCATCAAG
GGATGCAGAAGATAAATGACACAAAGTTGCCTTACCCGCAGTGTTCTCTGCTAT
TTGAGAGAACTATTCAGGTCTGTTTCCAATCAACTAAGGCTGTTTCTTAAGAACT
CAGTGACAGTAGGATTCAGCATATCATTGTTGGTACACGAAGGAACTTTCTATC
GCAATGATTAACCCTTTGTGTTTGGACTGTATCC

Cat

Inia boliviensis

TGACTTGCCCAGGAAG----------
GCCTCCGGGATCTTTTTAATGCCATTGCCACAGGCAACTACCCCTCCTGGACTTT
TTACATCCAGGTCATGACATTTAAACAGGCAGAAACTTTCCCATTTAATCCATTT
GATATCACCAAGGTGAGTCAGTTAACAACTAAACTGTTTTCTTTTTTAAGTGTCT
TTGCAACTAATTAAAAAAAATTGTGGTCAAGCATTCGTAAGTTGTATACAAAAC
ACAGTGGTACCACTTCAGAGTTTCCTAGCCTGTGCATGAGGGCTCACCAGCATTT
CCCTTCCAGTTCCTGTTTGATGAGTTTACTAAGTTCATCTGGGTGGCCTGATATA
TTGTTATTAGCAGGGAACAAATTTTGATGAGCTGATGTACTTTTGCCCAGGGAA
AGACTCAATGTTTACTGTTTACTGTTGTGAAAATTTTAACCAATGCACTCACCTT
AAGTTCTTATCATTTTGTTAGATTCAAAAATGTTATTTTCACTTATCATCATTGGC
TTCATTGTATTTGAAAGCTGATATTTTTATGCGTACTTTAATTTCCTTCTGTTGGT
TG

Inia geoffrensis (Peru)

TGACTTGCCCATGAAGATCCTGACTATGGCCTCCGGGATCTTTTTAATGCCATTG
CCACAGGCAACTACCCCTCCTGGACTTTTTACATCCAGGTCATGACATTTAAACA
GGCAGAAACTTTCCCATTTAATCCATTTGATATCACCAAGGTGAGTCAGTTAACA
ACTAAACTGTTTTCTTTTTTAAGTGTCTTTGCAACTAATTAAAAAAAATTGTGGT
CAAGCATTCGTAAGTTGTATACAAAACACAGTGGTACCACTTCAGAGTTTCCTA
GCCTGTGCATGAGGGCTCACCAGCATTTCCCTTCCAGTTCCTGTTTGATGAGTTT
ACTAAGTTCATCTGGGTGGCCTGATATATTGTTATTAGCAGGGAACAAATTTTGA
TGAGCTGATGTACTTTTGCCCAGGGAAAGACTCAATGTTTACTGTTTACTGTTGT
GAAAATTTTAACCAATGCACTCACCTTAAGTTCTTATCATTTTGTTAGATTCAAA
AATGTTATTTTCACTTATCATCATTGGCTTCATTGTATTTGAAAGCTGATATTTTT
ATGCGTACTTTAATTTCCTTCTGTTGGTTG

CHRNA

Inia boliviensis

TAACGTAAGCTCTGTGGCTTGAGATCTGCACCTCTTCTTTAAATGGTCAAATGCT
CGAGCACAGAGGGATGGGTTTGGCTTGATGGGAAGGTTGGCGTTCAAGGGGCA
GCTACTGACGTAAGATGTGCCCAGTGACCCCTTAGGCCATCTTAGCAAGTCATC
ATATTGTGAATAACCTATTTAAAAAATAAAGATCATAATGCCAGTGGAGGGATG
ATCAACAGATTGAAGGGCCCCTAGATGATGGATAGCACGAACATCGTGAGTCCG
TGATCGTCTTACGGAAATTCTAACATATTCCTCTCTCCCAGGCGGCCGAGGAATG
GAAGTACGTTGCAATGGTGATGGACCACATACTCCA

Inia geoffrensis (Peru)

TAACGTAAGCTCTGTGGCTTGAGATCTGCACCTCTTCTTTAAATGGTCAAATGCT
CGAGCACAGAGGGATGGGTTTGGCTTGATGGGAAGGTTGGCGTTCAAGGGGCA
GCTACTGACGTAAGATGTGCCCAGTGACCCCTTAGGCCATCTTAGCAAGTCATC
ATATTGTGAATAACCTATTTAAAAAATAAAGATCATAATGCCAGTGGAGGGATG
ATCAACAGATTGAAGGGCCCCTAGATGATGGATAGCACGAACATCGTGAGTCCG
TGATCGTCTTACGGAAATTCTAACATATTCCTCTCTCCCAGGCGGCCGAGGAATG
GAAGTACGTTGCAATGGTGATGGACCACATACTCCA

GBA

Inia boliviensis

TGCAATGCTACCTACTGTGACTCTCTTGACCCCCTGACCCTGCCTGACCCTGG
CACCTTCAGCCGCTTTGAGAGCACACGCAGTGGGCGCCGAATGGAGCTGAGTCT
GGGGACCATCCAGGCCAACCGCACAGGCACTGGTAACCACTACACCCCTCACGC
AGGCTGGGGTCCTCCTGGAGCTAAATCATGCCAGCAATCACCATGGAGTTTCTC
CCCTGTGCACTGACACCCTTTATTCCCTGC

Inia geoffrensis (Peru)

TGCAATGCTACCTACTGTGACTCTCTTGACCCCCTGACCCTGCCTGACCCTGGCA
CCTTCAGCCGCTTTGAGAGCACACGCAGTGGGCGCCGAATGGAGCTGAGTCTGG
GGACCATCCAGGCCAACCGCACAGGCACTGGTAACCACTACACCCCTCACGCAG
GCTGGGGTCCTCCTGGAGCTAAATCATGCCAGCAATCACCATGGAGTTTCTCCCC
TGTGCACTGACACCCTTTATTCCCTGC

IFN

Inia boliviensis

TCATGATTTCTGCTCTGACGATCTCCCAGGCACAAGGGCTGTACTTCTTCTCTTG
CAGATAGACAGTGATTCTGTGGAAGTATTTCCTCACAGCCAGGATGGAGTCCTC
CTTCAGCAGGGGAGTCCCTTCCAGCCCCGCCTCCTGCATCAGACAGGCTTGCAG
GTCAGTGAGCTGCTGATAAAGTGCAGTGCAGAACTTGTCCAGGAGGGTCTCATC
CCAAGCGGCAGCCGAGCCCTCCGTGCTGAAGAGCTGGAAGGTCTGCTGGATCAT
CTCCGTGGACCACAGCGATGGCTTGAGCCTTCTGGAACTGGTTGCCTCCAAACG
CCTCCTGGGGGAATCCAAAGTCATTTCTGTCCTTCAGGCAGGAGAAA

Inia geoffrensis (Peru)

TCATGATTTCTGCTCTGACGATCTCCCAGGCACAAGGGCTGTACTTCTTCTCTTG
CAGATAGACAGTGATTCTGTGGAAGTATTTCCTCACAGCCAGGATGGAGTCCTC
CTTCAGCAGGGGAGTCCCTTCCAGCCCCGCCTCCTGCATCAGACAGGCTTGCAG
GTCAGTGAGCTGCTGATAAAGTGCAGTGCAGAACTTGTCCAGGAGGGTCTCATC
CCAAGCGGCAGCCGAGCCCTCCGTGCTGAAGAGCTGGAAGGTCTGCTGGATCAT
CTCCGTGGACCACAGCGATGGCTTGAGCCTTCTGGAACTGGTTGCCTCCAAACG
CCTCCTGGGGGAATCCAAAGTCATTTCTGTCCTTCAGGCAGGAGAAA

Lactoalbumin

Inia boliviensis

GAGATGTCACAGATGTCCCTTGAGTGAGGGATCTGGTTGTCTCTGCACCAAATTT
TATTATTGATCTGGAAGAGTCCATATTCTGTGCTGCCATTGTTATTCACTATGGTT
TGTGTGTCACAACCACTAGTATGAAATACGGTACAGACCCCTGAAAGAAAGATG
AAAAAGAGATATCACAGGGATGTCCACATATAGCATAAATGTGGACAGACAGA
AAAAGGCTCTCCAGCCAATCTCCAAATACGGCCACAACCCCAGAGTATCCTGAT
AAGATTAAGAGTTCCATAAAACAATCAGACAATGGGAGAAAGGAGAGATAGGT
GAATAAATGTAATAAAAGAGTAAACAGATGAGCAGATAAGAGGATTATTAGTT
AATTGAGTTAAAAGTAGAGAAAAAAAGAGGAGGAAGAGAAGGGTGGGGAAGA
GAGAAGAAGATGAAGTATGGAACAAAGCAAGATAGCAGGGAACTCACATTCAG
GCAAAGTGATGCCTCCATAGCCATCCAGGTCTTTCAGCCTCTGGAACAACTCAC
ATTTTGTTAATTGTTCAGCCTGGATGGCATGGAACAGGATGCCCACCAAGAGCA
GAGAGACAAAG

Inia geoffrensis (Peru)

GAGATGTCACAGATGTCCCTTGAGTGAGGGATCTGGTTGTCTCTGCACCAAATTT
TATTATTGATCTGGAAGAGTCCATATTCTGTGCTGCCATTGTTATTCACTATGGTT
TGTGTGTCACAACCACTAGTATGAAATACGGTACAGACCCCTGAAAGAAAGATG
AAAAAGAGATATCACAGGGATGTCCACATATAGCATAAATGTGGACAGACAGA
AAAAGGCTCTCCAGCCAATCTCCAAATACGGCCACAACCCCAGAGTATCCTGAT
AAGATTAAGAGTTCCATAAAACAATCAGACAATGGGAGAAAGGAGAGATAGGT
GAATAAATGTAATAAAAGAGTAAACAGATGAGCAGATAAGAGGATTATTAGTT
AATTGAGTTAAAAGTAGAGAAAAAAAGAGGAGGAAGAGAAGGGTGGGGAAGA
GAGAAGAAGATGAAGTATGGAACAAAGCAAGATAGCAGGGAACTCACATTCAG
GCAAAGTGATGCCTCCATAGCCATCCAGGTCTTTCAGCCTCTGGAACAACTCAC
ATTTTGTTAATTGTTCAGCCTGGATGGCATGGAACAGGATGCCCACCAAGAGCA
GAGAGACAAAG

Figure 1. Autosomic and Y chromosome intron sequences (10) for the two Bolivian and two Peruvian pink river dolphins analyzed. - = indel (insertion-deletion).

Phylogenetically Informative Insertion-Deletion Events

The insertion-deletion (indel) events of some sequences such as DBY8 (151 bp) were phylogenetically important. All the animals sequenced from Bolivia had a deletion at position 6, with the exception of one individual collected from the upper-most sampling point within the Mamoré River, (Porvenir Lake) and which indisputably presented a C nucleotide. Additionally, the two Peruvian animals showed a G insertion. Therefore, the exemplars representative of *I. boliviensis* were characterized by a deletion gap or, in the upper Mamoré River, by the presence of a C. In contrast, in the dolphins representing *I. geoffrensis*, all exemplars presented a G in that position. We detected another indel in the same Y chromosome intron. Three animals from Bolivia (middle Mamoré river; Porvenir, Bella Unión and Bolivar Lakes) presented a deletion in this position, while two animals collected from the confluence of the Mamoré and Itenez (Guaporé) rivers, and the Peruvian animals showed an A. This would suggest that dolphins of the middle and upper Mamoré river areas originally had an A and later a deletion. We located another insertion-deletion at the 116 position. Again, the three animals from the middle Mamoré River presented a deletion in that nucleotide position, whereas the two animals from the confluence of the Mamoré and Itenez rivers presented a G insertion. In this case, the two Peruvian animals also showed the absence of this nucleotide. If we took the Amazon (non-Bolivian) animals as representing the ancestral population, therefore, at this position, the original state seems to be the absence of this position, and in the middle Mamoré River appeared exemplars with an inserted nucleotide (G). Finally, at DY8S, we found another insertion-deletion position (146 position) which is interesting from a phylogenetic point of view. In this case, the two animals from the Mamoré-Itenez confluence plus another exemplar from the middle Mamoré showed the presence of G, while the other two individuals from the middle Mamoré and the two Peruvian

animals had a deletion at this position. These data suggest that originally there was an absence of a nucleotide at this position and later, during the Bolivian isolation, animals of the middle and lower Mamoré river developed a (G) insertion. Another Y chromosome intron, UBEY7 (457 bp), also revealed a notable phylogenetic insertion-deletion trait. Different from all the Bolivian and Peruvian samples analyzed (*I. boliviensis* and *I. geoffrensis*) a specimen from the Putumayo River, in Colombia, had an AA insertion at the 32nd and 33rd positions. This suggests that originally there was an absence of this AA pair, followed by its insertion later, at least, in animals of the Putumayo River. Additionally, the nuclear CAT intron (580 bp) showed a conspicuous gap difference between the Bolivian and the Peruvian animals studied. The Peruvian exemplars presented a sequence of 12 bp (ATCCTGACTATG; 16-28 positions) nonexistent in the Bolivian dolphins.

The nuclear intron Actin (1039 bp) also exhibited interesting insertion-deletion traits. For example, at positions 22, 23 and 24, the Bolivian dolphins had a CAG sequence, that was absent in the Peruvian exemplars. Thus, in the Bolivian animals, an insertion occurred at this position. Furthermore, we observed a large deletion (nucleotides 521-562: GCGTCGTTTAAAAGACGGCCAGTCCACTACTTTAGGCAGATG) in the Bolivian but not the Peruvian animals.

Genetic Variability

Only the introns DY8S, SMY7 and CAT exhibited nucleotide changes (transitions or transversions). Therefore, the molecular evolution of *Inia* at the level of autosomic and Y chromosome genes, seems note worthly lower than that reported for the mitochondrial DNA (mtDNA), as we will discuss later. For DY8S, we detected two variable sites (a total of 2 mutations) in the first (a transversion, with a G and a T in the Bolivian and Peruvian exemplars, respectively) and fifth (a transition, with a C and a G in the Bolivian and Peruvian individuals, respectively) positions. Both sites are parsimoniously informative. For SMY7, we detected two polymorphic informative sites, thus implying two mutations (357 and 462 positions). The first was a transversion (a G in all the Bolivian individuals and an A in all the Peruvian and Orinoquian exemplars) and the second was a transition (an A in all the Bolivian and Peruvian dolphins and a T in the two Orinoquian animals). Also, position 465 presented a C in one Bolivian animal as well as in Peruvian exemplars, but presented a T in the remaining individuals regardless if they were from Bolivia, Perú or Orinoco. For CAT, we only detected one mutation at the twelfth position (a transversion; G in the Bolivian animals, T in the Peruvian ones). Our analysis of 4,544 bp in several exemplars of Bolivian (2) and Peruvian (2) pink river dolphins, located 6 nucleotide changes (0.13%) that demonstrated an extreme conservative molecular evolution in this Cetacean species at the autosomic and Y chromosome levels. Although, the haplotype diversity (h) among the sequences studied is relatively high (showing fixated differences in the minimal genetic divergence found among the *I. boliviensis* and the *I. geoffrensis*) ranging from 0.476 (DY8S) to 0.667 (SMY7), all the other genetic diversity measures were extremely small (Table 1). The main genetic diversity statistics results (Table 1) were as follows: π (nucleotide diversity) ranged from 0.0000001 (SMY7) until 0.0065 (DY8S); k (average number of nucleotide differences) oscillated from

0.795 (SMY7) to 1 (CAT); θ per site (with different methods) ranged from 0.00139 (SMY7) to 0.0066 (DY8S) as well as θ per sequence (with different procedures) oscillated from 0.5 (CAT) until 0.816 (DY8S). Taken simultaneously for all the sequences analyzed, these genetic measures were $\pi = 0.00134$, $k = 6$, θ (per site) = 0.00134 and θ (per sequence) = 6. Clearly, all these results support an extremely small genetic variability.

Genetic Differentiation, Gene Flow and Mutation Estimates

The absolute measures of genetic heterogeneity among the different dolphin populations were limited (Table 2). For example, D_{xy} and D_a showed small amounts of these measures (0.01361 at DY8S, 0.00216 at SMY7, 0.00176 at CAT and 0.00134 for the overall set of sequences). However, the relative genetic heterogeneity measures were extremely high among the diverse populations. For DY8S, all statistics measured (chi-square table, H_{st}, K_{st}, K^*_s, K^*_{st}, Z_s, Z^*_s and Snn) showed significant differences. Other genetic measures, such as G_{ST} , γ_{ST} , N_{ST} , F_{ST} and ϕ_{ST} offered the maximum differentiation of one possible. Their respective associated gene flow estimates were 0. The same was true for SMY7. All genetic heterogeneity measures were significant and all gene flow estimates, independent of the statistic calculated, were 0. For CAT, several heterogeneity statistics were not statistically significant, but all gene flow estimates were again 0. Therefore, these markers showed complete isolation between the Bolivian and Peruvian populations.

The situation was quite similar when indels were introduced in the analysis (fifth state). For CAT and Actin, several measures did not show statistical differences but all the gene flow estimates were identical to 0. The situation for DY8S was slightly modified. Several measures revealed significant differences whereas others did not. Nonetheless, G_{ST} (= 0.276; Nm = 2.62) , γ_{ST} (= 0.533; Nm = 0.88), and F_{ST} (= 0.75; Nm = 0.33) revealed a strong differentiation between Bolivian and Peruvian populations but incomplete isolation. However, all of the gene flow estimates were 0 when all the sequences were considered simultaneously. This clearly indicates that both populations are reproductively isolated. We also estimated $\tau (= 2\mu\tau)$ for DY8S, SMY7 and CAT. Based on the assumption that a generation in the pink river dolphin ranged from 6 to 10 years and the estimation that the time divergence between *I. boliviensis* and *I. geoffrensis* was about 6 millions of years ago (Banguera et al., 2002), we calculated the mutation rates per generation for these markers. In the case of DY8S, μ ranged from 3.2×10^{-7} to 5.4×10^{-7}; for SMY7, μ ranged from 3.9×10^{-7} to 6.6×10^{-7} and for CAT, μ ranged from 5×10^{-7} to 8.3×10^{-7}. However, these mutation rates could be substantially different if the real time split among *I. boliviensis* and *I. geoffrensis* was different to 6 millions of years ago.

Linkage Disequilibrium and Recombination Parameters

Of all the cases analyzed, only DY8S demonstrated a significant linkage disequilibrium. This occurred between the first and fifth positions (D = 0.204; D' = 1, r = 1; Fisher test =

0.048, $p < 0.05$; $\chi^2 = 7$, $p < 0.001$). The recombination statistics did not recognize this phenomenon.

Table 1. Several genetic diversity statistics estimated for the pink river dolphin (*Inia*) for the DY8S, SMY7S, CAT intron sequences as well as for 10 nuclear total intron sequences (TSS). Hd = haplotype diversity, SD = standard deviation, π =nucleotide diversity, πJ and C = π with Jukes and Cantor correction, k = average number of nucleotide differences and their respective stochastic (Vst(k)), sampling (Vs(k) and total variances (V(k)) assuming free1 and no recombination2; RS = raggedness statistic, RRR2 = Ramos-Onsins and Rozas R2 statistic (2002), and different estimates of θ (= 4Neμ) per site from π (θpπ, from S (number of polymorphisms) (θps), from Eta (total number of mutations with SD for free1 and no recombination2) (θpsEta) and per sequence from S (θs) with their respective variances assuming no-recombination (V(θs)NR) and free recombination (V(θ)FR)

Marker	No. Haplotype	Hd±SD	π±SD	πJ&C	θpsEta	θps^S ±SD[1]±SD[2]	$\theta_{p\pi}$	k
DY8S	2	0.476±0.171	0.0064±0.0023	0.00654	0.0055	0.0055±0.0043±0.0039	0.0065	0.952
SMY7S	3	0.667±0.078	0.0017±0.0031	0.00172	0.0014	0.0014±0.0011±0.0009	0.0017	0.795
CAT	2	1.000±0.500	0.0017±0.0009	0.00176	0.0018	0.0018±0.0018±0.0018	0.0018	1
TSS	2	1.000±0.500	0.00134±0.00067	0.00134	0.000134	0.00134±0.00102±0.0005	0.00134	6

Marker	Vst(k)[1,2]	Vs(k)[1,2]	V(k)[1,2]	θ_s	$V(\theta_s)_{NR}$	$V(\theta)_{FR}$	RS	RRR[2]
DY8S	0.399[1]	0.139[1]	0.538[1]	0.816	0.400	0.333	0.728	0.238
	0.317[2]	0.106[2]	0.423[2]					
SMY7S	0.322[1]	0.055[1]	0.376[1]	0.644	0.237	0.208	0.227	0.199
	0.265[2]	0.044[2]	0.309[2]					
CAT	0.333[1]	0.667[1]	1[1]	1	1	1	2	0.5
	0.333[1]	0.667[1]	1[1]					
TSS	5.333[1]	15.667[1]	21[1]	1	21	6	2	0.5
	2.000[2]	4.000[2]	6.000[2]					

Table 2. Genetic heterogeneity statistics applied to the pink river dolphin (*Inia*) for the DY8S, SMY7S, CAT intron sequences as well as for 10 nuclear total intron sequences (TSS). Dxy = average number of nucleotide differences per site between populations; DxyJC = the same with the Jukes and Cantor correction; SD = standard deviation; Da = number of net substitutions per site between populations; DaJC = the same with the Jukes and Cantor correction; χ^2 = a chi-square table; H_s, H_{st}, K_s, K_{st} = diverse intra and inter population genetic differentiation statistics; p = the probability obtained by a permutation test with 1000 replicates, G_{ST} , γ_{ST} , N_{ST} , F_{ST} and ϕ_{ST} = diverse relative genetic heterogeneity statistics; Nm = gene flow estimates. * = p < 0.05; ** = p < 0.01; *** = p < 0.001. NS = no significant

Marker	D_{xy}	D_{xy}JC ± SD	D_a	D_aJC±SD
DY8S				
Bolivia-Perú	0.01361	0.01373±0.00495	0.01361	0.01373±0.00495
SMY7S				
Bolivia-Perú	0.00216	0.00216±0.00005	0.00216	0.00216±0.0055
Perú-Orinoco	0.00216	0.00216±0.00072	0.00216	0.00216±0.0072
Bolivia-Orinoco	0.00473	0.00475±0.00178	0.0043	0.00432±0.00178
CAT				
Bolivia-Perú	0.00176	0.00176±0.00088	0.00176	0.00176±0.00088
TSS				
Bolivia-Perú	0.00134	0.00134±0.00067	0.00134	0.00134±0.00067

Marker	χ^2	p	H_s	H_{st}	p	k_s	k_{st}	p	G_{st}	Nm	γ_{ST}	Nm	N_{st}	Nm	F_{st}	Nm	ϕ_{st}	Nm
DY8S	7	**	0	1	*	0	1	*	1	0	1	0	1	0	1	0	1	0
SMY7S	26	***	0	1	***	0	1	***	1	0	1	0	1	0	1	0	1	0
CAT	4	**	0	1	NS	0	1	NS	1	0	1	0	1	0	1	0	1	0
TSS	8	**	0	1	*	0	1	*	1	0	1	0	1	0	1	0	1	0

Table 3. Different tests for detecting natural selection affecting the DY8S, SMY7S, CAT intron sequences as well as for 10 nuclear total intron sequences (TSS): Fu-Li D test, Fu-Li F test, Fu Fs test, SS = Strobeck test, Tajima D test. NS = no significant; * = p < 0.05; ** = p < 0.01

Marker	Fu-Li D	Fu-Li F	Fu Fs	SS	Tajima
DY8S	1.178 NS	1.145 NS	1.702 NS	0.514 NS	0.687 NS
SMY7S	0.952 NS	0.993 NS	0.313 NS	0.733 NS	0.655 NS
CAT	1.632 NS	1.276 NS	0.540 NS	0.818 NS	1.632 NS
TSS	2.156 *	2.072 NS	3.526 NS	0.238 NS	2.156 NS
cytb	2.254 **	2.298 *	5.480 **	0.075 NS	2.254 *

Table 4. Results of different genetic distances applied to the pink river dolphin (*Inia*) for the DY8S, SMY7S, CAT intron sequences as well as for 10 nuclear total intronsequences (TSS). These numbers reflect all of the completed comparisons. No differences were found for the diverse genetic distances employed.

Distance	DYS8	SMY7S	CAT	TSS
p-distance	0.006±0.004	0.002±0.001	0.002±0.002	0.001±0.001
Jukes-Cantor	0.007±0.005	0.002±0.001	0.002±0.002	0.001±0.001
Kimura 2p	0.007±0.005	0.002±0.001	0.002±0.002	0.001±0.001
Tarima-Nei	0.007±0.005	0.002±0.001	0.002±0.002	0.001±0.001
Tamura-Nei	0.007±0.005	0.002±0.001	0.002±0.002	0.001±0.001
Tamura 3p	0.007±0.004	0.002±0.001	0.002±0.002	0.001±0.001
Log Det	0.009±0.007	0.002±0.002	0.002±0.002	0.001±0.001

Detection of Natural Selection

Neither of the tests we used to detect natural selection (Fu and Li D* and F*, Fu's Fs, Strobeck's s and Tajima's D) showed significant evidence of this phenomenon acting upon the sequences analyzed at the DY8S, SMY7 and CAT markers (Table 3). However, when all sequences were analyzed together, one test detected possible significant action of natural selection (Fu and Li's D* test statistic = 2.156, P < 0.05).

Phylogenetic Analyses

Table 4 shows the average overall genetic nucleotide distances between the Bolivian and the Peruvian dolphins (and others geographic locations when applied). In all the cases, the genetic distances were small, or very small, reflecting the limited amount of genetic variability detected. Furthermore, all the methods employed revealed practically identical values. For DY8S, these values were around 0.007, for SMY7 and CAT around 0.002 and for the overall sequence set around 0.001. In two cases, we were able to construct phylogenetic trees (DY8S and SMY7) (Figure 2.). For DY8S, all the trees clearly differentiated the

Bolivian and the Peruvian exemplars with high bootstraps (higher than 85%), values independent of the genetic distance employed or the tree construction algorithm. For SMY7, all the trees revealed three clear clusters, two of which were part of a main group (the Peruvian and the Orinoco dolphins) while the third was an independent clade with all the Bolivian sequences. The bootstrap percentages were lower than in the previous case (40-70%). It seems clear that, although the gene differentiation is very small (which hardly contrast with mtDNA analyses; Banguera et al., 2002), the Bolivian (*I. boliviensis*) and the Peruvian (*I. geoffrensis*) animals formed two independent evolutionary clades, such as was previously confirmed with mtDNA.

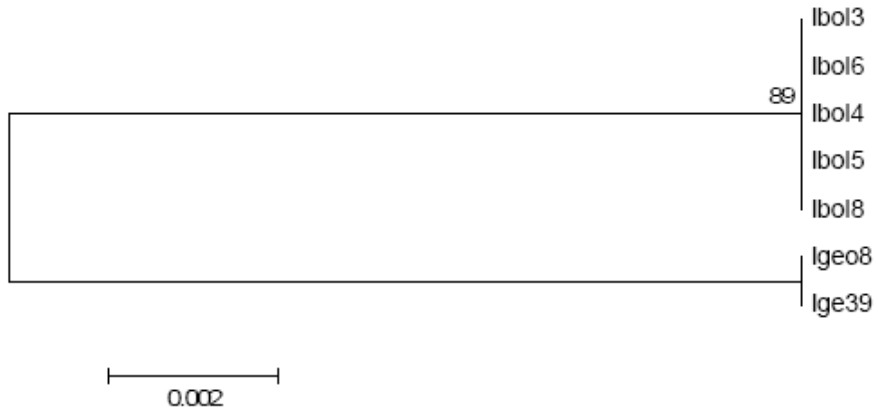

Figure 2a.

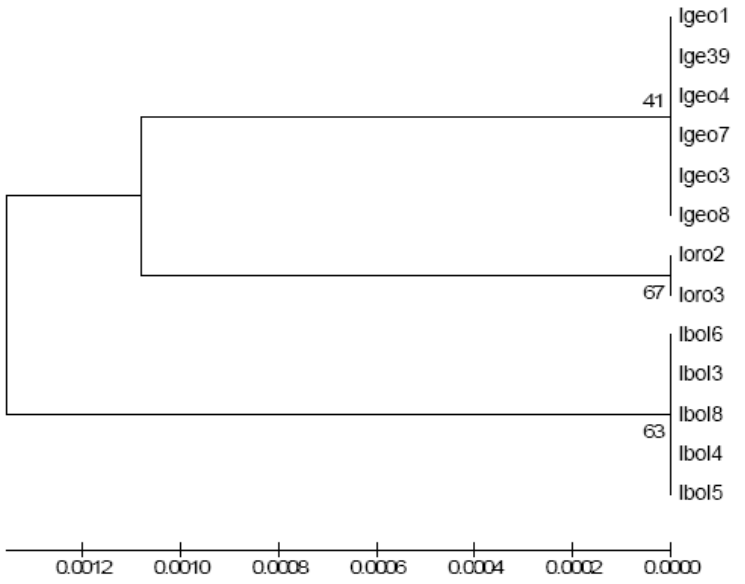

Figure 2b.

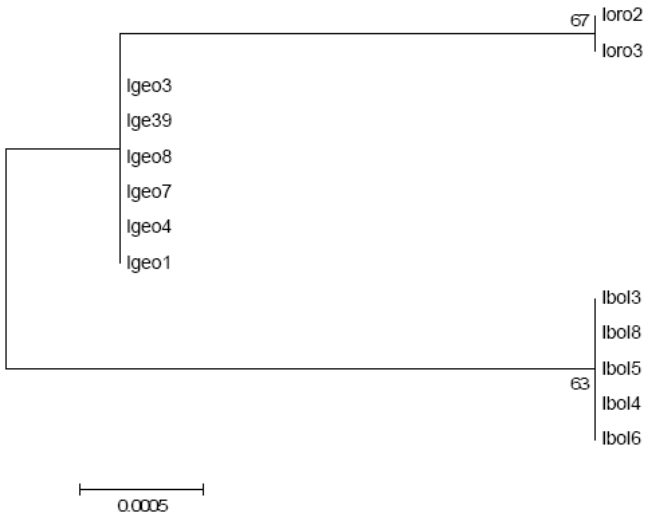

Figure 2c.

Figure 2. Phylogenetic relationships among diverse pink river dolphin populations analyzed by means of the DY8S and SMY7S intron sequences. Igeo = individuals from diverse Peruvian rivers (*Inia geoffrensis geoffrensis*). Ioro = individuals from the Orinoco basin (*Inia geoffrensis humboldtiana*). Ibol = individuals from Bolivia (*Inia boliviensis*). A/ Neighbor-joining tree with Log det distance for the DY8S sequences. B/ UPGMA tree with the Tamura genetic distance for the SMY7S sequences. C/ Neighbor-joining tree with Log det distance for the SMY7S sequences. Numbers on tree branches are the bootstrap percentages.

Discussion

Molecular Evolution Differences Between the Mitochondrial and Nuclear (Autosomic and Y Chromosome) DNA Sequences in the *Inia* Genus

One relevant difference garnered from a comparison of the present results with those published by Banguera et al., (2002) is the extremely low molecular evolution for the autosomic and Y chromosome introns compared to the absolute genetic differentiation found at the control region and at the cyt-b sequences of the mtDNA. The values of k for the control region and the cyt-b mtDNA sequences were 12.786 and 11.816, respectively while the values for the nuclear introns, which showed polymorphism, only ranged from 0.795 to 1. Therefore, this statistic was approximately 12-16 fold higher for the mtDNA compared to the nuclear introns. The levels of π were even proportionally higher for mtDNA relative to the nuclear introns. The control-region and cyt-b mitochondrial sequences offered a nucleotide diversity level of 0.05669 and 0.03376, respectively (Banguera et al., 2002) which was 337.600 to 566.900 times greater than the π value obtained for SMY7, 5 to 9 fold higher compared to the π value for DY8S, and 25 to 42 times greater than the π values of the complete intron sequence set. Therefore, the genetic variability for nuclear introns is clearly reduced in comparison to that of mtDNA in this cetacean. If we compared some absolute

genetic differentiation statistics, especially among the Amazon and the Bolivian populations, the situation is similar to that previously commented for the genetic diversity statistics. The values of D_a between the Amazon-Bolivian population pair were 0.0532 and 0.0299 for the mitochondrial control region and the cyt-b, respectively. These values were 4 to 30 times higher for the control region compared to that estimated for the polymorphic individual introns, as well as for all the introns taken together, and from 2 to 17 fold higher for cyt-b in regards to the nuclear introns. Thus, the nuclear sequence introns were of more limited phylogeographic and population genetics value for distinguishing pink river dolphin populations and their respective evolutionary trends than the mitochondrial markers. Why are there appreciably different evolutionary differentiation rates between mtDNA markers (Banguera et al. 2002) and nuclear introns (this study)?

There are several potential explanations.

1) *Constrictive mutation and negative purifying (background) selection affects Inia's nuclear genome.* For decades, several authors have detected that the levels of gene diversity (expected heterozygosity) were correlated with ecological and abiotic factors. For example, Nevo and Beiles (1991) showed that the gene diversity was significantly correlated with the habitat type in more than 300 Amphibian species. They determined that gene diversity was higher in the comparably instable terrestrial and arboreal areas than the aquatic or subterranean habitats. Nevo and Beiles (1989) reached the same conclusion when the studied snails (*Sphincterochila sp.*), reptiles (*Agama stellio*), and rodents (*Acomys cahirinus*) in Israeli deserts. Animal populations of these organisms presented higher gene diversity levels in the inner areas of these deserts, where the rain regimen was more undetermined, and the habitat was more instable. Similarly, Nevo et al., (1984) highlighted several significant relationships between genetic diversity and ecological traits. They demonstrated that terrestrial mammals and other vertebrates had statistically significant higher gene diversity levels (H = 0.069 and 0.052 for vertebrates, H = 0.062 and 0.023 for mammals) than the aquatic species (H = 0.046 for vertebrate and H = 0.018 for mammals). Vertebrate animals that were categorized as both aquatic and terrestrial, exhibited the highest gene diversity levels (H = 0.076) and fresh water vertebrates had lower gene diversity than marine species. Furthermore, specialist mammals (such as *Inia* compared to other mammals) showed significantly lower gene diversity levels (H = 0.032) than generalist mammals (H = 0.054). It's interesting to note that some previous authors have provided alternate explanations to understand the lower gene diversity levels in aquatic organisms (Schlotterer et al., 1991). The DNA of aquatic mammals could evolve more slowly because their environment is less mutagenic than terrestrial and atmospheric areas and because they are less exposed to important sources of radioactive irradiation such as the soil (granite rocks) and cosmic rays. Also, additional background selection associated with low recombination rates may impact the nuclear genome of cetaceans more than terrestrial mammals. Fisher (1930) demonstrated that genes, which encode for important proteins, are located in low recombination regions and by hitchhiking they can maintain other, linked genetic loci with restricted allele variation. Furthermore,

Begun and Aquadro (1992) and Aquadro et al., (1994) found a positive correlation between recombination rates and DNA sequence variation across the genome of *Drosophila melanogaster*. Similarly, Charlesworth et al., (1993) demonstrated that this correlation was consistent with the effects of a continual input of deleterious mutations in regions of low recombination, reducing neutral variability at linked loci. Thus, we could expect that *Inia's* nuclear gene diversity should be very limited because it's an aquatic mammal specialist within a homogeneous habitat. If the *Inia's* mtDNA evolution is less subject to selective constrictions (evolution is more neutral) its evolutionary rates should be higher than those found in nuclear genes. In fact, a large quantity of evidence supports an extremely slow nuclear DNA evolution in aquatic mammals. For instance, Simonsen et al., (1982a, b, c) determined extreme low electrophoretic gene diversity for aquatic mammals of diverse orders (Pinnipeda: *Pusa hispida*, *Pagophilus groenlandicus*, *Cystophora cristata* and *Odobenus rosmarus*; Cetacea: *Balaenoptera acutorostrata*). Schlotterer et al., (1991) cloned and sequenced several DNA microsatellites from 11 cetacean species and discovered an unusually high conservation of sequences for these microsatellites, averaging 3.2% differences over 35-40 million years among toothed and baleen whales. This percentage corresponds to a rate of nucleotide substitution of about 9×10^{-8}, the lowest found for any species group at that moment. The average divergence rate for nucleotide position in mammals usually ranges from 2×10^{-7} to 5×10^{-7}. Valsecchi and Amos (1996) isolated 12 microsatellite loci from sperm (*Physeter macrocephalus*) and humpback whales (*Megaptera novaeangliae*). Most of these microsatellites have been amplified in 30 cetacean species representing 23 genera and 10 families including *Inia* (Ruiz-Garcia et al., unpublished results). Similarly, Krutzen et al., (2001) cloned five polymorphic microsatellite loci for *Tursiops aduncus*, and showed high gene variability in so diverse cetacean species, such as *Stenella coeruleoalba* from the Mediterranean Sea and *Pontoporia blainvillei* from Southern coast of Brazil. This provides further, and undisputed evidence in favor of a highly conserved cetacean nuclear genome.

2) *Positive (diversifying) natural selection affects Inia's mtDNA*. The mtDNA in *Inia* showed striking levels of gene variability and genetic differentiation among the populations studied. It seems to be a particular characteristic of this DNA in other cetaceans as well. For instance, the brother clade of *Inia*, *Pontoporia blainvillei*, also showed an elevated gene diversity and genetic heterogeneity among only 20 animals studied coming from the coasts of Rio de Janeiro (Northern form) and Rio Grande (Southern form). Eleven haplotypes were found, five exclusive to the Rio Grande and six from Rio de Janeiro, with $\pi = 0.0038$ for the Northern population and 0.0101 for the Southern population (Secchi et al., 1998). Therefore, another possibility is that a form of diversifying natural selection is affecting parts of the mtDNA or, that positive natural selection counteracts a fraction of the negative natural selection that potentially affect all kinds of DNA in cetaceans. It is logical to think that the transition from a terrestrial to a fully aquatic lifestyle requires a complete transformation of many biological systems and possibly an alteration of mtDNA as well. If so, we must expect several differences in the cetacean mtDNA in regards to

that found in terrestrial mammals. Hoelzel et al., (1991) determined that indels were less common in the cetacean D-loop region than in other mammals and that the presumptive sequences most conserved in cetaceans were different from those reported in terrestrial mammals. On the other hand, the cetacean D-loop sequences showed a greater than random level of DNA sequence simplicity (indicative of the operation of DNA slippage within the sequence) than in other mammalian D-loop sequences. Thus, a different selection balance could be acting upon the cetacean mtDNA. Our results could be in agreement with this possibility. We applied different tests for detection of natural selection to all the mitochondrial cyt-b sequences obtained from Banguera et al., (2002), in addition to new cyt-b sequences we obtained from other Bolivian and Peruvian samples. The Tajima's D (2.254, $P < 0.05$), the Fu and Li's D* (2.254, $P < 0.02$) and the Fu and Li's F* (2.298, $P < 0.05$) tests all offered significant positive values and thus support the presence of positive (over dominant or diversifying) natural selection. According to Simonsen et al., (1995), the Tajima test is the most powerful of the three tests in detecting positive selection, while the Fu and Li's (1993) tests are superior in detecting background selection. The fact that the Fu and Li's tests detected positive natural selection supports the possible role of this phenomenon affecting the mitochondrial cyt-b gene among the Bolivian and the remainder *Inia* populations.

3) *Differential sex dispersion and migration*. Obviously, mtDNA only reflects the genetic differentiation among the different female lineages. In contrast, the nuclear autosomic genome displays the genetic influence of both sexes while the Y chromosome only reflects the influence of males. Martin and da Silva (2004a, b) concluded that, at least, in the pink river dolphin of Mamirauá (central Brazilian Amazon), there is sexual segregation with females and their calves occupying the most remote areas of várzea and the adult males occurring in more open areas, moving frequently between river systems. Thus, males could be superior gene dispersers compared to females. A greater number of males conforming to the founder group from more diverse and distant geographical origins than females, could result in lower genetic differentiation (reduced action of founder events and gene drift) among males compared to females in Bolivia and other geographical areas of this species. However, in disagreement, Martin and da Silva (2004a) stated that the Mamirauá population's sex-ratio was around 1:1. Therefore, the sex proportion seems similar and not necessarily more males than females had colonized the Beni-Mamoré river basin potentially six millions of years ago.
4) *Is the divergence between Inia boliviensis and Inia geoffrensis 5-6 millions old?* We have assumed that the separation among *Inia boliviensis* and *Inia geoffrensis* occurred during the formation of the rapids between Guayaramerin and Porto Velho (Grabert, 1984; Lundberg et al., 1998), in the Beni-Mamore and Madeira rivers, 5-6 millions of years ago. However, another way to explain the extremely small nuclear genetic sequence differentiation between these two populations is that this temporal divergence occurred more recently, during the last Pleistocene. There is substantial new evidence that supports this hypothesis. For example, we have previously demonstrated that the ancestral mitochondrial control region haplotype occurs in the

Amazon river and its main tributaries. Assuming a mutation rate for this mtDNA similar to that determined for human beings, we estimated that the Bolivian and Amazon forms separated around 40,000-120,000 years ago. Additionally, the new data obtained for Caballero et al., (2007) for the South American river dolphin, *Sotalia*, agree quite well with this hypothesis of a recent divergence between the pink river dolphin populations (although the Bolivian form is completely reproductively isolated from the other forms). These authors demonstrated the existence of two *Sotalia* forms, *Sotalia fluviatilis*, exclusive to the Amazon freshwater systems, and *Sotalia guianensis* in the Amazon delta and marine coasts of Brazil and other Latin American countries. They analyzed the same 10 autosome and Y nuclear introns and estimated a net divergence sequence of 0.07% between the coastal and riverine *Sotalia* (4,312 bp). They also estimated that a separation of both *Sotalia* forms occurred 1-1.2 millions of years ago. If we assume that there are similar intron mutation rates within *Inia* and *Sotalia* dolphin genera (both in freshwater systems) the net divergence in Inia (0.13 %) represents a maximum temporal divergence between *Inia boliviensis* and *Inia geoffrensis* of 1.8 millions years ago. Even if when we omitted the net divergence of DY8S (0.66 %), which had the highest value for *Inia*, the new average net divergence between *Inia boliviensis* and *Inia geoffrensis* for the remaining 9 introns was only 0.038 %, which corresponded to a temporal split of 543,000 years ago. By using 10 DNA microsatellites, Ruiz-Garcia et al., (2008) also showed a relatively small temporal divergence between the *Inia* forms (50,000-500,000 years ago) when they assumed similar microsatellite mutation rates to those estimated for *Sotalia* and *Pontoporia*. Additionally, the new discovery of an *Inia* fossilized skull in the Brazilian Amazon (Cozzuol, unpublished) belonging to a very differentiated form of *Inia* (less than 50,000 years ago) supports the possibility that the nuclear DNA is more conservative and constricted than the mtDNA in *Inia*. Probably, this last hypothesis is the most probable.

Small Nuclear Intron Sequence Gene Diversity, but Phylogenetic Differentiation of the Bolivian *Inia* Population

Nonetheless, the relative genetic differentiation statistics, as well as the phylogenetic procedures employed with the polymorphic sequence introns, practically revealed a total isolation of the Bolivian and the Amazon-Orinoco populations. Most of the G_{ST} , F_{ST} and ϕ_{ST} values were identical to 1, which revealed complete genetic isolation, with the respective gene flow estimates equal to 0. This agrees quite well with the results obtained for mtDNA. Therefore, we present additional molecular evidence that favors the independent evolutionary history of the Bolivian population. This reinforces our previous claim (Banguera et al., 2002) to subdivide *Inia* into two evolutionary significant units (ESUs) following the concept of Moritz (1994), although its divergence may not be as temporally distant as was claimed previously.

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