Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

First report of major histocompatibility complex class II loci from the Amazon pink river dolphin (genus *Inia*)

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Genet. Mol. Res. 5 (3): 421-431 (2006)
Received December 7, 2005
Accepted June 9, 2006
Published July 3, 2006

ABSTRACT. We report the first major histocompatibility complex (MHC) DQB1 sequences for the two species of pink river dolphins (*Inia geoffrensis* and *Inia boliviensis*) inhabiting the Amazon and Orinoco River basins. These sequences were found to be polymorphic within the *Inia* genus and showed shared homology with cetacean *DQB*-1 sequences, especially, those of the Monodontidae and Phocoenidae. On the other hand, these sequences were shown to be divergent from those described for other riverine dolphin species, such as *Lipotes vexillifer*, the Chinese river dolphin. Two main conclusions can be drawn from our results: 1) the *Mhc* DQB1 sequences seem to evolve more rapidly than other nuclear sequences in cetaceans, and 2) differential positive selective pressures acting on these genes cause concomitant divergent evolutionary histories that derive phylogenetic reconstructions

that could be inconsistent with widely accepted intertaxa evolutionary relationships elucidated with other molecular markers subjected to a neutral dynamics.

Key words: *Mhc* DQB1 sequences, *Inia*, Molecular evolution, River dolphins

INTRODUCTION

All gnatostomates (jawed vertebrates) possess major histocompatibility complex (*Mhc*) genes that encode proteins responsible for binding and displaying self and foreign peptides to T-cells to either eliminate self-reactive T-cells early in development in the thymus or elicit immune responses against pathogens (Doherty and Zinkernagel, 1975; Klein and Takahata, 1990). Specifically, *Mhc* class II *DQB* genes are expressed only by antigen-presenting cells, and encode cell surface proteins with a peptide-binding region (PBR) that bind and present self and foreign peptides to CD4 T-cells, to trigger the immune response (Brown et al., 1993). *DQB* genes comprise 6 exons. Exon 1 encodes the leader peptide, and exon 2, part of the variable PBR. This region is of biological importance because this is where the rest of the polypeptide chain (between 13 to 18 amino acids) of the foreign peptide is linked (Brown et al., 1988). Most of the polymorphism in the *Mhc* molecules occurs at the PBR, creating variation for binding specificity (Edwards and Hedrick, 1998). Additionally, exons 3, 4 and 5 encode the extracellular, transmembrane and cytoplasmic domains, respectively (Brown et al., 1993). A substantial amount is known about *Mhc* polymorphism and evolution in humans and certain captive primate populations (e.g., Klein et al., 1993; Bergström et al., 1999; Bontrop et al., 1999). In humans, particular *Mhc* variants are related to disease resistance (Hill et al., 1991; Jepson et al., 1997; Hill, 1999; Carrington et al., 1999). Such studies suggest that species with low *Mhc* variation may be more susceptible to infectious disease, and that some endangered species living in small, isolated populations could face additional threats of extinction from exposure to pathogens and parasites (Evermann et al., 1988; Lyles and Dobson, 1993; Mikkos et al., 1999; Murray et al., 1999; Lukas et al., 2004). *Mhc* polymorphism results from high-nonsynonymous nucleotide substitution rates, which shuffle the amino acids of histocompatible proteins, and ultimately shift the physiochemical properties of Mhc proteins. Such high-nonsynonymous substitution rates are mostly attributed to positive selection, like that exerted by pathogen diversity, load and virulence (Hughes and Nei, 1989; Garrigan and Hedrick, 2003). Additionally, a group of functional genes known to be more polymorphic within the *Mhc* has been demonstrated to be related to male sexual discrimination on the part of females. For instance, Potts et al. (1991) and Manning et al. (1992) performed different experiments demonstrating that *Mhc* genotypes can affect mate choice prior to fertilization in rodents. These mammals can distinguish *Mhc* genotypes on the basis of differential odors in the urine. The first study showed that female mice significantly preferred to settle in areas held by a male with unlike *Mhc* genotypes. Moreover, females tended to search for males that had *Mhc* genotypes different from that of the males of her own territory for extraterritorial copulations. The second study demonstrated that mice were able to distinguish

full-siblings from half-siblings on the basis of *Mhc* genotypes (*Mhc* genotypes as a signal for direct kin recognition). Additionally, the same study could demonstrate that mice were capable of dispensing altruistic behavior depending on the *Mhc* genotypes of the individuals. In fact, Wedekind et al. (1995) showed that the human females gave higher marks for men's odors for pleasantness, to men with *Mhc* genotypes dissimilar to their own (HLA-A, HLA-B and HLA-DR). It seems that if women were to choose a mate whose *Mhc* genotype is different than her own, they would enjoy better fittness as a result of higher rates of offspring survival and reduced inbreeding (Potts and Wakeland, 1993). On the other hand, Ober et al. (1992) showed in a Hutterite community of South Dakota that when human couples shared alleles at HLA-DR and HLA-B, they had lower fertility and higher spontaneous abortion rates than couples with no shared *Mhc* alleles.

Mhc DQB genes have been used as molecular markers in studies of population genetics, evolution, molecular ecology, and conservation genetics because they are the most polymorphic group of functional genes known from vertebrate genomes (Yuhki and O'Brien, 1990; Hambuch and Lacey, 2002) and because they are submitted to the direct influence of natural selection.

The marine mammals are considered a group of special interest within evolutionary genetics due to their special characteristics since they reflect a fast adaptation, in evolutionary time, to an aquatic lifestyle (Milinkovitch and Thewissen, 1997; Heyning and Lento, 2002). This supposes important morphological and physiological modifications and implies that these animals could be subjected to different pathogen pressures compared to those that afflict terrestrial mammals.

Most research on cetacean *Mhc* diversity and evolution is in an exploratory phase (e.g., Trowsdale et al., 1989; Flores-Ramírez et al., 2000, 2004). First assessments of *Mhc* polymorphism in marine mammals reported low variation in southern elephant seals (*Mirounga leonina*) and rorquals (*Balaenoptera physalus* and *B. borealis*) using MHC restriction fragment length polymorphisms, and were attributed to a supposed weak pathogenic pressure (Trowsdale et al., 1989; Slade, 1992). Later sequencing analyses revealed considerable levels of *Mhc*-I and *Mhc*-II polymorphism, due to frequent nonsynonomous substitutions, in beluga whales (*Delphinapterus leucas*), narwhal (*Monodon monoceros*), four species of pinnipeds, and gray whales (*Eschrichtius robustus*) (Murray et al., 1995; Murray and White, 1998; Hoelzel et al., 1999; Flores-Ramírez et al., 2000, 2004), suggesting that Darwinian selection drives *Mhc* polymorphism in marine mammals, and like for terrestrial vertebrates, this might reflect on their population dynamics (Yuhki and O'Brien, 1990). Only two published studies have been performed at the population level in cetaceans, comprising beluga whales (*Delphinapterus leucas*) and narwhals (*Monodon monoceros*) (Murray et al., 1995; Murray and White, 1998). Therefore, *Mhc* analyses on aquatic mammals have concentrated on marine species. Until now, there is only one published paper on a freshwater mammal. This is the case of the Chinese river dolphin (Yang et al., 2005). We report herein the first sequences of *Mhc* class II (DQB-1 locus) for the Amazon pink river dolphins (*Inia geoffrensis* and *Inia boliviensis*).

Some features of this dolphin genus are as follows: the pink river dolphin, also called boto, bufeo or bugeo, has a unique natural history. Therefore, it is noteworthy to explore the significance of *Mhc* diversity in cetaceans living in freshwater Rivers at the Amazon and Orinoco basins. The genus *Inia* contains two species, *Inia boliviensis*, which inhabits the Bolivian Amazon (Mamoré and Guaporé (= Iténez) Rivers and effluents), and *Inia geoffrensis*, living in the main rivers of the Amazon in Ecuador, Peru, Colombia, and Brazil, and in the Orinoco River

basin in Colombia and Venezuela (Banguera-Hinestroza et al., 2002). These two species are separated by 400 km of falls in the Madeira River (from Guayaramerín, in Bolivia, to Porto Velho, in Brazil).

Among the river dolphins, the boto seems to have the most stable population sizes, although its IUCN status is vulnerable, as its habitat is over exploited (Best and da Silva, 1989). In spite of *Inia*'s extensive distribution, migratory patterns and reproductive behavior in general are not known, but certainly are very dynamic given the strong seasonal fluctuations of river margins which are surpassed during the rainy season and expand to cover thousands of kilometers of forest. In contrast, during the dry season, river margins and tributaries retract and the dolphins can be found isolated in small lagoons (Best and da Silva, 1989; Da Silva, 1994). Interestingly, Martin and da Silva (2004a,b) found evidence that adult dolphins are largely segregated by sex and reproductive status, except in low water. Thus, as water levels increase, females and calves move into the most remote parts of the inundated areas, while males move to the main channels and rivers. The overall sex ratio seems to be around one.

There are two different kinds of rivers in the Amazon and Orinoco basins. The black rivers, with low productivity because of their low pH range, a high concentration of tannins and few organic resources, and the white rivers, which have contrastingly high levels of suspended sediments and organic resources with high productivity. Formerly, it was believed that dolphins could not live in black rivers due to the low pH. However, recent studies documented the presence of dolphins in such rivers in relation to fish density and not to water pH. Thus, these animals live in two different habitats and probably the pathogen pressure on these dolphins in these two different river systems is different as well.

In summary, two *Inia* species appear to occupy quite distinct habitats separated by 400 km of inaccessible habitat dominated by waterfalls. Both habitats are intrinsically heterogeneous, displaying seasonal flooding and specific ecological settings in specific black or white tributaries. The latter has compelled us to hypothesize that dolphins inhabiting such divergent habitats, as those of the main and Bolivian Amazon basins, have been subjected to differential Darwinian selection associated with their distinct parasite diversity, virulence and load, which have shaped the evolution and polymorphism of their pathogen recognition system. As a consequence, we conducted this preliminary study aiming to isolate and characterize the first *Mhc DQB*-1 sequences from *Inia*, which would lead to future studies to elucidate the significance of *Mhc* polymorphism in phylogenetically related cetacean species under distinct selection regimes.

Finally, studies are scarce due to the almost impossible task of taking blood samples from cetaceans without disturbing and compromising the welfare of these highly protected or endangered animals, as with the river dolphins.

MATERIAL AND METHODS

To isolate and characterize *Inia*'s *Mhc DQB*-1 sequences, skin biopsies were obtained from two individuals, one *I. geoffrensis* (PB12) and one *I. boliviensis* (B3), that were respectively sampled in Peruvian and Bolivian Amazon tributaries. The exact geographic locations for these animals were as follows. The Peruvian dolphin was caught at Tipishca del Loro in the Curaray River. It was an adult male of 195 cm. The Bolivian specimen was an adult male 207 cm in length and was caught at the Porvenir Lagoon in the Mamore River. In the laboratory, total DNA was extracted from each sample using a standard organic extraction protocol (Sam-

brook et al., 1989). Primers previously designed to amplify human and homologous cetacean *DQB*-1 sequences (Murray et al., 1995; Munguía-Vega, 2002): 5'-CTG GTA GTT GTG TCT GCA CAC-3' and 5'-CAT GTG CTA CTT CAC CAA CGG-3', were used to amplify 172 bp of the variable *DQB*-1 exon 2 that encode the PBR and thus most of the functional residues of class II DQB molecules. PCR products of expected size were obtained using 0.5 mM of each primer, 1X buffer, 2.5 mM $MgCl_2$, 1 U Taq polymerase (Invitrogen), 0.2 mM dNTPs, and 10-50 ng DNA. PCR reactions were conducted in a Techne Genius thermocycler, according to a thermal profile consisting of 1 cycle at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products of expected size were cloned (pCR2,1 vector, Topo TA cloning kit, Invitrogen) and sequenced on both strands (Macrogene Inc., Seoul, Korea). The histocompatibility nature of the sequences obtained was confirmed based on their shared homology with vertebrate *DQB*-1 sequences deposited in GeneBank, using the NCBI BLAST search tool.

The sequences were aligned using Clustal W (Thompson et al., 1994). A gene tree analysis of boto DQB sequences was performed with the neighbor joining method (Saitou and Nei, 1987) using the Kimura's two-parameter distance (Kimura, 1980), and with the help of MEGA 2.1 software (Kumar et al., 1993).

RESULTS AND DISCUSSION

The two DQB DNA sequences obtained are shown in Figure 1 and were deposited in the GeneBank. The deduced amino acid translation of the sequences produced two different proteins with open-reading frames (Figure 2) that seem to be functional. A comparison with the GeneBank database sequences found that *Inia* DQB exon 2 sequences share high similarity with other Odontoceti, such as sperm whales (Physeteridae) and Monodontidae, while the amino acid translation sequence showed similarity with sperm whale and porpoise (Phocoenidae) sequences. Figure 3 shows the phylogenetic relationships among the cetacean sequences analyzed. Bootstrap values supported clustering between *Inia* and the Monodontidae (narwhales and beluga whales), one of the families of Delphinoidea. Maximum parsimony trees were also constructed; they showed the same topology as the tree presented in Figure 3.

Inia boliviensis

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  1 ACGGAGCGGG TGCGGTTCGT GAGCAGATAC ATCTATAACC GGGAGGAATA CGTGCGCTTC
 61 GACAGCGACG TGGGCGAGTA CCGGGCGGTG ACCGAGCTGG GCCGGCCGTA CGCCGAGTAC
121 TGGAACAGGC AGAAGGACAT CCTGGAGCAG ACACGGGCCG AGCTGGACAC G
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Inia geoffrensis

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  1 ACGGAGCGGG TGCGGTACAT GAGCAGATAC ATCTATAACC GGGAGGAATA CGTGCGCTTC
 61 GACAGCGACG TGGGCGAGTA CCGGGCGGTG ACCGAGCTGG GCCGGCCGTA CGCCGAGTAC
121 TGGAACAGGC AGAAGGACAT CCTGAGACGC AGACGGGCCG AGGTGGACAC G
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Figure 1. DQB MHC-II exon 2 sequences of *Inia geoffrensis* and *Inia boliviensis*.

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                    *          20         *          40         *
IniaB3         TERVRFVSRY IYNREEYVRF DSDVGEYRAV TELGRPYAEY WNRQKDILEQ TRAELDT 57
IniaPB12       .....L.... .......... .......... .......... ........RR R...V.. 57
AY333410Live   .....YMT.H V......... .......... .....RT... ..S...L... R...V.. 57
AB164228Glma   .....L.D.. .......... ......F... .....RT... ..S......E E..A... 57
AB164227Glma   .......D.. ......F... ......H... ......D... ..S....... K...... 57
AB164226Glma   .....L.I.R ......FL.. ......F... .....RI..N ..S....... E..YV.. 57
AB164225Laob   .......D.. ......F... ....DDF... .....RT... ..S.E....E E..A... 57
AB164224Laob   .....H.... ......F... ......F... ......D... ..S....... E..DV.. 57
AB164223Orbr   .......D.. ......F... ......F... ......D... ..S......R K...... 57
AB164219Neph   .....L.E.. .H........ .......... ......D.K. ..G....... K...... 57
AB164218Neph   .....L.E.. .......... .......... .....RT... ..G....... K...... 57
AB164217Neph   .......E.H .......... .......... ......D.K. ..G....... K...... 57
AB164216Neph   .......E.H ......FL.. .......... .....QI..N ..G....... K...... 57
AB164215Neph   .....L.E.H .......... .......... ......D.K. ..G....... K...... 57
AB164214Neph   .....L.E.. .......... .......... ......D.K. ..G....... K...... 57
AB164213Neph   .....L.E.. ......F... .......... ......D.K. ..G....... K...... 57
AB164212Neph   .......E.. .......... .......... ......D.K. ..G....... K...... 57
AB164211Phph   .......E.R .......... .......... .....RT... ..G....... K...... 57
AB164210Meca   .....S..S. ......FL.. ....D..... ......D... ..S......R ....... 57
AB164209Mest   .....S.N.. .......... ....D..... .....LD... ..S....... ......A 57
AB164208Phca   .....Y.T.. .......... .......... ......D... ..S......R ....... 57
AB164207Babo   .....L.E.H ......FL.. .......... S.....T.K. ..SR...... K...V.. 57
AB164206Babo   .....L.V.H .......A.. .......... S.....D.K. ..S...L..E S..AV.. 57
AB164205Babo   .....Y.T.. .......A.. .......... S.....S.K. ..S....... ....V.. 57
AB164201Baac   .....Y.T.. .......A.. .......... S.....D.K. ..S...F... ....... 57
AB164200Bamu   .....A.V.H .......... .......... ......D... ..S......K R...V.. 57
AB164199Baphy  .....Y.T.. .......A.. .......... S.....D.K. ..S......K ....... 57
AB164198Euau   .....L.T.. ......L... .......... S.....D... ..S....... ....... 57
AY170897Phsi   .....L.E.H ......F... .......... .....QI..N ..G....... K..A... 57
U16990Dele     .....L.T.. .......... .......... .....RT... ..S......R ....... 57
U16989Dele     .....L.T.. .......... .......... .....RT... ..S......R .....E. 57
U16988Dele     .....L.... ......L.H. .......... ......D... ..S......R ...K... 57
U16991Momo     .....L.... .......... .......... .....RT... ..S......R ....... 57
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Figure 2. Amino acid alignment of diverse cetacean MHC-II DQB loci. Baac, *Balaenoptera acutorostrata*; Babo, *Balaenoptera bonaerensis*; Bamu = *Balaenoptera musculus*; Baphy, *Balaenoptera physalus*; Euau, *Eubalaena australis*; Dele, *Delphinapterus leucas*; Glma, *Globicephala macrorhynchus*; Laob, *Lagenorhynchus obliquidens*; Live, *Lipotes vexillifer*; Meca, *Mesoplodon carlhubbsi*; Mest, *Mesoplodon stejnegeri*; Momo, *Monodon monoceros*; Neph, *Neophocaena phocaenoides*; Orbr, *Orcaella brevirostris*; Phca, *Physeter catodon (macrocephalus*); Phph, *Phocoena phocoena;* Phsi, *Phocoena sinus*.

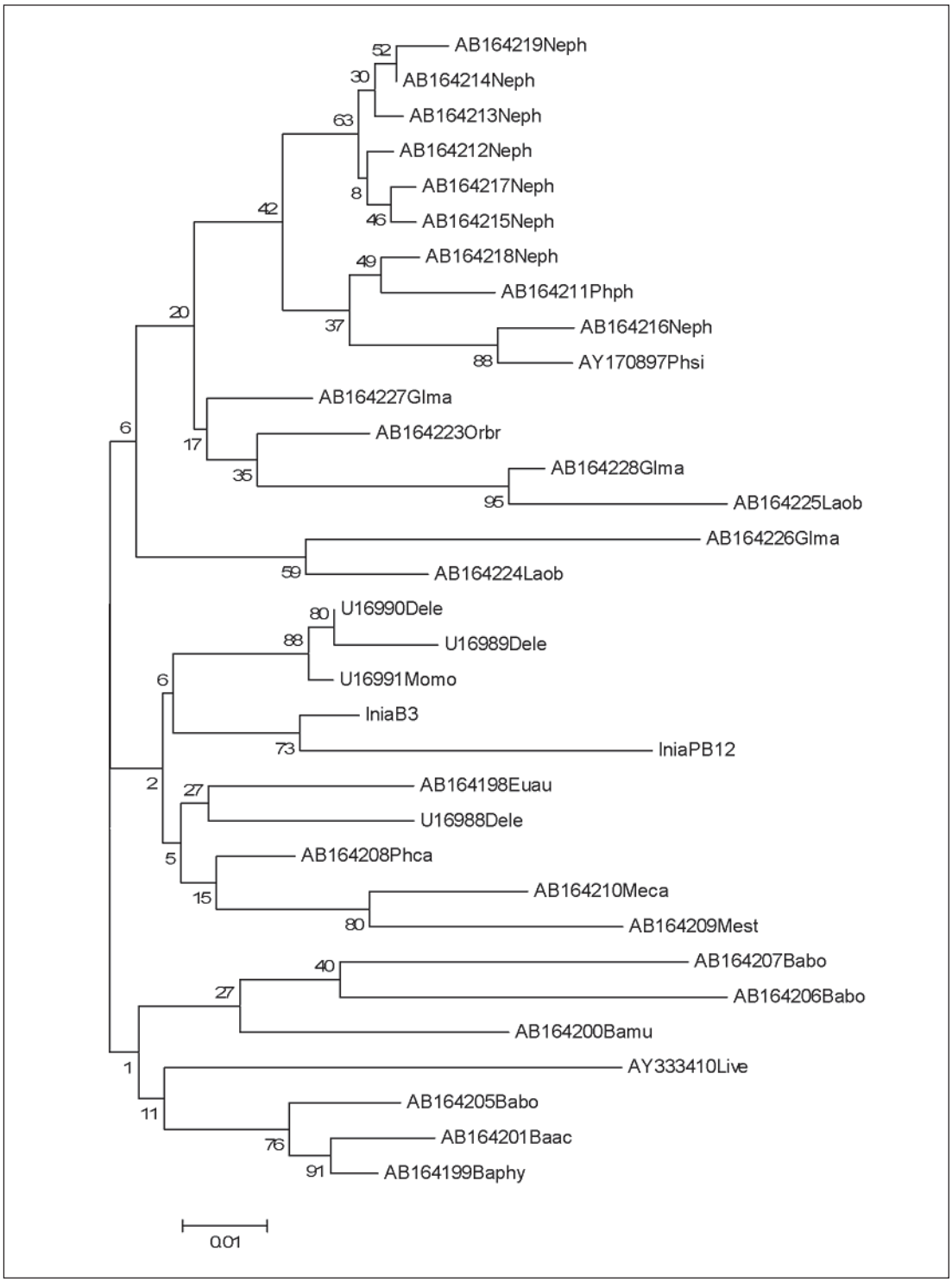

Figure 3. Phylogeny of *Inia* DQB MHC-II exon 2 sequences, based on alignments of cetacean DQB sequences constructed using the neighbor joining method in the MEGA program (Kumar et al., 1993). See Figure 2 legend for abbreviations.

These results could be compared with some previously published cetacean phylogenies incorporating several river dolphins (Milinkovitch et al., 1994; Cassens et al., 2000; Hamilton et al., 2001). Milinkovitch et al. (1994) were the first to demonstrate, using 1352 bp of two mitochondrial ribosomal and cytochrome b genes, that *Inia* is a sister species of the superfamily Delphinoidea and within this superfamily, *Delphinapterus leucas* (Beluga) is the most closely related to *Inia*. In fact, the possible sister phylogenetic relationship between *Inia* and the Delphinoidea had been previously claimed based on morphometric studies (Heyning and Mead, 1990) and myoglobin data (McKenna, 1987). Also, Cassens et al. (2000), employing mitochondrial 12S and 16S rRNA and cyt-b genes as well as two nuclear genes (the gene encoding the interphotoreceptor retinoid-binding protein and lactalbumin gene), showed that *Inia* is the sister genus of another partially freshwater river dolphin (*Pontoporia blainvillei*) and both are the sister clade of Delphinoidea, with Monodontidae and Phocoenidae being the families least divergent from *Inia*. The divergence time of *Inia* and *Pontoporia* from Delphinoidea was around 31 millions of years ago, based on a maximum likelihood tree. The molecular trees of Arnason and Gullberg (1996) and Hamilton et al. (2001) also revealed that *Iniidae* is the sister clade of Delphinoidea, being less differentiated from Monodontidae and Phocoenidae than the Delphinidae, which radiated more recently. However, the phylogenetic position of the Baiji (*Lipotes vexillifer*), the Yangtze river dolphin, is questionable. Several studies from a morphological and paleontological perspective (Heyning, 1989; Barnes, 1990) and based on molecular evidence (Yang and Zhou, 1999) have postulated that *Lipotes* (Lipotidae) is the sister clade of Iniidae and Pontoporidae. Our results disagree with this view point, because *Lipotes* clustered in a different clade. Our *Mhc* sequences are more in agreement with the morphological studies of Muizon (1991) and Messenger and McGuire (1998), as well as with the molecular analyses of Cassens et al. (2000) and Hamilton et al. (2001), which showed that the real sister clade of Iniidae and Pontoporidae is Delphinoidea. Nonetheless, *Mhc* has a trans-specific mode of evolution; therefore, the genetic lineages usually are more ancient than the species split (Nei and Huges, 1991). Moreover, the selective patterns affecting the *Mhc* genes of *Lipotes* in the Chinese rivers could be quite different from the selective patterns regulating the *Mhc* genes in the Amazon and Orinoco basins. Another marked feature is the relevant amount of nucleotide diversity and genetic differentiation among the diverse *Mhc* cetacean sequences analyzed and within the genus *Inia* as well. Contrarily, there is an extremely conservative evolution of other molecular markers, such as autosome and Y chromosome introns in *Inia geoffrensis* and *Inia boliviensis*, where only six nucleotide substitutions were determined among 4544 bp analyzed (Ruiz-García et al., 2006a). Identically, DNA microsatellites seem to be extremely conserved in cetaceans; Schlötterer et al. (1991) estimated a rate of neutral nucleotide substitution of about 0.09% per million years, which is quite low. In an identical fashion, Ruiz-García et al. (2006b) estimated average microsatellite mutation rates for three cetaceans, *Inia boliviensis*, *Pontoporia blainvillei* and *Sotalia fluviatilis*, all of them living in South American freshwater basins. The estimates obtained ranged from 9.71×10^{-6} to 3.7×10^{-5}, which were around the lower limit found in mammals. This means that some restrictive natural selection forces are acting upon the cetacean genomes. Contrarily, *Mhc* DQB-1 sequences seem to diverge more quickly than other nuclear sequences, supporting the possibility of different selective patterns affecting the MHC genes in these organisms, which could explain supposed inconsistencies in the cetacean phylogeny obtained by means of the MHC sequences.

In conclusion, we present the first DQB-1 sequence relationships of this river dolphin genus with other cetaceans. Additionally, we provided evidence that these sequences are polymorphic within the *Inia* genus. The sequences obtained for *Inia boliviensis* and *Inia geoffrensis* were different but highly related. This could indicate that similar pathogen pressures act upon the animals of the different Amazon rivers. These data are not yet, however, conclusive; more results could give important insights into understanding the genetic split of the Bolivian species from the other pink river dolphin as well as the different selective pressures affecting the diverse boto populations sampled by us in Peru, Colombia, Ecuador, Brazil, and Venezuela.

ACKNOWLEDGMENTS

Economic resources to carry out this study were obtained through Colciencias (Grant 1203-09-11239; Filogeografía, estructura poblacional y diversidad genética en dos especies de delfines de río, *Inia boliviensis* e *Inia geoffrensis*, mediante el uso de marcadores moleculares) and the Fondo para la Acción Ambiental (120108-E0102141; Estructura y conservación genética de los delfines de rio, *Inia* y *Sotalia*, en las cuencas de la Amazonía y Orinoquía). Additional thanks go to Dr. Fernando Trujillo (Omacha Fundation), Hugo Gálvez (Iquitos, Perú), Mariana Escovar (La Paz, Bolivia), Ariel Rodríguez (Colombia), Esteban Payán (Colombia), Dr. Diana Alvarez (Colombia), Pablo Escobar-Armel (Colombia), Armando Castellanos (Quito, Ecuador), and Isaias and his sons (Requena, Perú). Many thanks go to the Bolivian, Peruvian, Ecuadorian Ministries of Environment to facilitate obtaining the CITES and capture permissions, and especially to the Colección Boliviana de Fauna (Dr. Julieta Vargas) in La Paz (Bolivia). Thanks also go to the Pontificia Universidad Javeriana (Colombia) and to the Universidad de Baja California Sur for respective economic assistance.

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