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MHC class II expression in skin biopsies from the franciscana dolphin *Pontoporia blainvillei* and the southern right whale *Eubalaena australis*

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*Presently, there is no information about the functionality of the immune system in the franciscana dolphin (*Pontoporia blainvillei*), a small dolphin from southern Brazil. As in the case of the franciscana, the population of southern right whale (*Eubalaena australis*) which inhabits Brazilian waters has not yet been surveyed in what concerns MHC expression. For the first time it was possible to observe the DQB gene expression in skin of these two species of cetacean using RT-PCR. Skin pieces of the animals were collected to RNA extraction. The fragment corresponding to DQB exon 2 was amplified, cloned and sequenced. A total of five alleles were found, one to franciscana and four to southern right whale. The observed* d_N/d_S *ratio of 2.77 (*$P = 0.03$*) suggests that this gene is under balancing selection pressure (positive Darwinian selection). The sequences indicate that, in both species analysed, the DQB gene is functional.*

Keywords: major histocompatibility complex (MHC), skin biopsies, *Pontoporia blainvillei*, *Eubalaena australis*

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INTRODUCTION

The major histocompatibility complex (MHC) comprises a family of closely related genes and it is considered the most gene-dense and polymorphic region of the human genome. There are 224 genes in this region, and nearly half of the 128 expressed genes are related to immune responses (MHC Sequence Consortium, 1999). The highly polymorphic MHC molecules are expressed in cell surface and play a key role in the initiation and in the maintenance of immune responses by presenting antigens to the effectors cells (Kumánovics *et al.*, 2003). There are two major subfamilies of MHC genes (classes I and II), whose products are involved in distinct antigen presentation pathways. In humans, the classical class II genes (DP, DQ and DR) are constitutively expressed basically in professional antigen-presenting cells (such as macrophages, dendritic cells, B cells and on the thymic epithelium).

In marine mammals, studies focusing on the genomic organization and levels of diversity of the MHC system have been conducted with increased interest in the last years (Lehman *et al.*, 2004; Yang *et al.*, 2005; Baker *et al.*, 2006). The reduced MHC diversity described for some cetaceans and pinnipeds is approached sometimes as an indication of a less effective immune system as compared to terrestrial mammals (Slade, 1992; Murray *et al.*, 1995). Nevertheless, more information about the expression of MHC genes is essential to characterize the immune system of marine mammals as well as to improve our understanding of the interactions between these animals and their environment. One of the most studied cetaceans in Brazil is the franciscana, *Pontoporia blainvillei* (Gervais & D'Orbigny, 1844), a small coast dolphin. The population inhabiting the coast of Rio Grande do Sul (the southernmost state of Brazil) and Uruguay has been recently classified as vulnerable by the *IUCN Red List of Threatened Species* (IUCN, 2006). Studies about the behaviour and ecology of *P. blainvillei* have been conducted (e.g. Siciliano *et al.*, 2002; Secchi *et al.*, 2003; Danilewicz *et al.*, 2004), but limited data are available about the genetic diversity and phylogenetic relationships of this species (e.g. Secchi *et al.*, 1998; Lázaro *et al.*, 2004). Another cetacean observed in southern Brazil is the southern right whale, *Eubalaena australis* (Desmoilins, 1822), a conservation dependent species (IUCN, 2006). This is a migratory large whale, mating and calving off Brazil during winter and part of spring (IWC, 2001).

Analyses of MHC class II gene expression in cetacean skin were initially performed in *Tursiops truncatus* (bottlenose dolphin) using immunohistochemistry by Zabka & Romano (2003). Their study revealed a pattern of DQB genes expression that reflected the cellular structural and functional characteristics expected to this kind of protein. Besides this work, analyses of MHC expression in cetaceans were basically performed in lymphatic tissues in the harbour porpoise

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*Phocoena phocoen*a (Beineke *et al.*, 2007) and in blood lymphocytes from beluga whale *Delphinapterus leucas* (Bernier *et al.*, 2000). In the present study skin samples from franciscana dolphin and right whale were used in order to test the MHC class II DQB gene expression in cetacean skin using a more accurate method.

MATERIALS AND METHODS

Collecting samples

Samples from both cetacean species described in the present study were collected on the northern coast of Rio Grande do Sul, southern Brazil. In a rare event of live stranded animal (January, 2005), a skin sample (~5 mm) of a newborn franciscana dolphin was collected (Tramandaí, 29°58′S 50°07′W). Skin samples (~10 mm) from a free-ranging mother and calf pair of southern right whales were collected with a biopsy dart on July 2004 (Torres, 29°19′S 49°43′W). The samples from both species were stored in 4 ml of RNAlater solution (Ambion, Tx, USA) at 4°C until processing (ranging from 2–3 weeks).

RNA extraction and amplification protocols

Total cellular RNA was isolated by silica-based gel membranes combined with microspin technology to skin samples (RNeasy Mini Kit, QIAGEN, Germany) and stored at −20°C. The full length cDNA synthesis was performed using a SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the manufacturer's specifications. Universal primers were used to amplify a fragment of 212 base pairs of the DQB exon 2 peptide-binding region (PBR) as described by Murray *et al.* (1995). Briefly, the PCR reactions were carried out on 50 ng of cDNA in a total volume of 50 µl containing 20 pmol of each primer, 1.5 mM $MgCl_2$, 20 mM Tris pH8.4, 50 mM KCl, 1.5 units of recombinant *Taq* DNA polymerase (Invitrogen, Carlsbad CA, USA) and 2.5 mM of each dNTP. The amplification followed a touchdown protocol, starting with 94°C for 5 minutes, followed by 20 cycles with 94°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute, decreasing 1°C at each two cycles, followed by 20 cycles with 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and ending with an extension step of 72°C for 5 minutes. Glyceraldehide-3P-dehydrogenase (GAPDH) gene amplification was used as a positive control of expression, since it is considered to be a reliable control for gene expression studies on samples from cetacean skin biopsies (Spinsanti *et al.*, 2006). To confirm the absence of DNA on the purified samples, the RNA from each specimen was used as template for amplification, prior to the cDNA synthesis. Also, as a negative control, RNA purified from K562, a cell lineage defective on MHC expression, was used in each test. The amplification products were loaded in a 1% agarose gel and visualized under UV by ethidium bromide staining (Figure 1). A unique fragment of 212 bp, corresponding to the DQB exon 2, was amplified from each cDNA sample, excised from the gel and purified by *GFX PCR* DNA Purification Kit (GE Healthcare, NJ, USA). Purified PCR products were cloned into TOPO TA vector (Invitrogen). To confirm the sequences, 5 colonies per each sample were picked directly from the LB agar plate and the insert was amplified using the M13 primers (Invitrogen). Amplified fragments of the correct size were sequenced in a MegaBACE 1000 (Amersham Pharmacia Biotech, NJ, USA) (Figure 2). Data were confirmed by sequencing in both directions.

Statistical analyses

Several analyses were performed in order to establish if sequences corresponded to functional genes. The nucleotide sequences were analysed using the sequence alignment software programs CHROMA (Goodstadt & Ponting, 2001) and STADEN (Bonfield *et al.*, 1995), and compared using the NCBI BLAST program (Altschul *et al.*, 1990). To detect molecular-level evidence of selection, pairwise comparisons of nucleotide substitutions between alleles were conducted on a segment of 171 bp (57 complete codons) and relative frequencies of non-synonymous (d_N) and synonymous (d_S) substitutions were calculated following the Jukes & Cantor correction of modified Nei & Gojobori (1986) in MEGA4 (Tamura *et al.*, 2007). The ratio of non-synonymous to synonymous divergence was tested for departure from the neutral expectation of unity using the z statistic available in MEGA4. The amino acids' physicochemical properties were analysed using a ProtParam program from ExPASy (Gasteiger *et al.*, 2003) and their conservation properties followed Taylor's classification (Taylor, 1986).

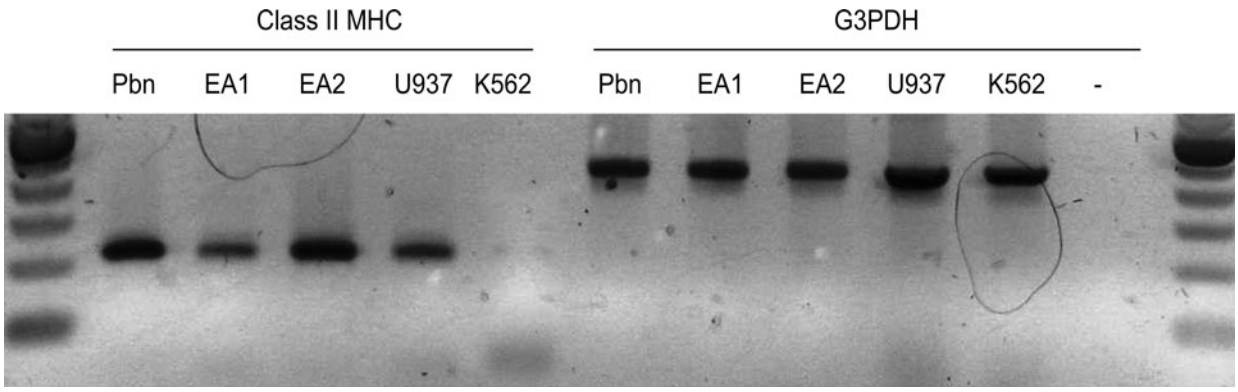

Fig. 1. Amplification of MHC fragments from franciscana and right whale cDNA. Exon 2 DQB amplified from franciscana (Pbn), right whale (mother identified as EA1 and her calf identified as EA 2), cDNA from cell lineage U937, corresponding to 212 bp. K562 cell lineage defective to MHC expression. Positive control was primers to human GAPDH (440 bp). Ladder 100 bp.

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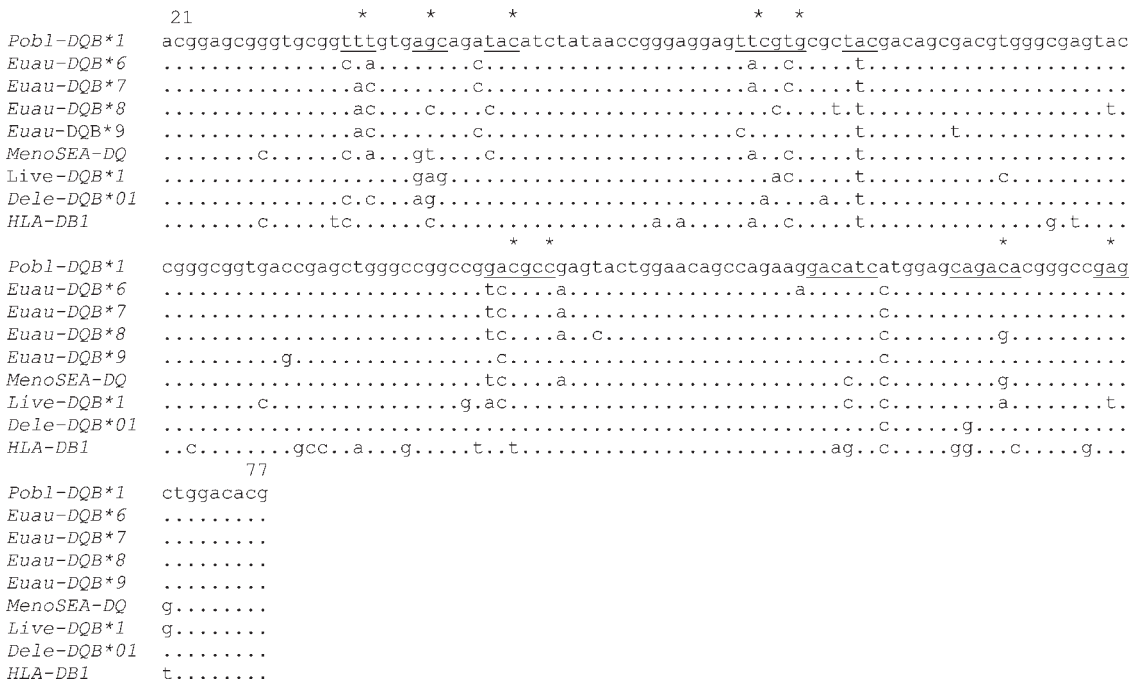

Fig. 2. DQB nucleotide sequences. In non-variable codons a dot is used to display identity. Abbreviations for individual species MHC sequences are as follows: Pobl, franciscana (*Pontoporia blainvillei*); Euau, right whale (*Eubalaena australis*); Live, baij (*Lipotes vexilifer*); Dele, beluga whale (*Delphinapterus leucas*); Meno, humpback whale (*Megaptera novaengliae*); HLA-DQB (*Homo sapiens*). Underlined codons indicate positions polymorphic in human MHC molecules and an asterisk (*) indicates codons postulated to be important to peptide binding (Brown *et al.*, 1993).

RESULTS

The analyses concerned evidence of selection and departures from neutrality for d_N/d_S ratio were carried out to southern right whale. Since we have just one franciscana sample we preferred not to include that in these analyses. The remaining analyses were conducted among the three individuals (all alleles) with the aim to identify characteristics expected to functional MHC sequences. We followed the standard four-letter abbreviations for species recommended for MHC nomenclature and adapted from Baker *et al.* (2006). We observed five alleles, one from franciscana (*Pobl-DQB*1*) and four different alleles from right whales (*Euau-DQB* 6–9*). The mother and calf southern right whales shared one allele (*Euau-DQB*7*). Besides that, both have exclusive alleles, the calf has *Euau-DQB*9* and the mother has *Euau-DQB* 6* and *8*. The sequences were deposited in the GeneBank (access numbers EU048216–EU048220). This elevated number of alleles found in right whale was expected since the existence of three copies of the DQB gene in the southern right whale genome was already suggested (Baker *et al.*, 2006). The nucleotide and deduced amino acid sequences of the amplified products were typical of transcripts from mammalian class II genes (Figures 2 & 3). The five alleles identified in the two species do not show insertions, deletions or stop codons, suggesting a functional role *in vivo*. Nevertheless, we were able to identify nucleotide substitutions in all possible three codon positions. Nine nucleotide substitutions could be assigned to both first and second bases and four to the third base of codons. Pairwise comparisons of nucleotide substitutions also indicated a functional status of southern right whales alleles. The average of d_N and d_S 0.075 and 0.027, respectively, resulting in a ratio of $d_N/d_S = 2.77$ significantly different from unity ($P = 0.03$). This high substitution ratio suggests a balancing selection pressure (positive Darwinian selection), which was expected from this particular region of the gene as shown in the functional PBR in humans (Huges & Nei, 1988). There are 14 predicted positions as important to peptide binding (28, 30, 32, 37, 38, 47, 56, 60, 61, 65, 68, 70, 71 and 74) in humans (Brown *et al.*, 1993). As expected, the sequences derived from *P. blainvillei* and *E. australis* differed from other MHC sequences mainly in and around the sites predicted to be important to peptide binding and recognition (Figures 2 & 3). The franciscana and right whale amino acid sequences correspond to sites 21–77 of the beluga DQB1 sequences (Murray *et al.*, 1995).

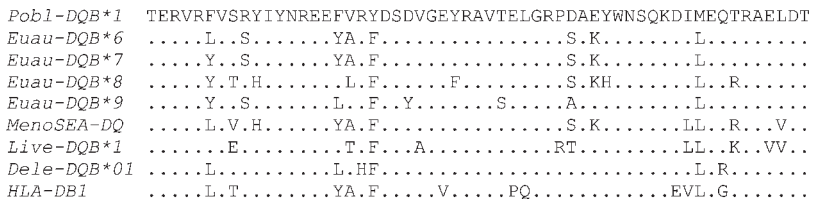

Fig. 3. Predicted amino acid sequences of DQB alleles. To ease comparison no character is used to display identity. The identification of sequences follows the abbreviations used for DQB nucleotide sequences. For physiochemical characteristics information see the text.

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Polymorphism was observed in residues 28, 37, 38, 47, 60, 65, 68 and 71, as well as in positions considered polymorphic within human PBR but not described as relevant to peptide recognition and binding (26, 29, 39, 40, 43, 51, 58 and 59). This same situation was previously described in other marine mammal sequences (Murray *et al.*, 1995; Yang *et al.*, 2005).

Two of the most important physiochemical amino acid characteristics that determine the protein structure are volume and type (hydrophobicity). In the positions 29 (Arg/Ser), 38 (Val/Ala/Leu), 40 (Tyr/Arg) and 43 (Asp/Tyr) the different amino acids observed at the different alleles presented a weak conservation of these physiochemical characteristics. In positions 28 (Ser/Thr), 30 (Tyr/His), 57 (Asp/Ser/Ala) and 59 (Gln/Lys) the replacements lead to a conservation degree varying from moderate to weak, concerning type and volume of amino acid, whereas in positions 26 (Phe/Leu/Tyr) and 37 (Phe/Leu/Tyr) the substitutions involved amino acid residues with quite similar characteristics (strong conservation). The strong conservation of physiochemical amino acid characteristics was also observed in the predicted positions outside the peptide recognition sites. A similar comparison clustering the four alleles from the southern right whale provided similar results, suggesting that this substitution pattern is related to the MHC molecule itself, reinforcing that the described alleles will lead to functional MHC molecules. This pattern suggests that the bias in amino acid usage with less conserved physiochemical characteristics is important to the molecule function. It is expected in a functional system that these sites influence the diversity of molecules' topologies allowing a less restrictive interaction with antigens within a population. The five new alleles were compared to previously published DQB exon 2 sequences of mammals and revealed a high sequence similarity with human (88%), bovine (91%) and canine (89%) as well as with sequences derived from other marine mammals: *Delphinapterus leucas* (94%), *Balaenoptera musculus* (94%) and *Zalophus californianus* (91%).

DISCUSSION

Marine mammals are subject to characteristic selective pressures in the sea environment, such as physiological constraints due to the availability of oxygen, low temperatures and high pressure that are considered to be immunosuppressive to other species (King *et al.*, 2001). In addition, over the last decades, these species have been subjected to environmental degradation, exposure to contaminants and climate changes that have been implicated in the emergence of new marine diseases (Harvel *et al.*, 1999). In a study with two species of southern American river dolphins, *Inia geoffrensis* and *Inia boliviensis* (Martínez-Agüero *et al.*, 2006), the authors observed different sequences, identified as DQB alleles, on each species. The authors hypothesized that the difference observed could reflect a different selection pressure generated by differences in environment, as well as distinct parasite diversity, virulence and load. Nevertheless this can be corrected; it is necessary to improve the knowledge about marine mammals' immune system and its potential to elicit a response against pathogens and in developing resistance to them before more assumptions can be made. There is little information about MHC class II expression in marine mammals. The skin is a natural barrier against injuries and pathogens and it is known that important genes related to immunity can be expressed at this tissue, e.g. classical class I MHC genes. Even considering the scarcity of studies demonstrating association between specific MHC alleles and diseases or parasite prevalence in wild populations, the identification of new alleles as well as the analysis of MHC gene expression will provide important tools for population management. The knowledge about marine mammals' immune system will be useful to identify the diversity of natural populations, explain such specific immune processes and to perform comparative evolutionary studies. In conclusion, the detection of MHC class II genes expression in skin of cetaceans by molecular methods provides an accurate tool for immunological studies with cetaceans and gives support to the utilization of skin when the use of other tissues are less feasible.

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REFERENCES

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410.

Baker S.C., Vant M.D., Delabout M.L., Lento G.M., O'Brien S.J. and Yuhki N. (2006) Diversity and duplication of DQB and DRB-like genes of the MHC in baleen whales (suborder: Mysticeti) *Immunogenetics* 58, 283–296.

Beineke A., Siebert U., Stott J., Muller G. and Baumgärtner W. (2007) Phenotypical characterization of changes in thymus and spleen associated with lymphoid depletion in free-ranging harbor porpoises (*Phocoena phocoena*). *Veterinary Immunolgy and Immunopathology* 117, 254–65.

Bernier J., De Guise S., Martineau D., Beland P., Beaudet M. and Fournier M. (2000) Purification of functional T lymphocytes from splenocytes of the beluga whales (*Delphinapterus leucas*). *Developmental and Comparative Immunology* 24, 653–662.

Bonfield J.K., Smith K.F. and Staden R. (1995) A new DNA sequence assembly program. *Nucleic Acids Research* 24, 4992–4999.

Brown J.H., Jardetzhy T.S., Gorga J.C., Stern L.J., Urban R.G., Strominger J.L. and Wiley D.C. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 364, 33–39.

Danilewicz D., Claver J.A., Pérez Carrera A.L., Secchi E. and Fontoura N.F. (2004) Reproductive biology of male franciscanas (*Pontoporia blainvillei*) (Mammalia: Cetacea) from Rio Grande do Sul, southern Brazil. *Fishery Bulletin* 102, 581–592.

Gasteiger E., Gattikerm A., Hoogland C., Ivanvi Appel R.D. and Bairoch A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research* 31, 3784–3788.

Goodstadt L. and Ponting C.P. (2001) CHROMA: consensus-based coloring of multiple alignments for publication. *Bioinformatics* 17, 845–846.

Harvel C.D., Kim K., Burkholder J.M., Colwel R.R., Epstein P.R., Grimes D.J., Hofmann E.E., Lipp E.K., Osterhaus O.D., Overstreet R.M., Porter J.W., Smith G.W. and Vasta G.R. (1999) Emerging marine disease—climate links and anthropogenic factors. *Science* 285, 1505–1510.

Huges A.L. and Nei M. (1988) Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. *Nature* 335, 167–170.

IUCN (2006) *Red List of Threatened Species: A Global Species Assessment*. Gland, Switzerland and Cambridge, UK: IUCN. Available from http://www.iucnredlist.org/search/details.php/41761/all (accessed December 2006).

IWC (2001) Report of the Workshop on the Comprehensive Assessment of Right Whales: A Worldwide Comparison. *Journal of Cetacean Research and Management* (Special Issue) 2, 1–35.

King D.P., Aldridge B.M., Kennedy-Stoskopf S. and Stott J.L. (2001) *CRC handbook of marine mammal medicine*. 2nd edition. Boca Raton, FL: CRC Press.

Kumánovics A., Takada T. and Lindhal K.F. (2003) Genomic organization of the mammalian MHC. *Annual Review of Immunology* 21, 629–57.

Lázaro M., Lessa E.P. and Hamilton H. (2004) Geographic genetic structure in the franciscana dolphin (*Pontoporia blainvillei*). *Marine Mammal Science* 20, 201–214.

Lehman N., Decker D.J. and Stewart B.S. (2004) Divergent patterns of variation in major histocompatibility complex class II alleles among Antarctic phocid pinnipeds. *Journal of Mammalogy* 85, 1215–1224.

MHC Sequencing Consortium (1999) Complete sequence and gene map of a human major histocompatibility complex. *Nature* 401, 921–923.

Martínez-Agüero M., Flores-Ramírez S. and Ruíz-Garcia M. (2006) First report of major histocompatibility complex class II loci from the Amazon pink river dolphin (genus *Inia*). *Genetics and Molecular Research* 5, 421–431.

Murray B.W., Malik S. and White B.N. (1995) Sequence variation at the major histocompatibility complex locus DQB in beluga whales (*Delphinapterus leucas*). *Molecular Biology and Evolution*, 12, 582–593.

Nei M. and Gojobori T. (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Molecular Biology and Evolution* 3, 418–426.

Secchi E.R., Wang J.Y., Murray B.W., Rocha-Campos C.C. and White B.N. (1998) Population differentiation in the franciscana (*Pontoporia blainvillei*) from two geographic locations in Brazil as determined from mitochondrial DNA control region sequences. *Canadian Journal of Zoology* 76, 1622–1627.

Secchi E., Ott P.H. and Danilewicz D. (2003) Effects of fishing bycatch and the conservation status of the franciscana dolphin, *Pontoporia blainvillei*. In Gales N., Hindell M. and Kirkwood R. (eds) *Marine mammals: fisheries, tourism and management issues*. Collingwood, Australia: CSIRO Publishing, pp. 174–191.

Siciliano S., Di Beneditto A.P. and Ramos R. (2002) A toninha, *Pontoporia blainvillei* (Gervais & d'Orbigny, 1844) (Mammalii, Cetacea, Pontoporiidae) nos estados do Rio de Janeiro e Espírito Santo, costa sudeste do Brasil: caracterizações dos habitats e fatores de isolamento das populações. *Boletim do Museu Nacional, Zoologia* 476, 1–15.

Slade R.W. (1992) Limited MHC polymorphism in the southern elephant seal: implications for MHC evolution and marine mammal population biology. *Proceedings of the Royal Society of London, Series B* 249, 163–171.

Spinsanti G., Panti C., Lazzeri E., Marsili L., Casini S., Frati F. and Fossi C.M. (2006) Selection of reference genes for quantitative RT-PCR studies in striped dolphin (*Stenella coeruleoalba*) skin dolphins. *BMC Molecular Biology* 7, 32.

Tamura K., Dudley J., Nei M. and Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596–1599.

Taylor W. (1986) The classification of amino acid conservation. *Journal of Theoretical Biology* 119, 205–218.

Yang G., Yan J., Zhou K. and Wei F. (2005) Sequence variation and gene duplication at MHC DQB loci of baiji (*Lipotes vexilifer*) a Chinese river dolphin. *Journal of Heredity* 96, 310–317.

and

Zabka T. and Romano T. (2003) Distribution of MHC II (+) cells in skin of the Atlantic bottlenose dolphin (*Tursiops truncatus*): an initial investigation of dolphin dendritic cells. *The Anatomical Record, Part A* 273A, 636–647.

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