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Molecular Characterization of Major Histocompatibility Complex Class II *DQB* and *DRB* Genes in Bottlenose Dolphins (*Tursiops truncatus* and *T. aduncus*) from the Western Pacific

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Wei-Cheng Yang, Jer-Ming Hu, and Lien-Siang Chou (2007) Molecular characterization of major histocompatibility complex class II *DQB* and *DRB* genes in bottlenose dolphins (*Tursiops truncatus* and *T. aduncus*) from the western Pacific. *Zoological Studies* **46**(6): 664-679. Variability of the major histocompatibility complex is very suitable for investigating a wide range of open questions in evolutionary ecology and conservation because it reflects evolutionarily relevant and adaptive processes within and between populations. Herein, we present the first full-length expressed *DQB* and *DRB* gene products in the order Cetacea from 2 species of bottlenose dolphins (genus *Tursiops*, family Delphinidae) of the western Pacific that differ in their diet, microflora, and habitat. These species might represent a natural model through which we can examine the immunogenetic consequences of different pathogenic and environmental influences on the MHC of mammals. Blood samples from 2 *T. aduncus* (from Taiwan and Indonesia) and 2 *T. truncatus* (from Taiwan and Japan) were used for RACE-PCR. The presence of 1 *DQB* locus and 2 *DRB* loci in this genus was revealed. The presence of *Tutr-DQB*, *Tuad-DRB*, and *Tutr-DRB* suggests a functional role for these molecules in pathogen-specific immune responses. However, several features do not support a traditional role for *Tuad-DQB* molecules in peptide binding, and further study is needed. The phylogenetic analysis revealed that the sequence divergence of these 2 species might reflect different selective pressures between pathogens in oceanic (*T. truncatus*) and coastal (*T. aduncus*) waters. This study provides an essential foundation for analyzing variations in MHC genes and studying infectious disease ecology in bottlenose dolphins.
http://zoolstud.sinica.edu.tw/Journals/46.6/664.pdf

Key words: Major histocompatibility complex, Bottlenose dolphins, *Tursiops*.

The risk of exposure to novel pathogens in marine species has become elevated from changes in chemical and microbial environments due to anthropogenic intrusion (de Swart et al. 1996), as emerging infectious diseases in marine environments are becoming more widely recognized (Harvell et al. 1999). Therefore, studies on the genetics of functionally important immunological genes such as those of the major histocompatibility complex (MHC) are important in marine organisms. The MHC is comprised of a family of highly polymorphic genes encoding a set of transmembrane proteins that are critical for generating immune responses (Kennedy et al. 2002). Maintaining genetic diversity of the MHC thus plays an important role in responding to rapidly evolving infectious agents that periodically afflict natural populations (Klein and Sato 1998). This diversity is thought to be maintained by balancing selection, a broad term that identifies any kind of natural selection in which no single allele is absolutely most fit (Hughes and Yeager 1998, Meyer and Thomson 2001). Exactly how much MHC diversity is required to ensure long-term population viability remains a fundamental question in conservation genetics (Miller and Lambert 2004). It is claimed that a lack of variation in the MHC may increase the susceptibility of a population to

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infectious disease epidemics, with potentially catastrophic consequences (Yuhki and O'Brien 1990, Bontrop and Watkins 2005). For example, a link between MHC diversity and an effective response to both pathogenic and toxicogenic challenges was proposed (Acevedo-Whitehouse et al. 2003). However, not all MHC genes show high diversity. The most diverse and extensively studied MHC genes are the *DQB* and *DRB* genes. Diversity of *DQB* or *DRB* genes has been characterized in many mammalian species including primates, mice, domestic mammals, and marine mammals (e.g., Schook and Lamont 1996, Yuhki and O'Brien 1997, Hughes and Yeager 1998, Mikko et al. 1999, Wagner et al. 1999, Bowen et al. 2004, Baker et al. 2006). After these studies on non-model free-ranging species were carried out, intriguing questions were raised about whether and how selection operates on the MHC of natural populations characterized by distinct pathogens and demographic and environmental conditions (Bernatchez and Landry 2003, Sommer 2005). In this study, we selected the *DQB* and *DRB* genes in bottlenose dolphins (of the genus *Tursiops*) in order to expand our understanding of how different environments influence MHC evolution in marine mammals.

Tursiops is of particular interest because it is the most common and well-known dolphin genus, but data on abundances and distributions are still far from complete and validated, and the IUCN lists this genus as data deficient in their *Red List of Endangered Species* (IUCN 2006). Bottlenose dolphins occur in all tropical and temperate waters, including littoral zones, inshore lagoons, estuaries, and bays, as well as the offshore realm (Reeves et al. 2003). In the northwestern Atlantic, there are at least 2 ecotypes of *T. truncatus* (Montagu 1812) (coastal and offshore) that differ in distribution, morphology, hemoglobin profiles, parasitology, and diet (Leatherwood and Reeves 1990). Similarly, 2 morphotypes of *Tursiops* have been described in the western Pacific. Several studies have suggested that bottlenose dolphins existing in Chinese waters are 2 separate species (Wang et al. 1999 2000a b, Perrin and Brownell 2001). The larger form generally found in deep, offshore waters was described as *T. truncatus*. The smaller form, which inhabits shallow, tropical, coastal waters is generally known as *T. aduncus* (Ehrenberg 1832, Yang 1976, Zhou and Qian 1985, Gao et al. 1995). Because shallow waters along the coast are influenced by terrestrial runoff, the diversity and abundance of pathogens in the coastal waters likely differ from those in oceanic areas (Hayashi et al. 2006). It would be interesting to investigate differences in selective forces from pathogens on immune genes between these 2 species whose diets and microflora presumably differ (Wang 2003). This provides a natural model for examining the immunogenetic consequences of different pathogenic and environmental influences on the MHC of marine mammals. This is the 1st study characterizing the full-length *DQB* and *DRB* gene sequences in the order Cetacea, which is an important foundation for future investigations on the evolutionary ecology of MHC genes and studies of the population health of bottlenose dolphins.

MATERIALS AND METHODS

Blood samples from 2 individuals of each species (Tt: *T. truncatus* and Ta: *T. aduncus*) from Taiwan (Tt1: a stranded case and Ta1: an individual kept in Hong Kong Ocean Park, Hong Kong), Japan (Tt2: kept in Hualien Ocean Park, Taiwan), and Indonesia (Ta2: kept in Hong Kong Ocean Park, Hong Kong) were directly collected into PAXgene Blood RNA tubes (QIAGEN, Valencia, CA, USA) for RNA isolation. RNA was isolated by silica-based gel membranes supplied in the PAXgene Blood RNA kit. RNA from the 2 animals of the same species was pooled into a single sample and used as a template for cDNA synthesis. The cDNA library construction, 5' and 3' gene transcript amplifications of the *DQB* and *DRB* genes, amplicon visualization, cloning, and sequencing were performed using the procedures described in Bowen et al. (2002 2004). The nucleotide sequences of the amplicons were compared using MEGA vers. 3.0 (Kumar et al. 2004). For each of the *DRB* and *DQB* gene products, regions with sequence homology were identified and used to design bottlenose dolphin MHC-specific oligonucleotides (Table 1), which were used to amplify full-length *DQB* and *DRB* gene transcripts from the pooled RNA-derived complementary (c)DNA fragments. Gene product amplification reactions contained both TtDQBFL1/TaDQBFL1 and TtDQBFL2/TaDQBFL2 (for *DQB* gene products) or TtDRBFL1/TaDRBFL1 and TtDRBFL2/TaDRBFL2 (for *DRB* gene products). RACE-PCR, amplicon visualization, cloning, and sequencing were performed using the same procedures mentioned above. Multiple clones were sequenced to maximize the identification of all sequences present in the 2 animals examined. Furthermore, these sequences were confirmed by performing 2 inde-

pendent PCRs, by examining multiple clones, and by sequencing each clone in both directions, in compliance with human lymphocyte antigen (HLA) nomenclature rules (Marsh et al. 2005). The nucleotide sequences of the RACE products were analyzed using MEGA vers. 3.0 and compared with known sequences using the NCBI BLAST program (Altschul et al. 1990). Synonymous and non-synonymous divergences (d_S and d_N) and standard errors were calculated using the Jukes and Cantor correction of Nei and Gojobori (Nei and Gojobori 1986). Sequences of *DRB* and *DQB* genes from human, cattle, and sea lion were selected, along with those identified in this study, for phylogenetic analyses. The nucleotide sequences obtained were aligned with other related mammalian MHC sequences using the CLUSTAL W method (Thompson et al. 1994) taking into account the deduced amino acid sequences. We followed Bowen et al. (2002, 2004) in identifying each exon. Phylogenetic analysis was carried out using the Neighbor-joining (NJ) method implemented in MEGA 3.0 with Kimura's 2-parameter model, and support for nodes was evaluated by 5000 bootstrap replications. Furthermore, since exon 2 includes the peptide-binding region (PBR) which usually shows much higher levels of genetic variation than other exons (Schook and Lamont 1996), we conducted 3 different datasets for tree reconstruction and comparison: full-length, only exon 2, and only exon 3-6 sequences. HLA-*DPB*1 was used as the outgroup in all analyses.

RESULTS

Characterization of bottlenose dolphin *DQB* and *DRB* cDNA clones

We obtained clones containing full-length bottlenose dolphin *DQB* and *DRB* sequences from the RACE cDNA products of 2 *T. truncatus* and 2 *T. aduncus* individuals. The nucleotide and deduced amino acid sequences of the 780- (*DQB*-primer derived) and 801 bp (*DRB*-primer derived) products were typical of transcripts from mammalian class II genes. These transcripts were characterized as *Tutr-DQB*, *Tuad-DQB*, *Tutr-DRB*, and *Tuad-DRB* based on alignments with *DQB* and *DRB* sequences of HLA, bovine lymphocyte antigen (BLA), and California sea lion lymphocyte antigen (GenBank accession nos.: HLA-*DRB*1, AY961074; BLA-*DRB*3, NM_001012680; *Zaca-DRB*, AY491464; HLA-*DQB*1, NM_002123; BLA-*DQB*, Y18202; and *Zaca-DQB*, AF503406) (Fig. 1). Furthermore, we verified that the nucleotides encoding amino acids 196 and 201 (threonine and aspartate, respectively) within exon 3 could be used to distinguish *DQB* from *DRB* products in bottlenose dolphins by following previous studies (Bowen et al. 2002, Robinson et al. 2003) (Figs. 1, 2). The longest bottlenose dolphin *DQB* and *DRB* gene products encoded molecules of 259 and 266 amino acids (aa), respectively (Fig. 2). DQβ molecules consisted of a 36-aa leader sequence, a 90-aa β1 domain, a 94-aa β2 domain, a 37-aa transmembrane domain, and a 2-aa cytoplasmic domain. DRβ sequences consisted of a 29-aa leader sequence, a 95-aa β1 domain, a 103-aa β2

Table 1. MHC class II-specific primers

Name	Primer sequence	Specificity	Amplicon
DQB-U172[a]	CYGAGGGCAGAGACTCKCC	Mammalian *DQB*	597 bp; exons 1-4
DQB-L769[a]	CTCTGGGMAGATTCAGACTG		
DRB-U182[a]	CGGGACSGAGCGGGTKC	Mammalian *DRB*	512 bp; exons 2-4
DRB-L729	CACTCAKCATCTTGCTCTG		
TtDQBFL1	ATGTCTGGGACGGTGGCTCT	*T. truncatus DQB*	797 bp; 5' exon 1-3'UTR
TtDQBFL2	TCCCCGTCAAAGCGTCCTCA		
TaDQBFL1	CTGGGTGTGAGCTGTGTTCACT	*T. aduncus DQB*	852 bp; 5'UTR-3'UTR
TaDQBFL2	TGAGGACGCTTTGACGGGGA		
TtDRBFL1	TTGCCTGCTCCCCTCACAGC	*T. truncatus DRB*	997 bp; 5'UTR-3'UTR
TtDRBFL2	GTCAAGGGGCAGGGGCTGAG		
TaDRBFL1	GGCTCCTGGATGGCAGCTCTGA	*T. aduncus DRB*	1001 bp; 5'UTR-3'UTR
TaDRBFL2	AAGGGGCAGGGGCTGAGGAA		

[a]From Bowen et al. 2002 2004.

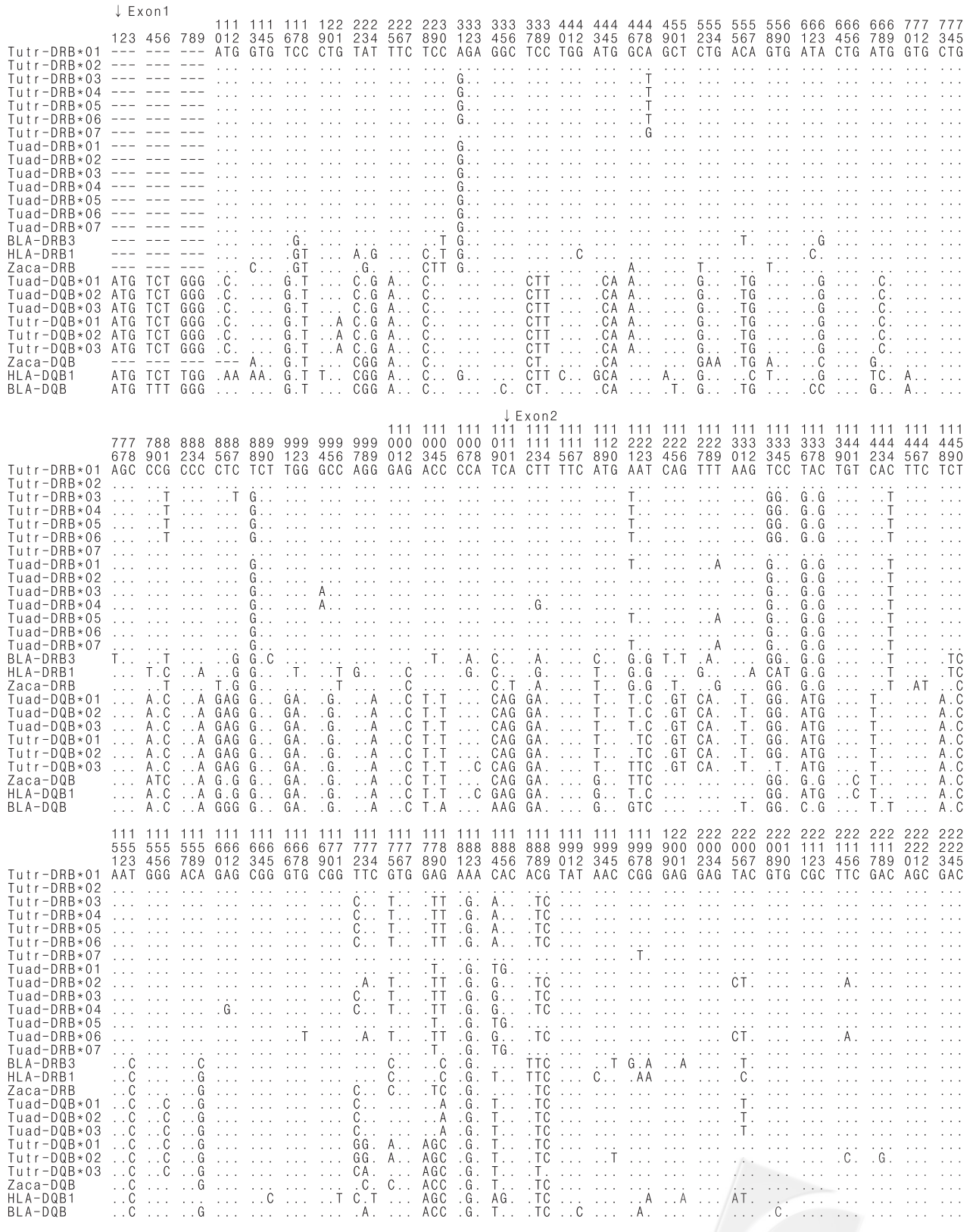
↓ Exon1
↓ Exon2
Tutr-DRB*01
Tutr-DRB*02
Tutr-DRB*03
Tutr-DRB*04
Tutr-DRB*05
Tutr-DRB*06
Tutr-DRB*07
Tuad-DRB*01
Tuad-DRB*02
Tuad-DRB*03
Tuad-DRB*04
Tuad-DRB*05
Tuad-DRB*06
Tuad-DRB*07
BLA-DRB3
HLA-DRB1
Zaca-DRB
Tuad-DQB*01
Tuad-DQB*02
Tuad-DQB*03
Tutr-DQB*01
Tutr-DQB*02
Tutr-DQB*03
Zaca-DQB
HLA-DQB1
BLA-DQB

Fig. 1. (Cont.)

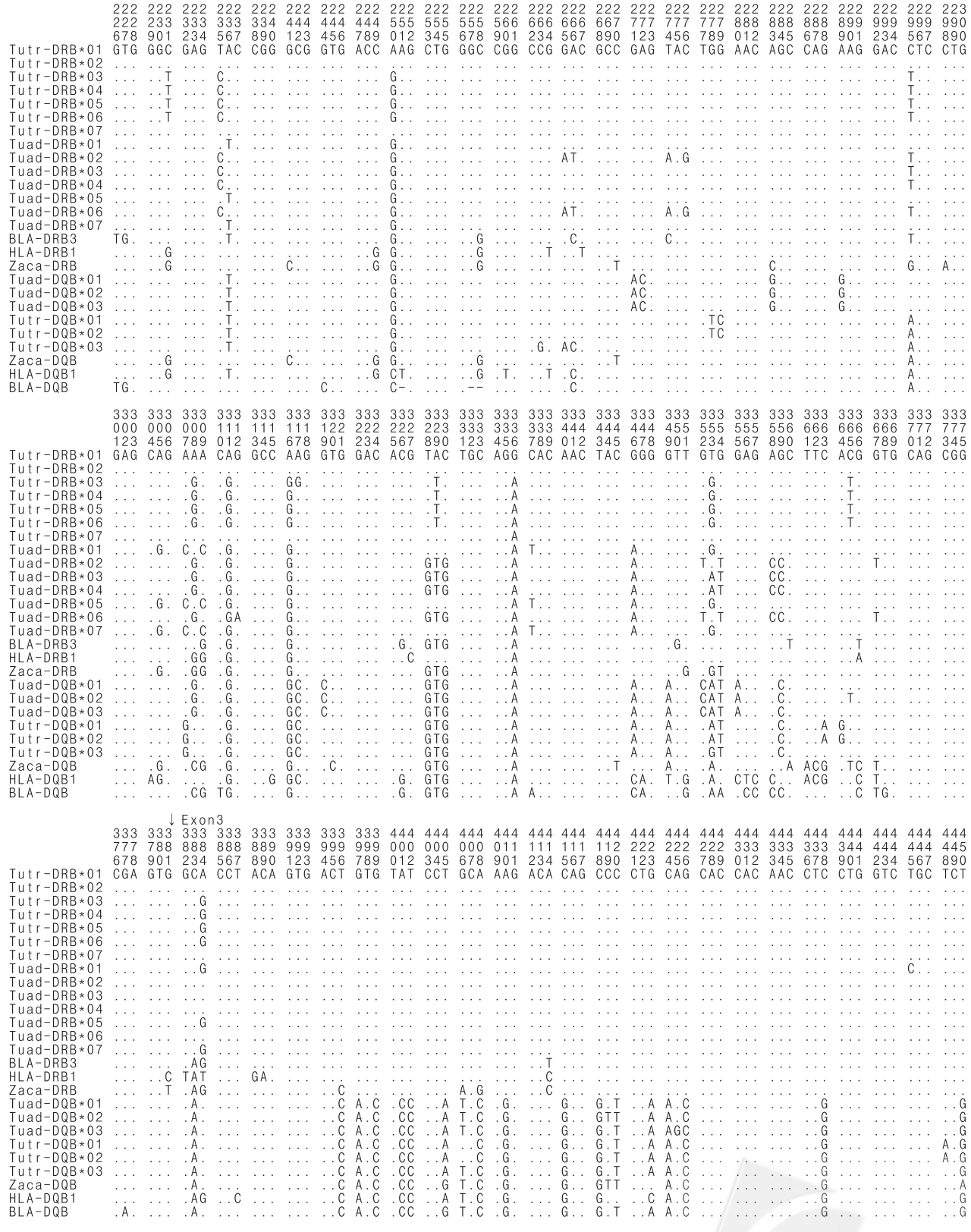

Fig. 1. (Cont.)

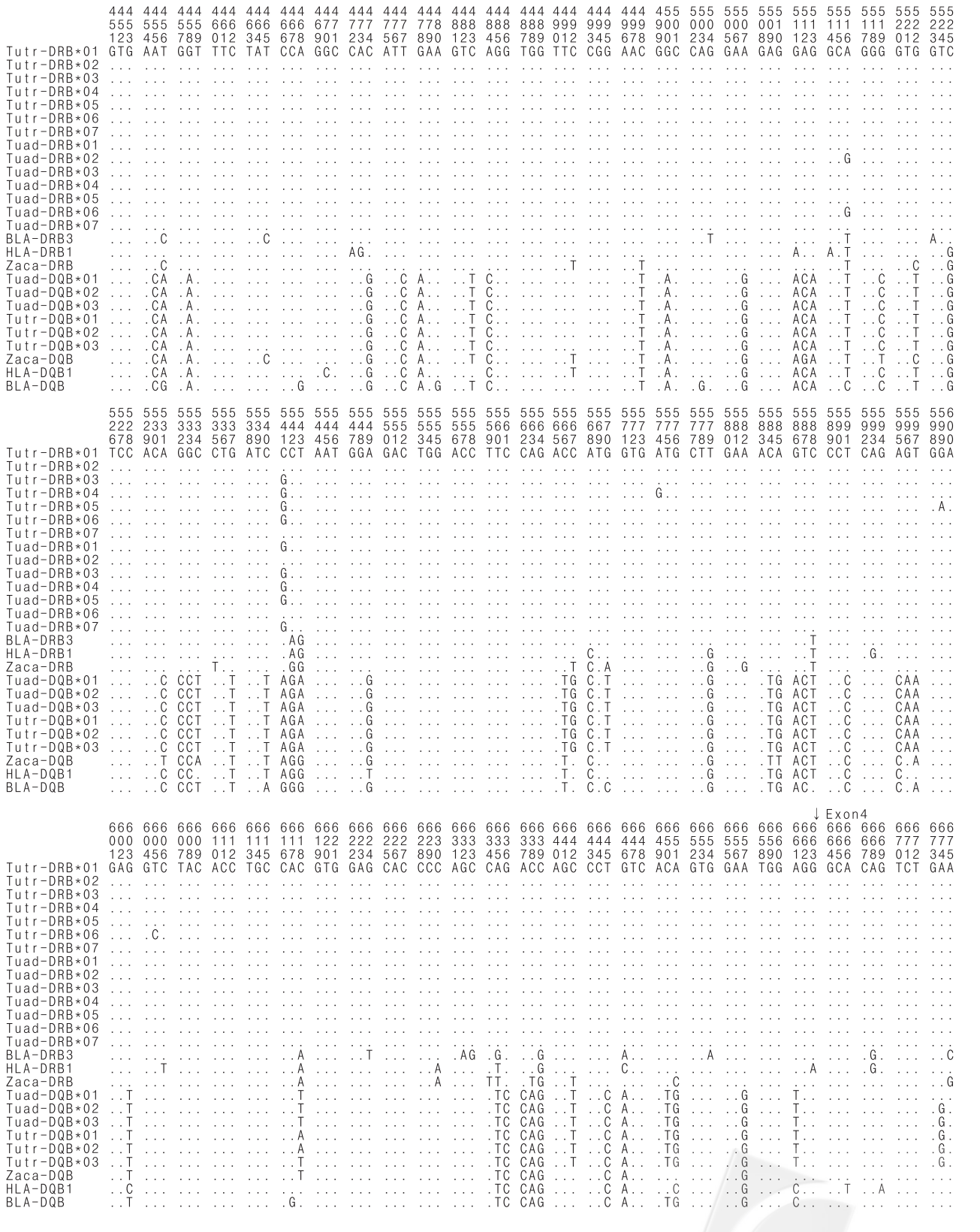
444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 444 455 555 555 555 555 555 555 555 555
555 555 555 666 666 666 677 777 777 778 888 888 888 999 999 999 900 000 000 001 111 111 111 222 222
123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345
Tutr-DRB*01 GTG AAT GGT TTC TAT CCA GGC CAC ATT GAA GTC AGG TGG TTC CGG AAC GGC CAG GAA GAG GAG GCA GGG GTG GTC
Tutr-DRB*02
Tutr-DRB*03
Tutr-DRB*04
Tutr-DRB*05
Tutr-DRB*06
Tutr-DRB*07
Tuad-DRB*01
Tuad-DRB*02
Tuad-DRB*03
Tuad-DRB*04
Tuad-DRB*05
Tuad-DRB*06
Tuad-DRB*07
BLA-DRB3
HLA-DRB1
Zaca-DRB
Tuad-DQB*01
Tuad-DQB*02
Tuad-DQB*03
Tutr-DQB*01
Tutr-DQB*02
Tutr-DQB*03
Zaca-DQB
HLA-DQB1
BLA-DQB
555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 555 556
222 233 333 333 334 444 444 444 555 555 555 566 666 666 667 777 777 777 888 888 888 899 999 999 990
678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890
Tutr-DRB*01 TCC ACA GGC CTG ATC CCT AAT GGA GAC TGG ACC TTC CAG ACC ATG GTG ATG CTT GAA ACA GTC CCT CAG AGT GGA
Tutr-DRB*02
Tutr-DRB*03
Tutr-DRB*04
Tutr-DRB*05
Tutr-DRB*06
Tutr-DRB*07
Tuad-DRB*01
Tuad-DRB*02
Tuad-DRB*03
Tuad-DRB*04
Tuad-DRB*05
Tuad-DRB*06
Tuad-DRB*07
BLA-DRB3
HLA-DRB1
Zaca-DRB
Tuad-DQB*01
Tuad-DQB*02
Tuad-DQB*03
Tutr-DQB*01
Tutr-DQB*02
Tutr-DQB*03
Zaca-DQB
HLA-DQB1
BLA-DQB
↓Exon4
666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666 666
000 000 000 111 111 111 122 222 222 223 333 333 333 444 444 444 455 555 555 556 666 666 666 777 777
123 456 789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 123 456 789 012 345
Tutr-DRB*01 GAG GTC TAC ACC TGC CAC GTG GAG CAC CCC AGC CAG ACC AGC CCT GTC ACA GTG GAA TGG AGG GCA CAG TCT GAA
Tutr-DRB*02
Tutr-DRB*03
Tutr-DRB*04
Tutr-DRB*05
Tutr-DRB*06
Tutr-DRB*07
Tuad-DRB*01
Tuad-DRB*02
Tuad-DRB*03
Tuad-DRB*04
Tuad-DRB*05
Tuad-DRB*06
Tuad-DRB*07
BLA-DRB3
HLA-DRB1
Zaca-DRB
Tuad-DQB*01
Tuad-DQB*02
Tuad-DQB*03
Tutr-DQB*01
Tutr-DQB*02
Tutr-DQB*03
Zaca-DQB
HLA-DQB1
BLA-DQB

Fig. 1. (Cont.)

domain, a 23-aa transmembrane domain, and a 16-aa cytoplasmic tail. Residues at putative N-linked glycosylation sites and the CD4 binding site were highly conserved. The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database with the following accession numbers: EF017815, EF507877, and EF507878 for *Tutr-DQB*; EF017816, EF507875, and EF507876 for *Tuad-DQB*; EF017817 and EF507863-EF507868 for *Tutr-DRB*; and EF017818 and EF507869-EF507874 for *Tuad-DRB*.

Variations in *DQB* sequences

Three different *Tuad-DQB* and 3 different *Tutr-DQB* sequences were identified from the RACE-PCR clones. This suggested that the *DQB*-specific primers amplified products from 1 locus if each sequence was considered a unique *DQB* allele.

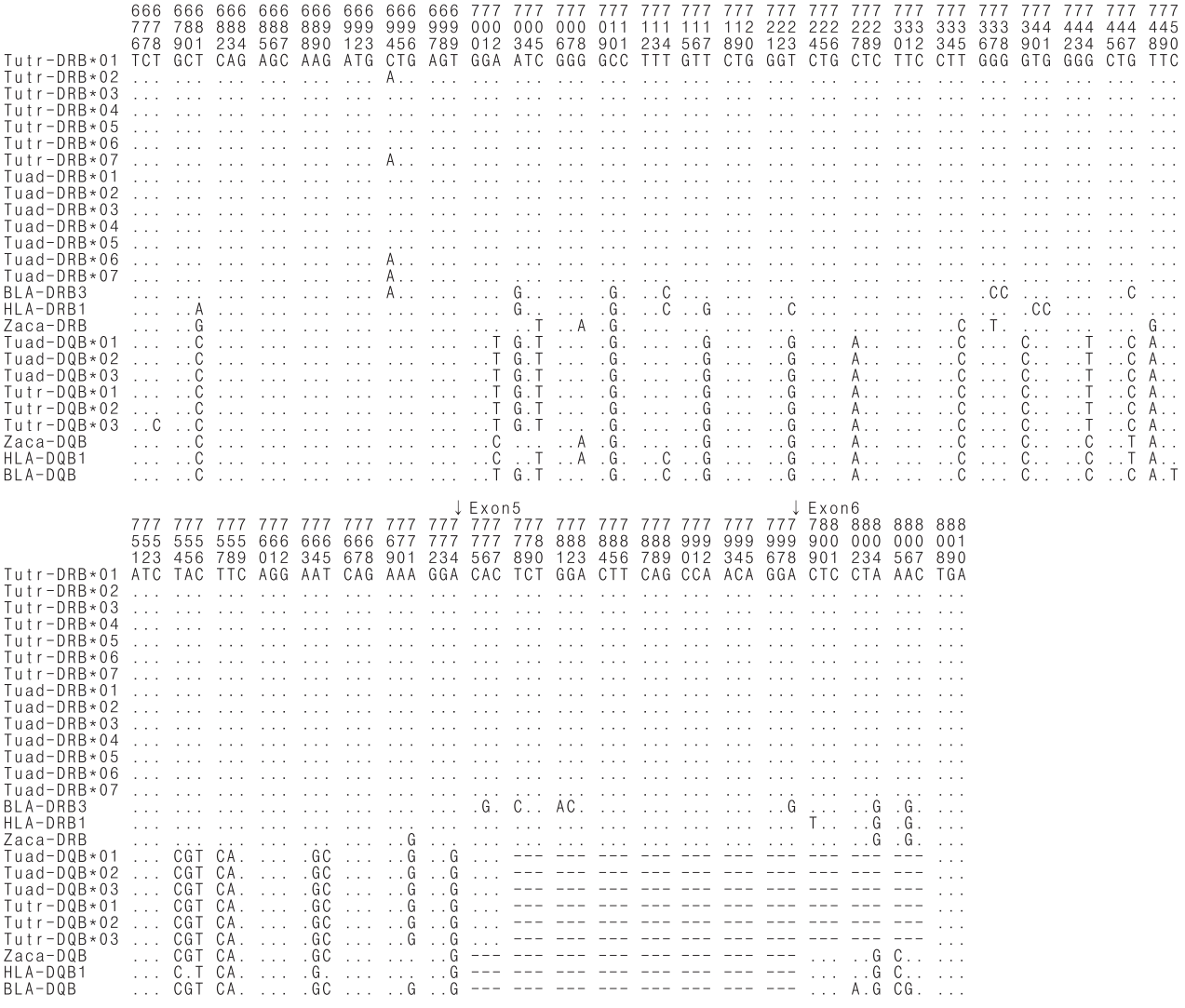

Fig. 1. Nucleotide sequence alignment of coding regions of the *DQB* and *DRB* genes among bottlenose dolphins, human, cattle, and California sea lion. Abbreviations for individual species MHC molecules are as follows: *Tutr*, common bottlenose dolphin (*Tursiops truncatus*); *Tuad*, Indo-Pacific bottlenose dolphin (*Tursiops aduncus*); *Zaca*, California sea lion (*Zalophus californianus*); HLA, human lymphocyte antigen; and BLA, bovine lymphocyte antigen. The complete nucleotide sequence of *Tutr-DRB*01* is shown. Single letters and dots below the nucleotide sequence represent nucleotides that are respectively distinct from or identical to *Tutr-DRB*01*. Boxes indicate codons used to distinguish *DQB* from *DRB*. Borders of each domain were assigned based upon the sequence homology between *Tutr-DRB*01* and *Zaca-DRB* (California sea lion *DRB*, GenBank accession no. AY491464). Allelic numbers for bottlenose dolphin sequences were assigned according to multispecies guidelines (Klein and Takahata 1990).

None of the sequences showed signs of pseudogenes. The similarity between *Tuad-DQB* sequences was extremely high, with each containing only 2 or 3 unique nucleotides. The deduced amino acid sequences indicated that all 4 polymorphisms represented non-synonymous nucleotide substitutions (Table 2). Of the 4 polymorphic amino acid residues, 1 was found in the β1 domain, which encodes the putative MHC class II peptide-binding groove (Brown et al. 1993, Stern et al. 1994), 2 were found in the β2 domain, and 1 in the transmembrane domain. In comparison, variations among the *Tutr-DQB* sequences were relatively high (23 nucleotide positions). Of these 23 variable sites, the majority were in exon 2 (18/23), with the remainder distributed between exons 1 (1/23), 3 (3/23), and 4 (1/23). The deduced amino acid sequences indicated that over 90% of the nucleotide substitutions (21/23) were non-synonymous (Table 2). The divergence of non-synonymous substitutions was significant at the codons of the PBR (Table 3; $p < 0.01$). The polymorphic *Tutr*-DQβ amino acid residues (n = 16) were located in the β1 (13/16) and β2 domains (3/16). The relative positions of these polymorphic residues were examined using the *HLA-DR* model for class II peptide binding (Brown et al. 1993, Stern et al. 1994). *Tutr*-DQβ polymorphism was observed at residues 9, 13, 26, 27, 31, 41, 40, 56, 57, 61, 86, 89, 90, 104, 118, and 174. While 13 of these polymorphic sites were located in the putative peptide-binding groove, 6 (residues 9, 13, 56, 61, 86, and 89) coincided with those described in humans (Brown et al. 1993, Stern et al. 1994).

Table 2. Sequence polymorphism of *Tuad-DQB*, *Tutr-DQB*, *Tuad-DRB*, and *Tutr-DRB*, delineated by exon. Abbreviations for individual species MHC molecules are as described in the legend to figure 1

Exon	1	2	3	4	5	6
Tuad-DQB						
Synonymous	0	0	0	0	0	0
Non-synonymous	0	1	2	1	0	0
Tutr-DQB						
Synonymous	1	1	0	0	0	0
Non-synonymous	0	17	3	1	0	0
Tuad-DRB						
Synonymous	0	9	2	0	0	0
Non-synonymous	1	29	2	1	0	0
Tutr-DRB						
Synonymous	3	3	1	0	0	0
Non-synonymous	2	24	4	1	0	0

Variations in *DRB* sequences

Seven different *Tuad-DRB* and 7 different *Tutr-DRB* sequences were identified from the RACE-PCR clones. These results suggested that the amplified products came from at least 2 loci in *DRB* genes per individual. All sequences had features comparable with classical MHC class II transcripts in other species. Variations among the *Tuad-DRB* sequences were relatively high (44 nucleotide positions) compared to those of *Tuad-DQB*. Of these 44 variable sites, the majority were in exon 2 (38/44), with the remainder being distributed in exons 1 (1/44), 3 (4/44), and 4 (1/44). The deduced amino acid sequences indicated that the substitutions clearly tended to be clustered around the PBR (Table 2). The divergence of non-synonymous substitutions was significant at the codons of the PBR (Table 3; $p < 0.005$). The polymorphic *Tuad*-DRβ amino acid residues (n = 25) were located in the leader peptide (1/25), the β1 domain (21/25), the β2 domain (2/25), and the transmembrane domain (1/25). *Tuad*-DRβ polymorphisms were observed at residues -1, 6, 9, 11, 22, 26, 27, 30, 31, 37, 40, 47, 57, 60, 67, 70, 71, 78, 81, 86, 88, 91, 117, 149, and 200. While 19 of these polymorphic sites were located in the putative peptide-binding groove, 12 (residues 9, 11, 30, 37, 47, 60, 70, 71, 78, 81, 86, and 88) coincided with those described in humans. Variations among *Tutr-DRB* sequences were also high (38 nucleotide positions), among which the majority were found in exon 2 (27/38), with the remainder being distributed in exons 1 (5/38), 3 (5/38), and 4 (1/38). The deduced amino acid sequences indicated that the substitutions clearly tended to be clustered around the PBR (Table 2). The divergence of non-synonymous substitutions was significant at codons of the PBR (Table 3; $p < 0.005$). The polymorphic *Tutr*-DRβ amino acid residues (n = 26) were located in the leader peptide (2/26), the β1 domain (19/26), the β2 domain (4/26), and the transmembrane domain (1/26). *Tutr*-DRβ polymorphisms were observed at residues -22, -3, 9, 13, 14, 26, 27, 28, 29, 30, 31, 34, 47, 52, 67, 71, 72, 74, 78, 86, 90, 149, 160, 168, 170, and 200. While 19 of these polymorphic sites were located in the putative peptide-binding groove, only 9 (residues 9, 13, 28, 30, 47, 71, 74, 78, and 86) coincided with those described in humans.

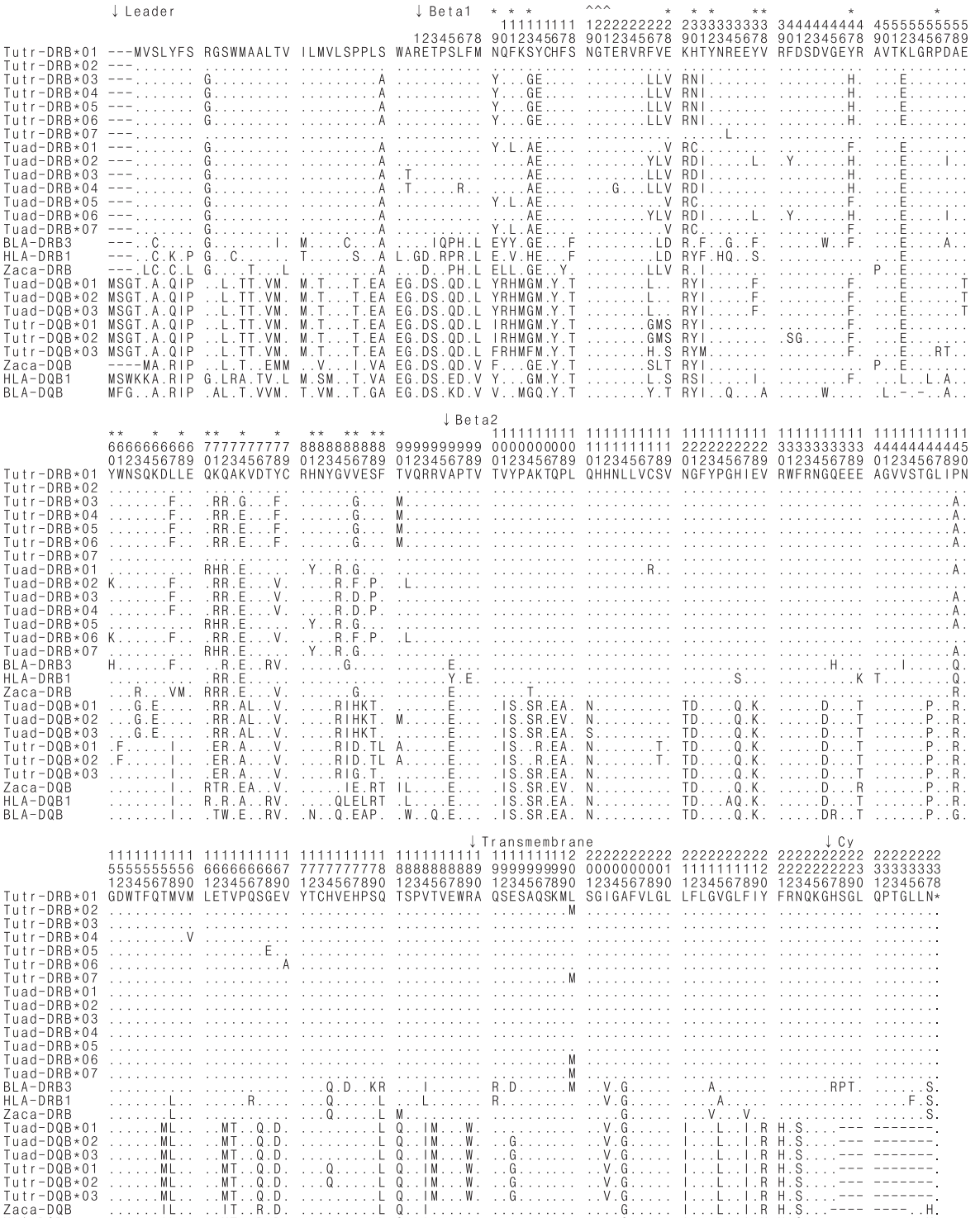

Fig. 2. Amino acid sequence alignment between bottlenose dolphins, human, cattle, and California sea lion DQβ and DRβ sequences. Abbreviations for individual species MHC molecules are as described in the legend to figure 1. The complete amino-acid sequence of *Tutr-DRB*01* is shown. Single letters and dots below the amino-acid sequence represent amino acids that are respectively distinct from or identical to *Tutr-DRB*01*. Asterisks indicate putative positions corresponding to the peptide-binding region as shown by Brown et al. (1993). A box indicates the putative CD4 binding site. Carets (^) indicate putative *N*-linked glycosylation sites. Borders of each domain were assigned based upon the sequence homology between *Tutr-DRB*01* and *Zaca-DRB* (California sea lion *DRB*, GenBank accession no. AY491464).

Phylogenetic analyses

The phylogeny based on the full-length coding region of *DQB* and *DRB* sequences from *T. aduncus*, *T. truncatus*, cattle, human, and California sea lion revealed 2 clades of genes corresponding to *DQB* and *DRB*, respectively (Fig. 3a). Sequences from dolphins formed a monophyletic group separated from other mammals with strong bootstrap support (100%) in both the *DQB* and *DRB* clades. This tree also showed that dolphins were more closely related to cattle than to humans or sea lions in both the *DQB* and *DRB* phylogenies. In the *DQB* clade, the monophyly of *T. aduncus*- and *T. truncatus*-specific alleles was supported by strong bootstrap values. Two well-supported dolphin groups could be seen in the *DRB* clade, and the monophyly of *T. aduncus*- and *T. truncatus*-specific alleles was also supported by strong bootstrap values in both groups, indicating that there are likely 2 *DRB* loci in bottlenose dolphins. The NJ tree based on exon 2 only (Fig. 3b) showed a similar phylogenetic relationship to figure 3a. In comparison, the NJ tree based on exons 3-6 (Fig. 3c) showed a different phenomenon from the other 2 trees. Although the *DQB* and *DRB* sequences of dolphins still formed monophyletic groups respectively separated from other mammals with strong bootstrap support (100%), there was no support for groups uniting sequences of the same dolphin species in either the *DQB* or *DRB* clade.

DISCUSSION

Previous studies of the cetacean MHC mainly examined exon 2, which encodes the functionally important PBR, from individual MHC class II genes (Murray et al. 1995, Murray and White 1998, Hayashi et al. 2003 2006, Yang et al. 2005, Baker et al. 2006, Martinez-Aguero et al. 2006). While those studies present valuable information, variation at 1 part of a gene, or 1 gene, is not an appropriate measure of variation for the entire MHC (Murray and White 1998). It is possible that low diversity at the MHC has been observed only because short fragments (usually about 200 bp) were amplified and the functionality of the alleles was not taken into account (e.g., Sommer 2003, Amills et al. 2004), which might lead to a misinterpretation of the results (Axtner and Sommer 2007), with the possible consequence that a severe population bottleneck is inferred (Baker et al. 2006). Therefore, characterization of full-length expressed sequences of MHC genes is very important for making valid evolutionary inferences on non-model species like bottlenose dolphins. Such information is also crucial for evaluating MHC diversity at the population level.

In this study, we used RACE technology to amplify full-length expressed class II gene sequences. This is the first identification of sequence polymorphisms throughout the entire length of both *DQB* and *DRB* gene products in bottlenose dolphins that are potentially subject to different pathogen pressures. This allows investigations into the effects of different environmental influences on MHC diversity in ongoing studies. Furthermore, we describe the bottlenose dolphin MHC-specific oligonucleotides for full-length amplification that will be useful in future work on bottlenose dolphin MHC research. We present 20 novel full-length expressed *DQB* and *DRB* alleles

Table 3. Number of synonymous substitutions per synonymous site (d_S), and the number of non-synonymous substitutions per non-substitution site (d_N) at the peptide-binding region (PBR), non-PBR, and all regions of MHC *DQB* exon 2. Abbreviations for individual species MHC molecules are as described in the legend to figure 1

	PBR		Non-PBR		All regions	
	d_S	d_N	d_S	d_N	d_S	d_N
Tuad-DQB	0 ± 0	0 ± 0	0 ± 0	0.6 ± 0.3	0 ± 0	0.5 ± 0.2
Tutr-DQB	0 ± 0	8.9 ± 3.5*	1.1 ± 0.6	1.2 ± 0.4	0.9 ± 0.5	2.5 ± 0.6
Tuad-DRB	4.7 ± 2.4	16.9 ± 4.7*	0.8 ± 0.4	1.1 ± 0.4	1.5 ± 0.5	3.5 ± 0.7
Tutr-DRB	2.1 ± 1.5	10.5 ± 2.7*	1.9 ± 0.7	1.4 ± 0.4	1.9 ± 0.6	2.8 ± 0.6

d_N and d_S are indicated as percentages. Standard errors were calculated using 500 bootstrap replications. * $p < 0.05$ denotes values of d_N that are significantly greater than d_S.

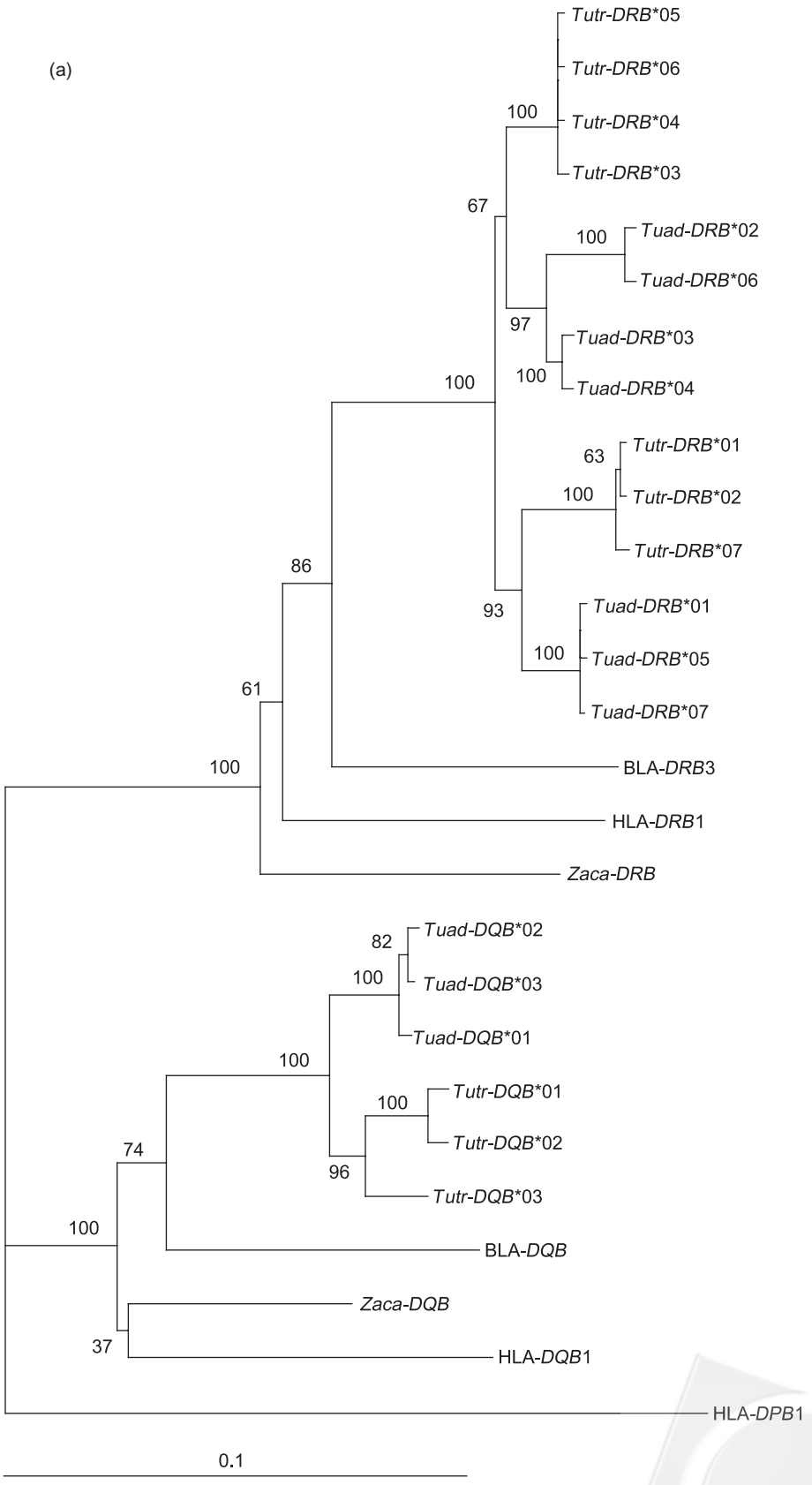
(a)
Tutr-DRB*05
Tutr-DRB*06
Tutr-DRB*04
Tutr-DRB*03
Tuad-DRB*02
Tuad-DRB*06
Tuad-DRB*03
Tuad-DRB*04
Tutr-DRB*01
Tutr-DRB*02
Tutr-DRB*07
Tuad-DRB*01
Tuad-DRB*05
Tuad-DRB*07
BLA-DRB3
HLA-DRB1
Zaca-DRB
Tuad-DQB*02
Tuad-DQB*03
Tuad-DQB*01
Tutr-DQB*01
Tutr-DQB*02
Tutr-DQB*03
BLA-DQB
Zaca-DQB
HLA-DQB1
HLA-DPB1
100
67
100
97
100
100
63
100
86
93
100
61
100
82
100
100
100
74
96
100
37
0.1

Fig. 3. (Cont.)

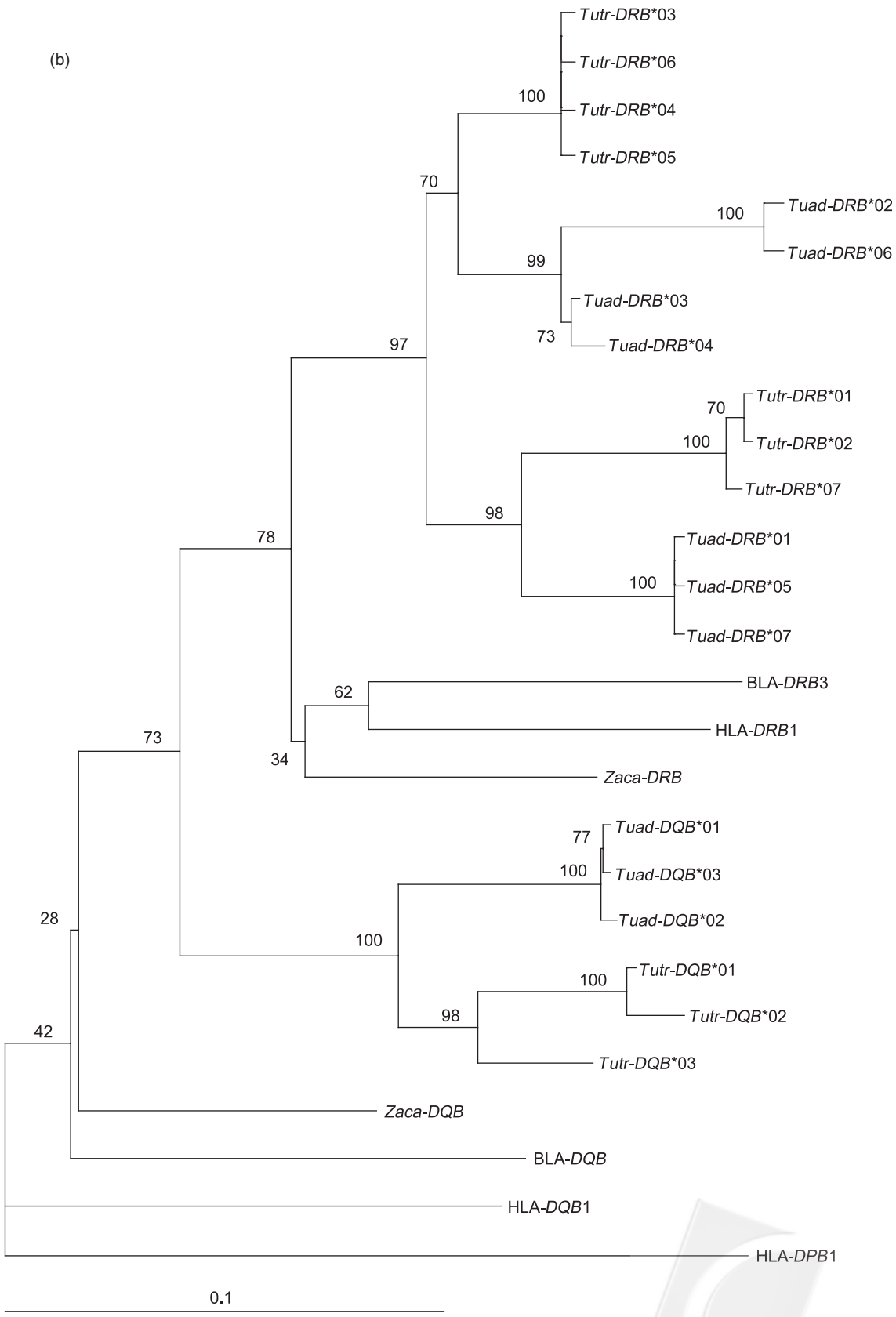
(b)
Tutr-DRB*03
Tutr-DRB*06
100
Tutr-DRB*04
Tutr-DRB*05
70
100
Tuad-DRB*02
Tuad-DRB*06
99
Tuad-DRB*03
73
Tuad-DRB*04
97
70
Tutr-DRB*01
100
Tutr-DRB*02
Tutr-DRB*07
98
78
Tuad-DRB*01
100
Tuad-DRB*05
Tuad-DRB*07
62
BLA-DRB3
HLA-DRB1
73
34
Zaca-DRB
77
Tuad-DQB*01
100
Tuad-DQB*03
28
Tuad-DQB*02
100
100
Tutr-DQB*01
98
Tutr-DQB*02
42
Tutr-DQB*03
Zaca-DQB
BLA-DQB
HLA-DQB1
HLA-DPB1
0.1

Fig. 3. (Cont.)

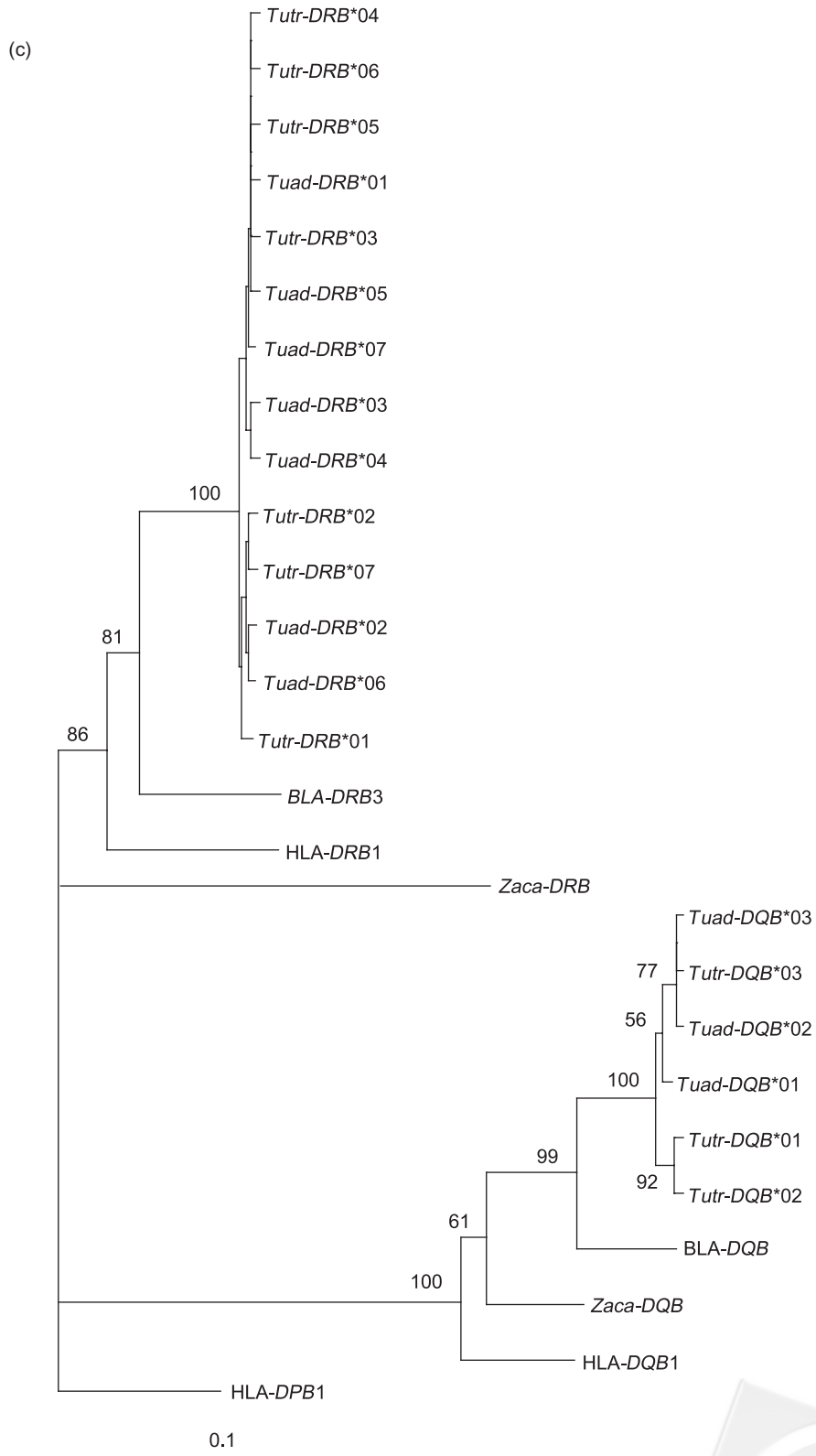

Fig. 3. Neighbor-joining trees for *DQB* and *DRB* genes with Kimura's 2-parameter model based on 5000 bootstrap replications. HLA-*DPB* (GenBank accession number NM_002121) was used as the outgroup. The datasets for tree construction are (a) the full-length coding region; (b) only exon 2; and (c) exons 3-6. Abbreviations for individual species MHC molecules are as described in the legend to figure 1.

from 2 *T. truncatus* and 2 *T. aduncus* individuals collected from the western Pacific. The high proportions of non-synonymous nucleotide substitutions in the putative peptide-binding regions of *Tutr-DQB*, *Tuad-DRB*, and *Tutr-DRB* suggest positive selection pressure on these gene loci (Hughes and Yeager 1998) and imply functional roles for these molecules in pathogen-specific immune responses. However, several features do not support a traditional role for *Tuad-DQB* molecules in peptide binding, including the extremely low number of non-synonymous residue substitutions in exon 2 and the overall lack of sequence variation. Sampling error is unlikely an explanation, since the samples were collected from 2 different regions (Taiwan and Indonesia). This unusual pattern of non-synonymous and synonymous substitutions closely resembles that used to describe non-classical MHC class I genes. The products of non-classical genes are not directly involved in peptide binding and are less polymorphic although they are evolutionarily related and structurally similar to classical genes (Meyer and Thomson 2001). It is important to conduct studies on differences in the immunological functionalities of the *DQB* gene in *T. aduncus* by comparing other genotypes between distinct populations before further conclusions can be drawn regarding the immunological importance of *Tuad-DQB* molecules.

Until now, the analyses of MHC class II genes studied in marine mammals have suggested that immunogenetic diversity is generated by polymorphisms at several specific loci, a reasonable assumption based on established knowledge in terrestrial species (Bowen et al. 2004). Previous surveys reported a single *DQB* locus in the beluga whale, narwhal (Murray et al. 1995), and finless porpoise (Hayashi et al. 2006). The beluga whale has also been reported to have 2 *DRB* loci (Murray and White 1998) whose sequences are closely related to the *DRB*3 gene of cattle. Our results revealed 1 *DQB* locus and 2 *DRB* loci in bottlenose dolphins, which is congruent with the results mentioned above. However, multiple copies of *DQB* were reported in the Baiji (Yang et al. 2005), humpback whale, and right whale (Baker et al. 2006). It was suggested that multiple *DQB* loci are an ancestral condition shared with ruminants in early-divergence cetacean species, and this condition has been lost in more-derived species such as small-toothed whales (Baker et al. 2006). In addition, baleen whales have been speculated to have only 1 *DRB* locus (Baker et al. 2006). Taking this evidence together, our results show that bottlenose dolphins and other small-toothed whales have very similar locus numbers in *DQB* and *DRB* genes corresponding to their evolutionary relationship.

The phylogenetic analyses of the full-length region and exon 2 of *DQB* and *DRB* showed no mixture but a clear division between *T. truncatus* and *T. aduncus*. This is an intriguing result compared to the general trans-specific pattern of evolution observed for MHC loci (Hughes 1999), which means that some alleles from an individual are more similar to those from other individuals of different species than to those of the same species. Hayashi et al. (2003) reported trans-specific patterns of the *DQB* gene in cetaceans, but their work included only 1 individual for 11 of 16 examined species, and their results require further investigation. The species-specific clustering of *DQB* or *DRB* loci has been described in a few species (South African antelope by van der Walt et al. 2001, cotton rats by Pfau et al. 1999) and several possible explanations have been proposed including sampling error, phylogeographic partitioning, and species-specific adaptations. The first 2 explanations are less likely in our case because we chose different sampling sites (Taiwan, Japan, and Indonesia) although the sample size is small. Since *T. truncatus* and *T. aduncus* have different diets, microflora, and distributions (Wang et al. 1999, Wang 2003), it is thus reasonable to assume that different selective pressures from pathogens exist in oceanic (*T. truncatus*) and coastal (*T. aduncus*) waters. The most likely explanation for our result is that species-specific alleles may have adaptive value for certain species and can be discriminately selected. When the selective advantage of MHC alleles differs among environments that vary in the diversity and abundance of pathogens, pathogen-driven directional selection could act differentially among individuals from distinct populations (Bernatchez and Landry 2003). This would have resulted in sequence divergence of exon 2 in bottlenose dolphins as observed in this study. A more-thorough analysis of geographic variations of polymorphisms, allelic frequencies, and evolution of the MHC should prove insightful because bottlenose dolphins have such a broad geographic distribution and are exposed to considerable environmental variation. The suggested directions of subsequent investigations include (1) studies of the sequence diversity of *DQB* and *DRB* genes with a larger number of bottlenose dolphin specimens from the western Pacific; and (2) the molecular characterization and genetic diversity of

MHC class II loci of 2 ecotypes of *T. truncatus* (coastal and offshore) in the northwestern Atlantic to determine how important the pathogen-driven directional selection has been in bottlenose dolphin MHC evolution. In addition, because all current regional and international conservation legislation recognizes only 1 species of *Tursiops* (Wang et al. 1999), understanding potential differences in MHC characterization among bottlenose dolphins will have important implications for effective conservation programs.

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REFERENCES

Acevedo-Whitehouse K, F Gulland, D Greig, W Amos. 2003. Inbreeding: disease susceptibility in California sea lions. Nature **422:** 35.

Altschul S, W Gish, W Miller, E Myers, D Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215:** 403-410.

Amills M, N Jimenez, J Jordana, A Riccardi, A Fernandez-Arias, J Guiral, JL Bouzat, J Folch, A Sanchez. 2004. Low diversity in the major histocompatibility complex class II *DRB*1 gene of the Spanish ibex, *Capra pyrenaica*. Heredity **93:** 266-272.

Axtner J, S Sommer. 2007. Gene duplication, allelic diversity, selection processes and adaptive value of MHC class II *DRB* genes of the bank vole, *Clethrionomys glareolus*. Immunogenetics (in press)

Baker CS, MD Vant, ML Dalebout, GM Lento, SJ O'Brien, N Yuhki. 2006. Diversity and duplication of *DQB* and *DRB*-like genes of the MHC in baleen whales (suborder: Mysticeti). Immunogenetics **58:** 283-296.

Bernatchez L, C Landry. 2003. MHC studies in nonmodel vertebrates: What have we learned about natural selection in 15 years? J. Evol. Biol. **16:** 363-377.

Bontrop RE, DI Watkins. 2005. MHC polymorphism: AIDS susceptibility in non-human primates. Trends Immunol. **26:** 227-233.

Bowen L, BM Aldridge, F Gulland, W Van Bonn, R DeLong, S Melin, LJ Lowenstine, JL Stott, ML Johnson. 2004. Class II multiformity generated by variable MHC-DRB region configurations in the California sea lion (*Zalophus californianus*). Immunogenetics **56:** 12-27.

Bowen L, BM Aldridge, F Gulland, J Woo, W Van Bonn, R DeLong, JL Stott, ML Johnson. 2002. Molecular characterization of expressed *DQA* and *DQB* genes in the California sea lion (*Zalophus californianus*). Immunogenetics **54:** 332-347.

Brown JH, TS Jardetzky, JC Gorga, LJ Stern, RG Urban, JL Strominger, DC Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature **364:** 33-39.

de Swart RL, PS Ross, JG Vos, AD Osterhaus. 1996. Impaired immunity in harbour seals (*Phoca vitulina*) exposed to bioaccumulated environmental contaminants: review of a long-term feeding study. Environ. Health Pers. **104(Supplement 4):** 823-828.

Gao A, K Zhou, Y Wang. 1995. Geographical variation in morphology of bottlenose dolphins (*Tursiops* sp.) in Chinese waters. Aquat. Mamm. **21:** 121-135.

Harvell CD, K Kim, JM Burkholder, RR Colwell, PR Epstein, DJ Grimes, EE Hofmann, EK Lipp, AD Osterhaus, RM Overstreet, JW Porter, GW Smith, GR Vasta. 1999. Emerging marine diseases-climate links and anthropogenic factors. Science **285:** 1505-1510.

Hayashi K, S Nishida, H Yoshida, M Goto, L Pastene, H Koike. 2003. Sequence variation of the *DQB* allele in the cetacean MHC. Mamm. Study **28:** 89-96.

Hayashi K, H Yoshida, S Nishida, M Goto, LA Pastene, N Kanda, Y Baba, H Koike. 2006. Genetic variation of the MHC DQB locus in the finless porpoise (*Neophocaena phocaenoides*). Zool. Sci. **23:** 147-153.

Hughes AL, M Yeager. 1998. Natural selection at major histocompatibility complex loci of vertebrates. Annu. Rev. Genet. **32:** 415-435.

Hughes AL. 1999. Adaptive evolution of genes and genomes. New York: Oxford Univ. Press. IUCN. 2006. 2006 IUCN Red list of threatened species. Available at http://www.iucnredlist.org/.

Kennedy LJ, R Ryvar, RM Gaskell, DD Addie, K Willoughby, SD Carter, W Thomson, WE Ollier, AD Radford. 2002. Sequence analysis of MHC DRB alleles in domestic cats from the United Kingdom. Immunogenetics **54:** 348-352.

Klein J, A Sato. 1998. Birth of the major histocompatibility complex. Scand. J. Immunol. **47:** 199-209.

Klein J, N Takahata. 1990. The major histocompatibility complex and the quest for origins. Immunol. Rev. **113:** 5-25.

Kumar S, K Tamura, M Nei. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. **5:** 150-163.

Leatherwood S, R Reeves. 1990. The bottlenose dolphin. San Diego, CA: Academic Press.

Marsh SG, ED Albert, WF Bodmer, RE Bontrop, B Dupont, HA Erlich, DE Geraghty, JA Hansen, CK Hurley, B Mach, WR Mayr, P Parham, EW Petersdorf, T Sasazuki, GM Schreuder, JL Strominger, A Svejgaard, PI Terasaki, J Trowsdale. 2005. Nomenclature for factors of the HLA system, 2004. Tissue Antigens **65:** 301-369.

Martínez-Agüero M, S Flores-Ramírez, M Ruiz-García. 2006. First report of major histocompatibility complex class II loci from the Amazon pink river dolphin (genus *Inia*). Genet. Mol. Res. **5:** 421-431.

Meyer D, G Thomson. 2001. How selection shapes variation of the human major histocompatibility complex: a review. Ann. Hum. Genet. **65:** 1-26.

Mikko S, K Roed, S Schmutz, L Andersson. 1999. Monomorphism and polymorphism at Mhc DRB loci in domestic and wild ruminants. Immunol. Rev. **167:** 169-178.

Miller HC, DM Lambert. 2004. Genetic drift outweighs balancing selection in shaping post-bottleneck major histocom-

patibility complex variation in New Zealand robins (Petroicidae). Mol. Ecol. **13:** 3709-3721.

Murray BW, S Malik, BN White. 1995. Sequence variation at the major histocompatibility complex locus DQ beta in beluga whales (*Delphinapterus leucas*). Mol. Biol. Evol. **12:** 582-593.

Murray BW, BN White. 1998. Sequence variation at the major histocompatibility complex *DRB* loci in beluga (*Delphinapterus leucas*) and narwhal (*Monodon monoceros*). Immunogenetics **48:** 242-252.

Nei M, T Gojobori. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:** 418-426.

Perrin WF, JRL Brownell. 2001. Appendix 1 [of Annex U]. Update on the list of recognized species of cetaceans. J. Cet. Res. Manage. **3(Supplement):** 364-365.

Pfau RS, RA Van Den Bussche, K McBee, RL Lochmiller. 1999. Allelic diversity at the Mhc-DQA locus in cotton rats (*Sigmodon hispidus*) and a comparison of *DQA* sequences within the family Muridae (Mammalia: Rodentia). Immunogenetics **49:** 886-893.

Reeves RR, BD Smith, EA Crespo, G Notarbartolo di Sciara. 2003. Dolphins, whales and porpoises: 2002-2010 conservation action plan for the world's cetaceans. IUCN/SSC Cetacean Specialist Group. Gland, Switzerland and Cambridge, UK: IUCN.

Robinson J, MJ Waller, P Parham, N de Groot, R Bontrop, LJ Kennedy, P Stoehr, SG Marsh. 2003. IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucleic Acids Res. **31:** 311-314.

Schook L, S Lamont. 1996. The major histocompatibility complex region of domestic animal species. Boca Raton, FL: CRC Press.

Sommer S. 2003. Effects of habitat fragmentation and changes of dispersal behaviour after a recent population decline on the genetic variability of noncoding and coding DNA of a monogamous Malagasy rodent. Mol. Ecol. **12:** 2845-2851.

Sommer S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Front. Zool. **2:** 16.

Stern LJ, JH Brown, TS Jardetzky, JC Gorga, RG Urban, JL Strominger, DC Wiley. 1994. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. Nature **368:** 215-221.

Thompson JD, DG Higgins, TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4680.

van der Walt JM, LH Nel, AR Hoelzel. 2001. Characterization of major histocompatibility complex *DRB* diversity in the endemic South African antelope *Damaliscus pygargus*: a comparison in two subspecies with different demographic histories. Mol. Ecol. **10:** 1679-1688.

Wagner JL, RC Burnett, R Storb. 1999. Organization of the canine major histocompatibility complex: current perspectives. J. Hered. **90:** 35-38.

Wang J, L Chou, B White. 2000a. Osteological differences between two sympatric forms of bottlenose dolphins (genus *Tursiops*) in Chinese waters. J. Zool. **252:** 147-162.

Wang J, L Chou, BN White. 2000b. Differences in the external morphology of two sympatric species of bottlenose dolphins (genus *Tursiops*) in the waters of China. J. Mammal. **81:** 1157-1165.

Wang JY, L Chou, BN White. 1999. Mitochondrial DNA analysis of sympatric morphotypes of bottlenose dolphins (genus: Tursiops) in Chinese waters. Mol. Ecol. **8:** 1603-1612.

Wang MC. 2003. Feeding habits, food resource partitioning and guild structure of odontocetes in Taiwanese waters. PhD dissertation, Taipei, Taiwan: National Taiwan Univ.

Yang G, J Yan, K Zhou, F Wei. 2005. Sequence variation and gene duplication at MHC *DQB* loci of Baiji (*Lipotes vexillifer*), a Chinese river dolphin. J. Hered. **96:** 310-317.

Yang H. 1976. Studies on the whales, porpoises and dolphins of Taiwan. Ann. Rep. Sci. **19:** 131-178.

Yuhki N, SJ O'Brien. 1990. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. Proc. Natl. Acad. Sci. USA **87:** 836-840.

Yuhki N, SJ O'Brien. 1997. Nature and origin of polymorphism in feline MHC class II *DRA* and *DRB* genes. J. Immunol. **158:** 2822-2833.

Zhou K, W Qian. 1985. Distribution of the dolphins of the genus *Tursiops* in the China Seas. Aquat. Mamm. **1:** 16-19.