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Sequence Variation of MHC Class II *DQB* Gene in Bottlenose Dolphin (*Tursiops truncatus*) from Taiwanese Waters

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ABSTRACT: The major histocompatibility complex (MHC) is a large multigene coding for glycoproteins that play a key role in the initiation of immune responses in vertebrates. For a better understanding of the immunologic diversity in thriving marine mammal species, the sequence variation of the exon 2 region of MHC *DQB* locus was analyzed in 42 bottlenose dolphins (*Tursiops truncatus*) collected from strandings and fishery bycatch in Taiwanese waters. The 172 bp sequences amplified showed no more than two alleles in each individual. The high proportion of non-synonymous nucleotide substitutions and the moderate amount of variation suggest positive selection pressure on this locus, arguing against a reduction in the marine environment selection pressure. The phylogenetic relationship among *DQB* exon 2 sequences of *T. truncatus* and other cetaceans did not coincide with taxonomic relationship, indicating a trans-species evolutionary pattern.

KEY WORDS: major histocompatibility complex, *DQB*, bottlenose dolphin, *Tursiops truncatus*.

INTRODUCTION

A significant number of marine mammal populations are in decline or have been classified as threatened (Bowen et al., 2004). Since infectious disease is an important cause of morbidity and mortality in many marine mammal species (Bowen et al., 2005), it follows that epidemiological genetic investigations should include examinations of functionally important genes such as those in the major histocompatibility complex (MHC). The MHC gene family includes highly polymorphic genes encoding a set of transmembrane glycoproteins that are critical to the generation of immune responses (Kennedy et al., 2002). In general, foreign proteins enter antigen-presenting cells either by infection or by phagocytosis. These foreign proteins are broken down into small peptides and loaded onto specific MHC molecules. The complex cascade of immune response is triggered when the T cells bind to the peptides presented by the MHC molecules on the antigen-presenting cells (Harding, 1997). While a single MHC molecule can bind multiple peptides that have common amino acids at particular anchor positions (Altuvia and Margalit, 2004), it is thought that pathogen recognition can be further promoted by polymorphic MHC genes (Brown et al., 1993). The contention that polymorphism is maintained by natural selection is supported by the evidence of the maintenance of alleles over long periods of evolutionary time and a high proportion of nonsynonymous change in the peptide-binding region (Hughes and Yeager, 1998).

Genetic and antigenic diversity of the MHC could be important in a host's ability to accommodate rapidly evolving infectious agents that periodically afflict natural populations (Klein and Sato, 1998). A lack of variation at the MHC may increase the susceptibility of an isolated population to infectious disease epidemics, with potentially catastrophic consequences (Bowen et al., 2002). Therefore, understanding the polymorphism of these genes, and their products, is vital for studying infectious disease ecology at the population level. This is particularly important in marine species whose chemical and microbial environment is increasingly influenced by anthropogenic encroachment, which increases marine species' risk of exposure to novel pathogens (Harvell et al., 1999). Since MHC polymorphism may reflect the pathogen environment of a given species (Hedrick, 2002; Wegner et al., 2003), it has been suggested that the pathogen environment of marine mammals may provide a diminished selective pressure for maintaining MHC polymorphism (Murray et al., 1995; Murray and White, 1998; Slade, 1992), due to the relatively low prevalence of

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infectious disease in the marine environment. For example, Murray et al. (1995) found that the genetic variability at the MHC *DQB* loci of the beluga was much lower than those of primates. There are several other hypotheses that have been put forward to explain the reduction of MHC diversity in marine mammals, such as population bottlenecks and random drift acting in small populations (Murray and White, 1998; Slade, 1992). In order to discriminate among the hypotheses, it would be most informative to assess MHC variation in delphinids with large populations and no evidence of historic population bottlenecks --- bottlenose dolphins for example.

Bottlenose dolphins (*Tursiops truncatus*) are of particular interest because they, along with other marine mammals, are long-lived, top-level predators on a wide variety of fishes and squids, occur in all tropical and temperate waters (Reeves et al., 2003) and are supposed to be excellent sentinels for disturbances in marine ecosystems globally (Wells et al., 2004). The current study is directed at investigating variation of *DQB* gene exon 2 locus, which has been shown to be highly polymorphic in many terrestrial carnivores and domestic animals (Schook and Lamont, 1996; Wagner et al., 1999; Yuhki and O'Brien, 1997). This is the first study on the variation of bottlenose dolphin *DQB* gene, and the information gained from this study will increase our understanding of how MHC genes in bottlenose dolphins can be influenced by selection.

MATERIALS AND METHODS

A total of 42 muscle samples from *T. truncatus* (11 males and 13 females from fishery bycatch in the coastal waters around Taiwan, and 10 males and eight females from strandings along Taiwan coasts) were collected by Cetacean Research Laboratory of National Taiwan University and Taiwan Cetacean Society from 1994 to 2005. Total cellular DNA was isolated from tissue by digestion with Proteinase K, followed by standard silica-gel-membrane technology in DNeasy Tissue System (QIAGEN, USA). Fragments of the *DQB* gene were amplified with polymerase chain reaction (PCR) under standard conditions: 1X PCR buffer II, 2.5 mM MgCl2, 0.5 U Advantage® 2 (Clontech, USA), 0.24 mM dNTPs, 0.2 μM primers, and approximately 25–100 ng of DNA. Amplifications were conducted with a PE Applied Biosystems (ABI, USA) model 9700 thermocycler. A 172-bp fragment (excluding primers) in exon 2 of the *DQB* gene was amplified with the universal primers DQB2 (5′-CATGTGCTACTTCACCAACGG-3′) and DQB1 (5′-CTGGTAGTTGTGTCTGCACAC-3′) as reported by Murray et al. (1995) with the following "touch-down" thermocycling profile: initial denaturation/activation at 94°C for 10 min; 10 cycles of 94°C denaturation for 30 s, 64°C annealing for 20 s, and 72°C extension for 30 s, with the annealing temperature decreasing by 1°C on each cycle; 25 cycles of 94°C for 40 s, 54°C for 30 s, and 72°C for 30 s. A final 10-min soak at 72°C was used to allow for complete extension of the PCR fragments.

Initially, 20 individuals (10 from by-catch and 10 from stranding) were randomly chosen for TA cloning. Purified PCR products were cloned into pGEM-T Easy vectors (Promega, USA) following manufacturer's recommendations and transformed into DH5α competent cells. Ten to 15 colonies were checked for the correct sized PCR product of 172 bp. After the insert check, at least 5 correctly sized products were sequenced for each individual using ABI 3100 automated sequencer with BigDye Terminator Chemistry (ABI). Sequences were aligned and edited using Sequencher (v4.1, Gene Codes Corporation, USA). Sequences differing by a single nucleotide from common allele sequences were assumed to be due to amplication error unless the variable site was shared with other alleles or found in more than one clone. Heterozygote and homozygote identifications for the remaining 22 individuals were determined by direct sequencing.

Sequences were aligned initially with the beluga *DQB* sequences from GenBank following Murray et al. (1995). Nucleotide diversity was calculated using the program MEGA3 (Kumar et al., 2004). Synonymous and nonsynonymous divergence (d_S and d_N) and standard errors were calculated using the Jukes and Cantor correction of Nei and Gojobori. The Hardy-Weinberg test for expected heterozygosity (H_e) (Nei and Tajima, 1981) and the test of statistical significance of heterozygote deficit were conducted using Genepop web version 3.4 (Raymond and Rousset, 1995) (http://wbiomed.curtin.edu.au/genepop/) as an indicator for genetic diversity. Tajima's D test (Tajima 1989) and Fu and Li's D* test (Fu and Li, 1993) of neutrality were conducted in DnaSP 4.0 (Rozas et al., 2003). Associations among MHC alleles and sample sources were examined by logistic regression. The deduced amino acid sequences were determined by the program, MacClade v.4.0 (Maddison and Maddison, 2000). Phylogenetic relationships among *DQB* sequences from this research and previous studies were reconstructed using neighbor-joining (NJ) or maximum parsimony (MP) methods, as implemented in the program PAUP* v4.0b10 (Swofford, 2003).

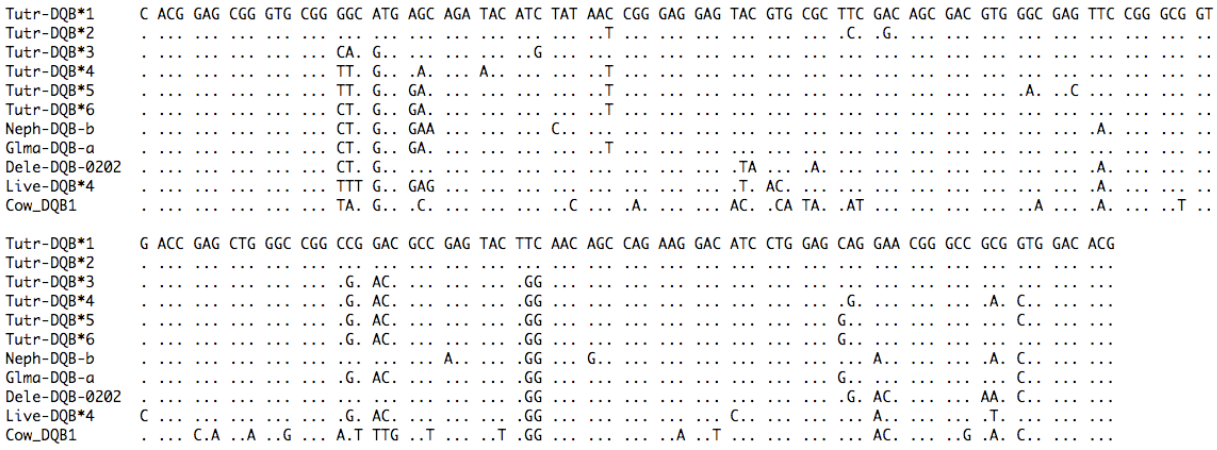

Fig. 1 Nucleotide sequence alignment of coding regions of *DQB* of bottlenose dolphin (*Tursiops truncatus*), other cetaceans and cow. The complete nucleotide sequence of *Tutr-DQB*1* is shown. *Single letters* and *dots* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *Tutr-DQB*1*. Species abbreviations and GenBank accession number: bottlenose dolphin *DQB* (Tutr-DQB*1-6, EF690293~8), finless porpoise *DQB* (Neph-DQB*b, AB164213), short-finned pilot whale *DQB* (Glma-DQB-a, AB164226), beluga *DQB* (Dele-DQB-0202, U16990), baiji *DQB* (Live-DQB*4, AY177153), and cow *DQB*1 (U77794).

Table 1. The number of synonymous substitutions per synonymous site (d_S), and the number of nonsynonymous substitutions per nonsubstituion site (d_N) at the antigen-recognition sites (ARS), non-ARS, and all sites of MHC *DQB* exon 2 analyzed for the *T. truncatus*.

	ARS	Non-ARS	All sites
d_N	16.5±6.8	5.1±2.4	8.5±2.5
d_S	4.6±5.2	1.4±1.5	1.1±1.1

For NJ reconstructions, genetic distances were adjusted using the best-fit model of substitution (F81+I+G) indicated by the hierarchical likelihood ratio test implemented in the program MODELTEST vs3.6 (Posada and Crandall, 1998), and branch support was assessed with 1,000 bootstrap replications. Parsimony analyses were performed using 100 replicates with random addition of taxa, tree-bisection-reconnection branch swapping, and transversion:transition weighting (2:1).

RESULTS

Sequences of the *DQB* exon 2 alleles of the first 20 individuals were determined following TA cloning. The analysis of multiple clones from each individual did not reveal more than two different alleles. Alignment of the 172 bp nucleotide sequences of the *DQB* alleles revealed 21 variable sites (Fig. 1), defining 6 alleles (*Tutr-DQB**1 to 6: GenBank accession numbers: EF690293~8). The allelic identifications of the remaining 22 individuals determined by direct sequencing showed no new substitution sites or allele. All the mutations were base substitutions and no indels were found. Amino acid alignments derived from the *Tutr-DQB* alleles (Fig. 2) showed that most of the substitutions were nonsynonymous (20/21). The nonsynonymous substitutions per nonsynonymous site (d_N) percentage was 8.5, while the synonymous substitutions per synonymous site (d_S) percentage was 1.1 (Table 1). The d_N percentage in putative antigen-recognition sites (ARS) and non-ARS were 16.5 and 5.1, respectively. Thus, the d_N percentage in ARS was 3.24 times higher than that in non-ARS. The number of pairwise nucleotide differences between pairs of 6 *Tutr-DQB* alleles ranged from 3 (*Tutr-DQB**1 vs *Tutr-DQB**2) to 16 (*Tutr-DQB**2 vs *Tutr-DQB**5), and the number for amino acid varied from 2 (*Tutr-DQB**1 vs *Tutr-DQB**2) to 12 (*Tutr-DQB**2 vs *Tutr-DQB**4 and *Tutr-DQB**2 vs *Tutr-DQB**5).

The results of Tajima's D test (D=0.03763, p>0.1) and Fu and Li's D* test (D=0.43558, p>0.1) revealed no significant departures from neutrality. The overall observed heterozygosity (H_O) was not significantly different from the expected heterozygosity (H_E) according to the Hardy-Weinberg test (p>0.05). The overall H_E was high, with a value of 0.77 (Table 2). The situation was similar when the data set is separated into two groups according to the sources. The most common allele in stranded samples was *Tutr-DQB**6, while in by-caught samples it was *Tutr-DQB**1. *Tutr-DQB**3~5 were the rare alleles in both sample sources. Compared between the stranded and by- caught cases using the odds ratio test, the

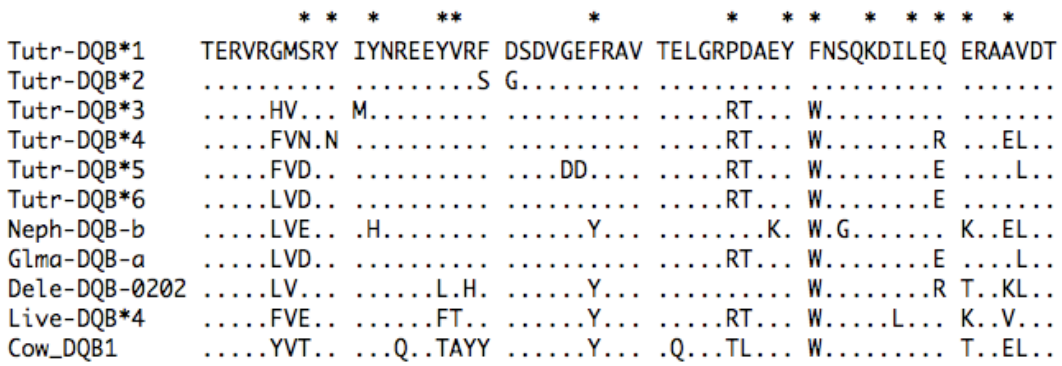

Fig. 2 Inferred reading frame of *DQB* exon 2 sequences from bottlenose dolphin (*Tursiops truncatus*) aligned to published sequences from other cetaceans and cow. The complete amino acid sequence of *Tutr-DQB*1* is shown. *Single letters* and *dots* below the amino acid sequence represent amino acids that are, respectively, distinct from or identical to *Tutr-DQB*1*. Positions thought to be involved in peptide binding (*) follow Hayashi et al. (2006). Species abbreviations and GenBank accession number: bottlenose dolphin *DQB* (Tutr-DQB*1-6, EF690293~8), finless porpoise *DQB* (Neph-DQB*b, AB164213), short-finned pilot whale *DQB* (Glma-DQB-a, AB164226), beluga *DQB* (Dele-DQB-0202, U16990), baiji *DQB* (Live-DQB*4, AY177153), and cow *DQB*1 (U77794).

Table 2. Sample sizes, allele frequencies, and heterozygosity analysis in each sampling source are shown. H_E is expected heterozygosity, H_O is observed heterozygosity. No significant difference (p<0.05) between H_E and H_O is observed in each sampling source.

Sources	No. of samples	Frequency in each allele						Heterozygosity	
		1	2	3	4	5	6	H_E	H_O
By-catch	24	16	13	4	3	4	8	0.77	0.65
Stranding	18	10	9	2	2	2	11	0.76	0.67
Total	42	26	22	6	5	6	19	0.77	0.66

individuals carrying *Tutr-DQB**6 showed a likely increased risk of stranding (odds ratio = 3.1429, 95% confidence interval (CI) = 0.8807-11.2154). The associations between stranding and the other *TutrDQB* alleles, genotypes, or sex were not evident (odd ratio = 1.0426~1.4773). No homozygote of *Tutr-DQB**6 from each sample source was found.

To investigate the phylogenetic relationship among cetacean *DQB* alleles, a further 12 published sequences of other toothed whales (including 4 families, 6 species) were added, giving a total of 18 *DQB* sequences. Among them, bottlenose dolphin, Pacific white-sided dolphin, Hector's dolphin, and short-finned pilot whale belong to the same family, Delphinidae. The phylogenetic reconstruction of the cetacean *DQB* sequences is shown with domestic cow *DQB1* sequence as outgroup (Fig. 3). The tree shows no or weak support (finless porpoise, 61%) for clades of same family or species. *Tutr-DQB**1 and *Tutr-DQB**2, which grouped together with strong bootstrap support (99%), were clustered with *Tutr-DQB**3 and Pacific white-sided dolphin *DQB*-2 with weak bootstrap support. *Tutr-DQB**4 was more similar to *DQB* sequences from Hector's dolphin than to others from *T. truncatus* with weak bootstrap support. *Tutr-DQB**5 and *Tutr-DQB**6 grouped with Pacific white-sided dolphin *DQB*-1 and short-finned pilot whale *DQB*-1, respectively, with moderate bootstrap supports (87% and 71%, respectively).

DISCUSSION

To date, MHC class II gene investigations in marine mammal species have presumed that immunogenetic diversity is generated by polymorphism at one or two specific loci (Murray et al., 1999), a reasonable assumption based on established knowledge in terrestrial species (Mikko et al., 1999; Wagner et al., 1999). In this study, the sequence analysis of 172 bp fragments amplified for the *T. truncatus* showed that there are no more than two alleles revealed in each individuals. This suggests that we succeeded in the amplification at a single *DQB* locus in this species. One single *DQB* locus has been reported in other toothed whales (Hayashi et al., 2003; Hayashi et al., 2006; Murray et al., 1995). However, duplicate *DQB* genes were described in the baleen whales (Baker et al., 2006) and baiji (Yang et al., 2005), which are early divergence of cetaceans. It is proposed that bearing multiple *DQB* genes is consistent with the retention of an ancestral condition shared with the ruminants, and it has been lost in the more derived cetaceans such as the true dolphins (Baker et al., 2006).

Previous studies of the level of MHC variation in marine mammals have found a low level of allelic variation in comparison with terrestrial mammals (Murray et al., 1995; Slade, 1992). It was hypothesized that the marine environment has a

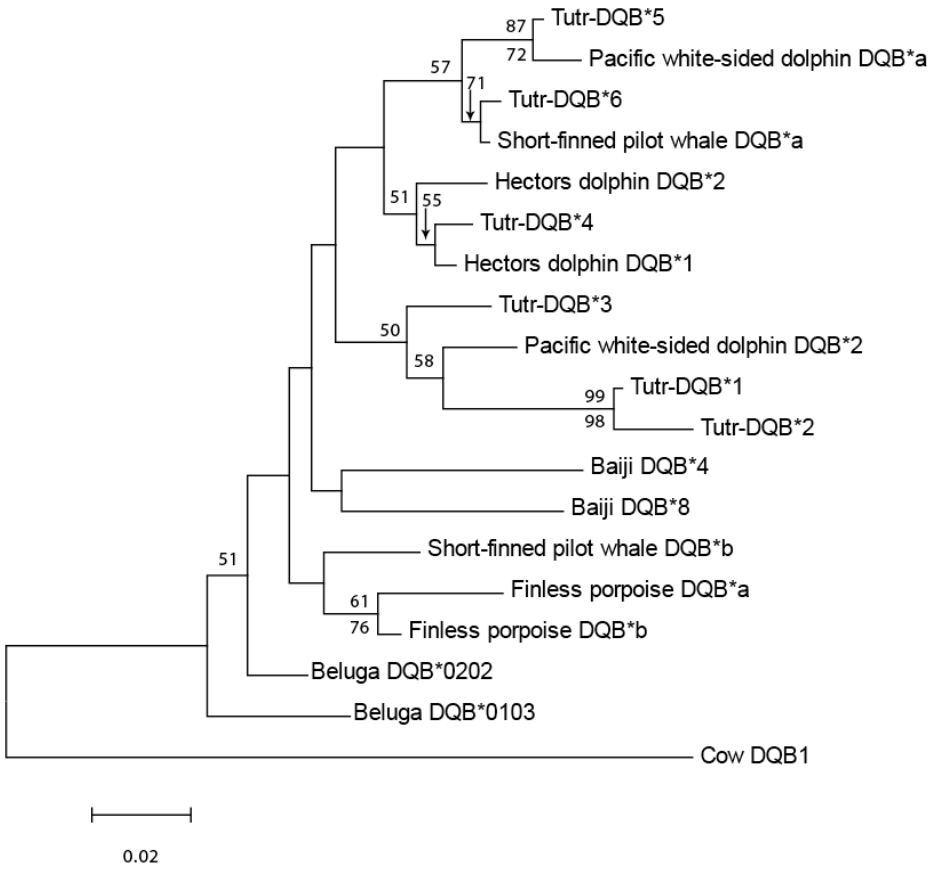

Fig. 3 Neighbor-joining tree based on F81+I+G model of *DQB* sequences with 1,000 bootstrap replications. Cow DQB1 sequence (GenBank accession number: U77794) was used as outgroup. Numbers above and below internal node indicate bootstrap values >50% from neighbor-joining analysis and maximum parsimony analysis, respectively. The sequences from other cetaceans with GenBank accession numbers: beluga U16989, U16990; baiji AY177153 AY177283; Hector's dolphin DQ354628, DQ354629, short-finned pilot whale AB164226, AB164227; finless porpoise AB164212, AB164213; Pacific white-sided dolphin AB164224, AB164225.

reduced level of micro-parasite diversity that affects marine mammals when compared with terrestrial mammals, and thus reduces the balancing selection pressure maintaining MHC diversity (Slade, 1992). This would mean that the substitution rate of nonsynonymous changes at ARS should be much lower in marine mammals than that observed in terrestrial mammals. Nonetheless, the proportion of non-synonymous nucleotide substitutions in exon 2 of *Tutr-DQB* is similar to that observed in terrestrial mammals (Murray and White, 1998). In addition, the amount of variation at the *Tutr-DQB* is significantly higher than that in beluga (only 5 *DQB* alleles and 11 nucleotide substitutions in 172 bp were found in 233 beluga) (Murray et al., 1995). These findings not only suggest a positive selection pressure on the bottlenose dolphin *DQB* locus (Hughes and Yeager, 1998) but also argue against a reduction in the marine environment selection pressure. Similar arguments were made in the studies on beluga *DRB*1 locus (Murray and White, 1998), North Atlantic right whale *DQB* locus (Murray, 1997), Baiji *DQB* locus (Yang et al., 2005), and finless porpoise *DQB* locus (Hayashi et al., 2006). However, in this study we detected no significant departures from neutrality and no deviation from Hardy-Weinberg expectations, suggesting that the effect of balancing selection in *Tutr-DQB* for short time periods might be weak and masked by other microevolutionary forces (e.g. gene flow, mutation, drift, and non-random mating). Similar conclusions were reached by Boyce et al. (1997) from bighorn sheep, Huang and Yu (2003) from the Southeast Asian house mouse in Taiwan, and Miller et al. (2004) from New Zealand robins. Nonetheless, there are other possible explanations, such as spatiotemporal variation of selection and demographic processes acting on small populations (reviewed by Piertney and Oliver, 2006). Therefore further investigation with a larger sample size and on other neutrally evolving loci is needed before we interpret the effect of selection over the history of the *T. truncatus* population around Taiwan.

In phylogenetic analysis, the relationship among the cetacean *DQB* alleles did not coincide with the taxonomic relationship, as previous studies on cetacean MHC have documented (Baker et al., 2006; Hayashi et al., 2003). It is noticed that some alleles from a species are more similar to the alleles of different species than each other, rather than the species-specific pattern. This scenario has been referred to as trans-species evolution (Klein, 1987), which is one of the characteristics of the MHC genes and has been identified in a wide range of taxa including primates, salmonids, ungulates, pinnipeds, rodents, geckos, and warblers (reviewed by Piertney and Oliver, 2006). It remains controversial whether such pattern of apparent transspecific sharing of alleles is due to common lineages or convergence of independent lineages (Yeager and Hughes, 1999). Coalescent and neutral theories predict that two species will share a proportion of alleles at any given locus immediately following divergence from their ancestral form. Over time, from a phylogenetic perspective we should see a gradual progression from polyphyly, through paraphyly, to monophyly. However, balancing selection, which acts on MHC genes, retains alleles among species for considerably longer periods of time and increases the time over which there is incomplete lineage sorting and delaying the time to monophyly (Piertney and Oliver, 2006). If the trans-species evolutionary pattern in cetaceans described in this study is due to common lineage, the sharing of similar alleles by a common ancestry between bottlenose dolphin and beluga or baiji would require their preservation in each of these lineages for at least 15 million years (Arnason et al., 2004) or 25 million years (Nikaido et al., 2001), respectively.

The correlations between MHC alleles and disease resistance (e.g. malaria, hepatitis B, leprosy, tuberculosis) and disease-susceptibility (cancer, parasite infestation) have been reported (reviewed by Sommer, 2005). In marine mammals, for example, MHC genotypes of California sea lions were associated with urogenital cancer (odds ratio = 3.64, 95% CI = 1.11-11.97) (Bowen et al., 2005). Compared with the research in California sea lion, the relation between *Tutr-DQB**6 and the risk of stranding revealed by the odd ratio test was only marginally significant (lower bound of CI is lightly smaller than 1). In addition, only five individuals carried *Tutr-DQB**6 were fresh enough for pathological examination in this study so that the subsequent statistical analysis of lesions could not be done. Therefore, further studies are needed to determine the potential mechanisms underlying the association between MHC alleles and cetacean strandings.

The sequence information from this study raises important questions regarding immunologic diversity in the bottlenose dolphins. The findings of this study are also particularly important in light of the ongoing debate regarding low MHC polymorphism in several marine mammal species. Nonetheless, we presume that the MHC variation in one locus cannot definitely represent the ability of pathogen defense of a species because MHC polymorphism in marine mammals arises from several loci. For example, a moderate to high degree of polymorphism is only found in *DRB* genes, not in *DQB* gene, in beluga and California sea lion (Bowen et al., 2004; Murray and White, 1998), and the situation reverses in humpback whale (Baker et al., 2006). Hence we should characterize the MHC diversity of marine mammals more rigorously before interpreting the interrelation between levels of MHC variation and capability of disease resistance in certain species. The directions of future investigations will be studying other MHC loci to accurately quantify the degree of MHC variability of bottlenose dolphins.

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臺灣海域瓶鼻海豚 (*Tursiops truncatus*) 第二型主要組織相容性複合體 DQB 基因之序列變異

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摘　　要

主要組織相容性複合體 (MHC) 為一多基因家族，其轉譯之蛋白質於脊椎動物免疫反應的啟動扮演重要的角色。為了更了解繁衍興盛的海洋哺乳類之免疫多樣性，我們採集 42 隻瓶鼻海豚 (*Tursiops truncatus*) 分析其 MHC DQB 基因座中第二外顯子之序列變異。增幅之序列長為 172 個鹼基對，且每一個個體僅有一至兩條序列。高比例的非同義核酸取代與中等程度的變異暗示此基因座受到正向選汰壓的影響，而此現象反駁了海洋環境選汰壓較低的說法。瓶鼻海豚與其他鯨類 DQB 第二外顯子序列之系統發生關係與其分類關係並不吻合，顯示這裡出現跨物種演化模式。

關鍵詞：主要組織相容性複合體、DQB、瓶鼻海豚 、*Tursiops truncatus*。

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