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ORIGINAL PAPER

Major histocompatibility complex class II variation in bottlenose dolphin from Adriatic Sea: inferences about the extent of balancing selection

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Abstract The bottlenose dolphin (*Tursiops truncatus*) is the most common cetacean species worldwide and the only marine mammal species resident in the Croatian part of the Adriatic Sea. To gain insight into genetic diversity of bottlenose dolphins at adaptively important loci relevant to conservation, we analysed the polymorphism of major histocompatibility complex (MHC) genes, which play a key role in pathogen confrontation and clearance. Specifically, we examined the diversity of MHC class II DRA, DQA and DQB alleles in 50 bottlenose dolphins from the Adriatic Sea collected between 1997 and 2011 and in 12 animals from other Mediterranean locations. Notable variation in DQA, DQB and three-locus haplotypes was found, with all 10 DQA and 12 DQB alleles encoding unique protein products. Analysis of the ratio of non-synonymous to synonymous substitution rates suggests that positive selection acts at both highly variable loci. Phylogenetic analyses revealed trans-species polymorphism at the DQB locus, strongly indicating the influence of balancing selection in the long term. In fact, the balancing selection observed in bottlenose dolphins is higher than that reported for most other cetaceans and comparable to that seen in terrestrial mammals.

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Introduction

The bottlenose dolphin (*Tursiops truncatus*) is the most abundant cetacean species; it inhabits primarily coastal and inshore regions of warm and temperate seas around the world (Jefferson et al. 1993). Although the species can migrate long distances, analyses based on mitochondrial DNA and microsatellite loci showed genetic differentiation among dolphin populations both across its worldwide range (Natoli et al. 2004) and on a regional geographic scale, e.g. along a contiguous distributional range from the Black Sea to the eastern North Atlantic (Natoli et al. 2005). The latter study revealed that population boundaries coincide with transitions between habitat regions (e.g. the strongest boundary is separating the Mediterranean and the Black Seas), implying that bottlenose dolphin populations adapt to local environments leading to the observed regional population structure. The results of Sharir et al. (2011), who found unique morphological characteristics of bottlenose dolphins from the easternmost region of the Mediterranean (Levant) to differ from those of western Mediterranean Seas, contribute to the local adaptation hypothesis.

The bottlenose dolphin is the only resident marine mammal species in the Croatian part of the Adriatic Sea, which forms a semi-enclosed basin in the northern Mediterranean

(Bearzi et al. 2004; Đuras Gomerčić et al. 2009). Population size was drastically reduced as a result of an intensive eradication campaign in the 1950s when culling was promoted as a means of mitigating conflict with fisheries (Bearzi et al. 2004), which may have induced a genetic bottleneck effect, though definitive evidence for this is lacking (Galov et al. 2011). Area census surveys in 1998 estimated the total number of bottlenose dolphins in the Croatian part of the Adriatic Sea at 218 individuals (Gomerčić et al. 2002). Today, the bottlenose dolphin is considered endangered and is protected under Croatian law. Microsatellite analysis did not indicate reduction of genetic diversity, implying that the Adriatic dolphins are not isolated and may be part of a larger eastern Mediterranean population (Galov et al. 2011), which was inferred by Natoli et al. (2005). However, data on the fine-scale genetic structure of the eastern Mediterranean bottlenose dolphin population are still missing.

Genetic studies on wild populations are mostly performed on neutral makers which are informative regarding population history or phylogenetic reconstructions. Nevertheless, if we are interested in adaptively important genetic diversity and selective processes that involve the interaction of individuals with their environment, the best candidate genes for analysis are the genes of the major histocompatibility complex (MHC) (Sommer 2005; Bernatchez and Landry 2003). MHC genes are the most polymorphic genes in vertebrates, and they encode cell-surface glycoproteins that bind and present antigens to T cells, triggering an appropriate immune response. Class II molecules are primarily expressed on antigen-presenting cells, where they bind peptides derived from the processing of extracellular pathogens such as bacteria or parasites.

The extreme variability at MHC loci is thought to determine the ability of individuals to respond to pathogens and parasites and thus to influence individual fitness and long-term population survival (Hughes 1991; Hughes and Nei 1992). Most of this polymorphism occurs in exon 2, which encodes the peptide-binding region (PBR) that recognises and binds antigens. MHC polymorphism is believed to be maintained by balancing selection, which has been proposed to occur in several forms: heterozygote advantage or overdominance, which predicts that heterozygous individuals can respond to a broader range of antigens than can homozygous individuals (Doherty and Zinkernagel 1975); negative frequency-dependent selection, also referred to as rare-allele advantage selection, which favours rare alleles presumably because they confer greater resistance to new pathogens (Takahata and Nei 1990), and diversifying selection, according to which heterogeneous selective pressures promote local adaptation and maintain MHC allele diversity on a local scale (Hedrick 2002). These forms of balancing selection are not mutually exclusive: several forms may be operating simultaneously to maintain MHC polymorphism.

MHC genes have been used as indicators of evolutionary and adaptive potential of populations and species in various studies of population genetics, evolution, molecular ecology and conservation genetics. In marine mammals, the levels of MHC polymorphism are unclear. On one hand, studies have revealed high MHC diversity in grey seal (*Halichoerus grypus*) (Cammen et al. 2011), California sea lion (*Zalophus californianus*) (Bowen et al. 2006), baleen whales (Mysticeti) (Baker et al. 2006) and finless porpoise (*Neophocaena phocaenoides*) (Xu et al. 2007). On the other hand, studies have reported low MHC diversity in fin whale (*Balaenoptera physalus*) (Nigenda-Morales et al. 2008), sei whale (*Balaenoptera borealis*) (Trowsdale et al. 1989), beluga (*Delphinapterus leucas*) (Murray et al. 1995), narwhal (*Monodon monoceros*) (Murray and White 1998) and vaquita (*Phocoena sinus*) (Munguía-Vega et al. 2007). The low MHC polymorphism in these marine species has been attributed to the low prevalence of infectious disease in the marine environment, since parasites and pathogens are thought to be the primary agents selecting for MHC diversity (Trowsdale et al. 1989; Slade 1992). Whatever its cause, this lower MHC polymorphism has led researchers to hypothesise that balancing selection on MHC loci is weaker in marine mammals than in terrestrial mammals. These inconsistent results about MHC genetic diversity in marine mammals makes it difficult to draw conclusions, especially since many studies are based on relatively few individuals per species (Hayashi et al. 2003; Xu et al. 2009). Limited number of studies has been performed on bottlenose dolphin MHC genes. Yang et al. (2007) detected one DQB and two DRB loci in *Tursiops*, while subsequent study of 42 *T. truncatus* individuals from Taiwanese waters showed moderate variation at the DQB locus (Yang et al. 2008). We are unaware of studies examining DRA and DQA polymorphism in bottlenose dolphins on a population level.

To help clarify and deepen our understanding of MHC genetic diversity in bottlenose dolphins, we investigated the diversity of MHC class II DRA, DQA and DQB loci in a bottlenose dolphin population from the Adriatic Sea. We determined MHC class II haplotypes and compared them with haplotypes found in other Mediterranean locations. We used our results to discuss and contribute to a better understanding of molecular signatures of natural selection in dolphin MHC evolution.

Materials and methods

Laboratory procedures

Tissue samples from 62 bottlenose dolphin carcasses were collected from 1997 until 2011 along the eastern (Croatian)

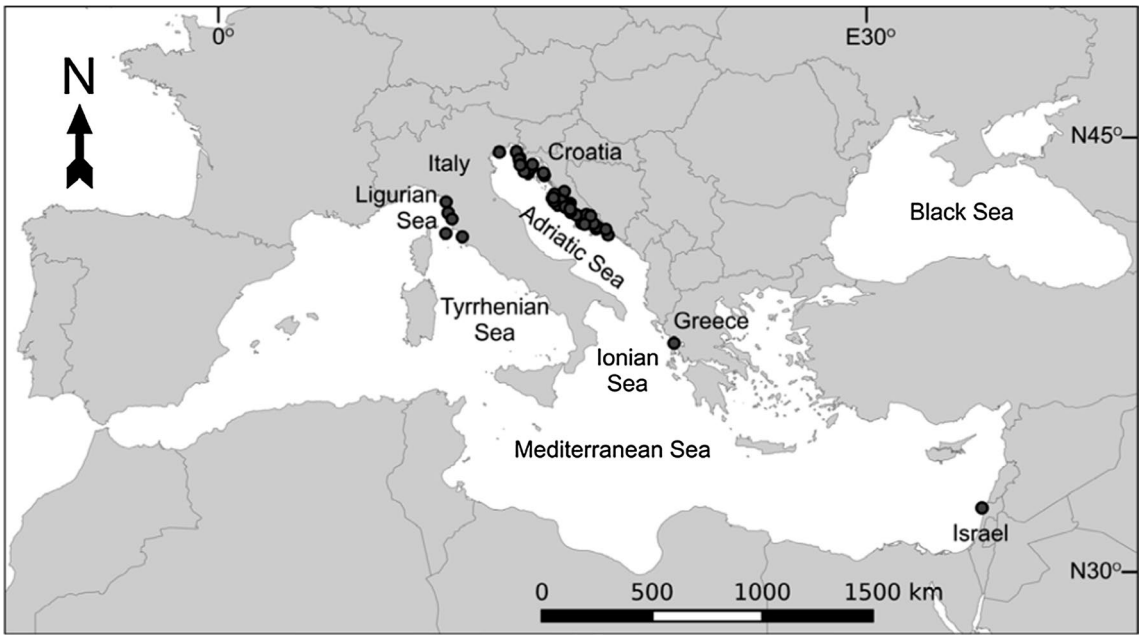


Fig. 1 The location of sampled individuals (*black dots*)

coast of the Adriatic Sea. Tissue decomposition prevented us from extracting useful DNA from all samples; in the end, we obtained samples from 48 individuals (Fig. 1). Two more Adriatic samples were obtained from animals that stranded on the western (Italian) coast. To allow comparison with these Adriatic samples, we also obtained tissue from bottlenose dolphins in other Mediterranean locations, including one individual from the Tyrrhenian Sea, five from the Ligurian Sea, one from the Ionian Sea and five off the coast of Israel.

Total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega). We amplified regions of exon 2 in the MHC II genes DRA, DQA and DQB using the following primers: aatcatgtgatcatccaagctgagttc (DRA1, forward) and tgtttggggtgttgttggagcg (DRA2, reverse; Xu et al. 2007); gctgaccatgttggctcctatg (DQAex2F, forward; Heimeier et al. 2009) and aagagggaggatgagggtcc (DQAR, reverse; this study); catgtgcaacttcaccaacgg (DQB1, forward) and ctggtagttgtgtctgcacac (DQB2, reverse; Murray et al. 1995). Amplifications were carried out by polymerase chain reaction (PCR) in a total volume of 25 µL containing 150–250 ng of genomic DNA, 0.2 µM of each primer and 1× QIAGEN HotStarTaq Master Mix (Qiagen, Hilden, Germany), consisting of 1× PCR buffer, 200 µM of each dNTP and 2.5 units HotStartTaq DNA polymerase. A negative control containing no DNA template was included in each amplification run to detect contamination. All amplifications were performed using a standard PCR protocol consisting of an initial incubation at 95 °C for 15 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at the appropriate temperature for 1 min (DRA, 55 °C; DQA, 50 °C; DQB, 58 °C) and polymerisation at 72 °C for 1 min. A final extension step was carried out at 72 °C for 10 min.

PCR products were visualised on 1 % agarose gels stained with SYBR Safe DNA gel stain (Invitrogen). Sequencing for typing was performed using the same primers as for PCR and in only the reverse direction for DQA but in both directions for DRA and DQB. Sequence processing and analysis were performed with BioEdit (Hall 1999). To identify each of the two alleles in heterozygous animals, we used Applied Biosystems SeqScape® software, which is designed for reference-based analysis. This program compares each individual sequence obtained from PCR with a library which is constituted of previously identified alleles to determine the allele or pair of alleles present in homozygotes and heterozygotes. The program reports the complete or the closest allele matches for the input sequence. For each locus investigated, we constructed the reference library using alleles available from GenBank and alleles that we identified by cloning. Sequencing to confirm new alleles was then performed in both directions, and further DNA cloning was performed. The PCR products were ligated into vectors and transformed into bacteria using the pGEM-T Vector System II (Promega). Plasmid DNA from 8 to 10 positive clones per individual was isolated and purified using the Promega Wizard Plus SV Miniprep DNA Purification System, and inserts were sequenced using the PCR primers described above. Comparison of the cloned sequences with directly sequenced PCR products allowed

the confirmation of allele sequences and controlled for artefacts generated during PCR or cloning. We assigned new alleles following the criteria established by the mammalian MHC nomenclature committees, which include DNA cloning and subsequent sequencing of several clones in both directions and/or sequencing of at least two PCRs from homozygous animals in both directions (Kennedy et al. 1999). Three-locus haplotypes were identified using Clark Empirical Method (Clark 1990; Kennedy et al. 2002). Three haplotypes in totally six animals were identified as homozygous (Supplementary table S1). Those three haplotypes were further found in 47 individuals: 17 dolphins were heterozygous for homozygous haplotypes, while 30 dolphins carried one of the homozygous haplotypes and the other haplotype was inferred by subtraction. During that procedure, we were able to infer further 11 haplotypes. Finally, the nine remaining dolphins were examined using 11 haplotypes inferred in the previous step. In five animals, we confirmed four of those haplotypes, and in two individuals, we inferred two new haplotypes. Haplotypes in two individuals remained ambiguous and unresolved. Additionally, we confirmed obtained haplotypes by reconstructing gametic phases of three-locus genotypes using ELB algorithm (Excoffier et al. 2003) implemented in Arlequin 3.11 (Excoffier et al. 2005). The frequencies of alleles and three-locus haplotypes present in the Adriatic population and individuals from other Mediterranean locations were compared using a set of common statistical tests (chi-squared test, Fisher's test, *G* test) with bootstrap (5,000 iterations) under the R software environment (R Core Team 2014), using package *boot* (Canty and Ripley 2014).

Genetic analysis

We used MEGA version 5 (Tamura et al. 2011) to choose the best nucleotide and amino acid substitution models according to the Bayesian information criterion, as well as to compute nucleotide and amino acid evolutionary distances (*d*). We tested for the presence of recombination using RDP3 Alpha 44 software (Martin et al. 2010). RDP3 simultaneously applies various statistical and phylogenetic methods to generate a weighted consensus about the presence and characteristics of recombination events and to identify potential recombinants. For each locus and for three-locus haplotypes, we tested deviation from Hardy–Weinberg proportions on 50 individuals from the Adriatic Sea using the exact test (Monte Carlo Markov Chain simulation) (Guo and Thompson 1992), as implemented in the program Arlequin 3.11 (Excoffier et al. 2005). DRA sequences were excluded from further analysis due to their low polymorphism.

Positive selection can be deduced when the non-synonymous mutation rate (d_N) exceeds the synonymous mutation rate (d_S). Due to the fact that it takes a long time to accumulate a statistically significant d_N to d_S ratio, this test does not explore the presence of positive selection in a contemporary population, but points to the selection that acts through evolutionary time (Garrigan and Hedrick 2003). We used four program packages to test whether positive selection was shaping the evolution of DQA and DQB genes in bottlenose dolphins. MEGA 5 was used to calculate relative values of d_N and d_S across the entire sequences according to Nei and Gojobori (1986) and after correcting for multiple substitutions (Jukes and Cantor 1969). Standard errors were obtained using 1,000 bootstrap replicates, and the significance of observed positive selection ($d_N > d_S$) was assessed using the one-tailed *Z* test. This MEGA analysis by itself is incomplete because selection is less likely to act across an entire gene and more likely to act at specific sites within the gene. Therefore, we used two program packages to analyse each codon individually using different statistical approaches; both programs allowed the d_N/d_S ratio to vary among codon sites in order to identify those likely to have evolved under the influence of positive selection. The CODEML program, a powerful maximum-likelihood algorithm within the Phylogenetic Analysis by Maximum Likelihood (PAML) 4.3b package (Yang 2007), was used to measure selective pressure at the codon level based on the ratio of non-synonymous to synonymous mutations ($\omega = d_N/d_S$); values of $\omega < 1$, $= 1$ and >1 indicate negative purifying selection, neutral evolution and positive selection, respectively. We used a Bayes empirical Bayes (BEB) approach to estimate mean ω and standard errors across codon positions. The models implemented in this study were M0 (one ratio: best average ω across all sites), M1a (nearly neutral: estimates the proportion of sites that best-fit $\omega = 0$ versus those best-fit by $\omega = 1$), M2a (positive selection: adds a third set of sites to M1a that have $\omega > 1$ and estimates associated proportion of sites, M3 (discrete: fits proportions and ω values assuming three classes of sites), M7 (beta: ω is beta-distributed) and M8 (beta and omega: a proportion of sites are beta-distributed). M0 does not allow for variation in ω across codon sites, and M1a and M7 allow only for neutral evolution and purifying selection at some proportion of sites, while M2a, M3 and M8 allow for the possibility of positive selection at a proportion of sites. Models M0, M1a and M7 served as null models for M3, M2a and M8, respectively. We compared M0 and M3 to test for the significance of heterogeneity in ω across sites, whereas M1a was compared with M2a and M7 with M8 to test for positive selection. Statistical evaluation to compare nested models was performed using the likelihood ratio test. Significant adaptive evolution was inferred if twice the difference in log-likelihood values was greater than the chi-squared critical value for the given degrees of freedom. The next three codon-based maximum-likelihood

methods that we applied were implemented in the HyPhy package (Kosakovsky Pond et al. 2005) on the Datamonkey web server (Kosakovsky Pond and Frost 2005). The fixed effects likelihood (FEL) method directly estimates the rates of non-synonymous and synonymous substitutions based on a codon substitution model and assesses the significance of a $d_N \neq d_S$ result using a likelihood ratio test. The random effects likelihood (REL) approach models the distribution of substitution rates across sites and then infers the rate at which each site evolves. REL can be considered an extension of PAML that allows variation in the synonymous mutation rate. Since REL can be unsuitable for closely related sequences, we analysed DQB sequences using the statistically more robust fast unconstrained Bayesian approximation (FUBAR; Murrell et al. 2013).

Deviations from neutral expectations of molecular evolution were tested using Tajima's *D* test (Tajima 1989), as implemented in DNASP 5.0 (Librado and Rozas 2009). This testing was carried out calculating the average Tajima's D and also using a sliding window option with nine nucleotides window length and a step size of three nucleotides. Tajima's *D* is based on the difference between genetic variation estimated from the number of segregating sites and the genetic variation estimated from the average number of nucleotide differences based on pairwise comparisons. In a population of constant size, Tajima's *D* is expected to be zero in the absence of selection (neutrality), positive in the presence of balancing selection and negative in the presence of purifying selection against deleterious mutations (Tajima 1989).

Phylogenetic analysis

DRA, DQA and DQB sequences identified in this study were used for phylogenetic analyses. Since allele sharing and/or similarity among closely related taxa are hallmarks of MHC genes, we also included publicly available sequences from the family *Delphinide*. To establish the phylogenetic relationship of bottlenose dolphin DRA, DQA and DQB alleles to those from other cetacean and mammalian species, we included sequences from more distantly related cetacean species (classification according to Rice (1998)), artiodactyl and equid sequences in our analyses. We found considerable number of cetacean DRA and DQB sequences to be deposited in GenBank, but only a limited number of DQA sequences, reported by Heimeier et al. (2009). The Bayesian inference as implemented in MrBayes 3.2.1. (Ronquist and Huelsenbeck 2003) was used to infer phylogenetic relationships among alleles as well as to detect possible trans-species polymorphism. Prior to analysis, the best-suited evolutionary model for each data set was determined using jMODELTEST 0.1.1 software (Posada 2008) and the Bayesian information criterion. Selected models were TPM3 + G, K80 + G and TVM + I + G for MHC class II DRA, DQA and DQB data sets, respectively. Bayesian phylogenetic analyses were conducted separately using the model selected for each data set. Two parallel runs with four chains were run for three million generations. Trees were sampled every 100th generation, and the first 20 % of the trees was discarded as a burn-in. In all analyses, the average standard deviation of split frequencies value fell below 0.01 well before the end of the search, indicating the convergence of the two runs. Statistical parsimony networks of MHC class II DQA alleles were constructed using TCS v. 1.21 (Clement et al. 2000) using 90 and 95 % parsimony connection limits.

Results

DRA, DQA and DQB diversity and three-locus haplotypes

In this study, 62 *T. truncatus* samples amplified successfully at all three loci analysed. Amplified sequences comprised the entire exon 2 of the DQA locus (246 bp) and part of exon 2 of DRA and DQB loci (189 and 172 bp, respectively; Table 1). None of the detected sequences contained any stop codons. Among individuals analysed, 20 were homozygous at the DRA locus, 14 at the DQA locus and nine at the DQB locus. Fourteen heterozygous individuals that were cloned to identify unknown alleles (with 8–10 clones per individual analysed) contained no more than two alleles at any of the loci analysed. These findings supported previous data reporting the presence of one locus (copy) in *T. truncatus* (Yang et al. 2007; Heimeier et al. 2009; Hayashi et al. 2003) and in family *Delphinidae* (Xu et al. 2009, Heimeier et al. 2009; Hayashi et al. 2003) for each of the genes analysed in this study. The number of unique exon 2 sequences detected at each locus was three for DRA, 10 for DQA and 12 for DQB (Figure S1). One of the DQA alleles contained a three-nucleotide deletion, which did not result in frame shifting (the way the sequence is read is not disrupted). Novel alleles, not present in the GenBank data base or found in literature, were identified at each locus: one at DRA, nine at DQA and three at DQB (Figure S1). New alleles were assigned official names according to the established MHC nomenclature (Ellis et al. 2006). DQA alleles were deposited with GenBank (accession numbers KF751585–KF751593), whereas DQB and DRA alleles were deposited in the Dryad Repository (http://doi.org/10.5061/dryad.1vt90).

Comparing the three loci analysed, DQA and DQB showed considerable polymorphism, while the three alleles detected at DRA displayed high sequence similarity. These three alleles had only three polymorphic sites, of which one was non-synonymous, leading to a change of one amino acid. The estimated diversity, in terms of the number of

Table 1 Number of variable nucleotide sites, average nucleotide and amino acid distances, number of unique amino acid sequences, $\Theta\pi$, Θs and Tajima's *D* values for DRA, DQA and DQB alleles identified in *T. truncatus* (62 individuals) from Mediterranean Sea

Locus	Length of amplified sequence	No. of variable nucleotide sites	Nucleotide distance		Amino acid distance		No. of unique amino acid sequences	$\Theta\pi$	Θs	Tajima's D[c]
			Nucleotide substitution model	*d*	Amino acid substitution model	*d*				
DRA(3)	189	3	JC	0.011	JTT	0.011	2	2.00	2.00	0
DQA(10)	246[a]	21	JC + G	0.047	JTT + G	0.093	10	7.51	7.42	−0.22
DQB(12)[b]	172	40	T92 + G	0.114	JTT + G	0.255	12	12.9	13.25	−0.12
			JC	0.080						

The number of alleles identified is given in parentheses

JC Jukes–Cantor substitution model, *T92* Tamura 3 parameter, *G* gamma distribution shape parameter, *JTT* Jones–Taylor–Thornton substitution model, *d* overall mean distance value

[a] For entire exon 2

[b] Two distance values were calculated for DQB: T92 + G, since it was the best nucleotide model proposed by MEGA, and JC, to enable comparison with other cetacean species (Villanueva-Noriega et al. 2013)

[c] $P > 0.1$ (all three loci)

Table 2 16 DRA/DQA/DQB haplotypes defined in 60* bottlenose dolphins

	DRA	DQA	DQB	h	Frequency % of haplotypes found in Adriatic Sea	Sampling locations outside Adriatic Sea
Haplotypes found exclusively in the Adriatic Sea	DRA*01	DQA*02	DQB*08		9.18	
	DRA*02	DQA*06	DQB*27		2.04	
	DRA*01	DQA*05	DQB*05		1.02	
	DRA*02	DQA*02	DQB*26		1.02	
	DRA*01	DQA*03	DQB*25		1.02	
Haplotypes found in the Adriatic Sea and on other Mediterranean locations	DRA*01	DQA*02	DQB*07	3	34.69	Tyrrhenian Sea, Ligurian Sea
	DRA*02	DQA*01	DQB*22	2	29.59	Israel, Greece, Tyrrhenian Sea
	DRA*02	DQA*03	DQB*25		14.28	Israel
	DRA*01	DQA*01	DQB*22		2.04	Israel
	DRA*02	DQA*04	DQB*01		2.04	Ligurian Sea
	DRA*03	DQA*04	DQB*01		2.04	Ligurian Sea
	DRA*02	DQA*07	DQB*06		1.02	Israel
Haplotypes found exclusively on other Mediterranean locations	DRA*02	DQA*02	DQB*06			Ligurian Sea
	DRA*02	DQA*08	DQB*20			Ligurian Sea
	DRA*02	DQA*09	DQB*10	1		Israel
	DRA*02	DQA*10	DQB*11			Ligurian Sea

Underlined alleles were found exclusively on locations specified in the first column

h absolute number of animals homozygous for a given haplotype

* We were unable to define the haplotypes in two individuals

variable nucleotide sites and nucleotide and amino acid distances, was higher for DQB than for DQA. All observed DQA and DQB alleles encoded products with unique amino acid sequences (Table 1). In 50 individuals of the Adriatic population, observed heterozygosity exceeded the expected value at all three loci, but it was not significantly different from Hardy–Weinberg expectations.

All alleles combined to form 16 DRA/DQA/DQB haplotypes (Table 2). Using the Clark Empirical Method, we could not define haplotypes in one individual from the Adriatic Sea and one from the Ligurian Sea due to ambiguous allele combinations. Both of these individuals had the same genotype (DRA*02;*03/DQA*02;*04/DQB*01;*07).

In individuals from the Adriatic Sea, we identified 12 DRA/DQA/DQB haplotypes, two of which were identified in four homozygous animals (Table 2). Four haplotypes were relatively common, occurring at frequencies of 9.18–34.69 %. Five DRA/DQA/DQB haplotypes, two

DQA alleles and four DQB alleles appeared to be specific for Adriatic Sea samples, as they were absent in individuals from other locations. In 12 individuals from other Mediterranean locations, we identified 11 DRA/DQA/DQB haplotypes (Table 2), seven of which were shared with the Adriatic population, including the three most frequent ones. Three DQA alleles and three DQB alleles found in other Mediterranean locations and not present in the Adriatic Sea gave rise to three unique haplotypes. Interestingly, the allele DQB*08, which occurred frequently in the Adriatic (9.18 %), was not found at other locations. The alleles DQA*09 and DQB*10 that formed the haplotype DRA*02/DQA*09/DQB*10 were not present in the Adriatic. This same haplotype was, however, found in the homozygous state in an individual from Israel, suggesting that it may be prevalent in or, even unique to, that part of the Mediterranean Sea. All bootstrapped statistical tests showed that the difference in frequencies of three-locus haplotypes between dolphins in the Adriatic and other Mediterranean locations is not coincidental ($P < 0.01$) and therefore cannot be attributed to small sample size. Similarly, differences in DQA and DQB allele frequencies in the Adriatic and other Mediterranean locations cannot be attributed to chance alone ($P < 0.01$).

Selection analysis

Since the presence of recombinant sequences in the data set significantly influences selection and phylogenetic analysis, we tested DQA and DQB sequences for the presence of intralocus recombinations. No recombination events were found. The global estimates of d_N/d_S averaged across all codon sites showed significantly higher non-synonymous substitution rates than synonymous substitution rates, indicating positive selection at DQA and DQB loci (Table 3). Results from maximum-likelihood analysis in CODELM confirmed that positive selection was acting on DQA and DQB loci: models allowing variable evolutionary rates across codon sites (M3, M2a, M8) fit our sequence data better than did models assuming one evolutionary rate (M0, M1a, M7) (Table 4).

All codon-based methods used in this study, which relied on d_N/d_S (ω) values, identified codons that were likely to be affected by positive selection (Table 5). The sets of codons under positive selection identified by different methods overlapped considerably, while the number of detected codons identified by each method correlated with the type of statistical test applied. Thus, the most conservative FEL predicted the smallest number of codons to be under positive selection (Table 5). Most codons predicted to be under positive selection are involved in antigen binding or lie adjacent to antigen-binding sites based on the

Table 3 Average rates of non-synonymous (d_N) and synonymous substitutions (d_S) used to calculate d_N/d_S values in order to test for the presence of selection

Locus	N	d_N	d_S	Type of selection	P
DRA	3	0.004 (0.004)	0.036 (0.028)	Neutral evolution $d_N/d_S = 0.11$	n.a.
DQA	10	0.036 (0.011)	0.004 (0.004)	Positive $d_N/d_S = 9.0$	0.001
DQB	12	0.103 (0.023)	0.016 (0.011)	Positive $d_N/d_S = 6.44$	0.001

d_N and d_S values were computed using the Nei and Gojobori (1986) method with the Jukes–Cantor correction implemented in MEGA software. Standard errors (in parentheses) were obtained through 1,000 bootstrap replicates

N number of alleles, *P* significance of positive selection

Table 4 Likelihood ratio test analyses to compare different models of codon evolution implemented in PAML for exon 2 sequences at the DQA and DQB loci in *T. truncatus*

Models compared	*df*	DQA (10)		DQB (12)	
		2Δl	*P* value	2Δl	*P* value
M3 versus M0	4	34.0	<0.0001	82.1	<0.0001
M2a versus M1a	2	39.8	0.0006	21.8	0.002
M8 versus M7	2	45.3	0.0005	23.8	0.002

Differences in log-likelihood values (2(lb–la)) were compared with degrees of freedom using χ^2 distribution. The number of sequences analysed is given in parenthesis

df the number of free parameters in the ω distribution, ω the selection parameter, *2Δl* the likelihood ratio statistic computed as 2(lb–la), where la and lb are the log-likelihood values for each of the models being compared (log-likelihood values for each model were extracted from CODEML output file)

Table 5 Codon sites under positive selection identified using codon-based maximum-likelihood methods based on d_N/d_S (ω) values: M2 and M8 (implemented in PAML), and FEL, REL and FUBAR (implemented in HyPhy)

Locus	Codons identified
DQA	**22***[b], **29**[b], **50***[n], 51[b], **64***[n], **66**[b], **67**[b], **71**[n], **74**[b]
DQB	**21**, **23**[b], **25**[b], **32**[b], 51[b], **52**[n], **62**[n], **65**[b], **66***[b], **69**[b], 70[n]

All sites indicated here were identified by models M2 and M8 with at least >95 % posterior probability, while sites in **bold** were identified by model M8 with posterior probabilities of >99 %

Underlined sites were identified by the REL method at the DQA locus (Bayes factor > 100) or by the FUBAR method at the DQB locus (posterior probability > 0.9). Sites marked with asterisks were identified by the FEL method ($P < 0.1$)

Codon numbering corresponds to exon 2 amino acid positions

[b] Predicted antigen-binding sites

[n] Sites neighbouring to predicted antigen-binding sites

three-dimensional structural model of the human ortholog (Figures S2–S3; Brown et al. 1993).

The average Tajima's D was close to zero for DQA and DQB exon 2 sequences ($D = -0.22$ and $D = -0.12$, respectively, $P > 0.1$) showing no deviation from neutral expectations (Table 1). Sliding window analysis of Tajima's D within exon 2 identified regions with a D value above the threshold value of $P < 0.05$ for both DQA and DQB loci, indicating an excess of high-frequency segregating sites and supporting the presence of positive selection at some sites. Moreover, regions of exon 2 flagged by Tajima's D as frequently segregating also contained two codons flagged by codon-based selection models: codon 50 at the DQA locus and codon 21 at the DQB locus (Figures S2 and S3).

Phylogenetic analysis

Phylogenetic analysis of DRA and DQA data sets revealed well-supported monophyletic Cetacea clade (Figs. 2a, 3a). Phylogenetic analysis of DQB sequences was, for the most part, consistent with the expectation that trans-species polymorphism occurs mainly among closely related species (Fig. 2b). We discovered three DQB alleles that were identical across species of the same family *Delphinidae*: genera *Tursiops* and *Sousa* and genera *Grampus* and *Stenella* (Fig. 2b). Most DQB alleles clustered according to families but not according to species: alleles in *T. truncatus* clustered mainly with other species or genera of the family *Delphinidae* (circles in Fig. 2b). We observed strong evidence (>0.99 Bayesian posterior probability) of trans-species and

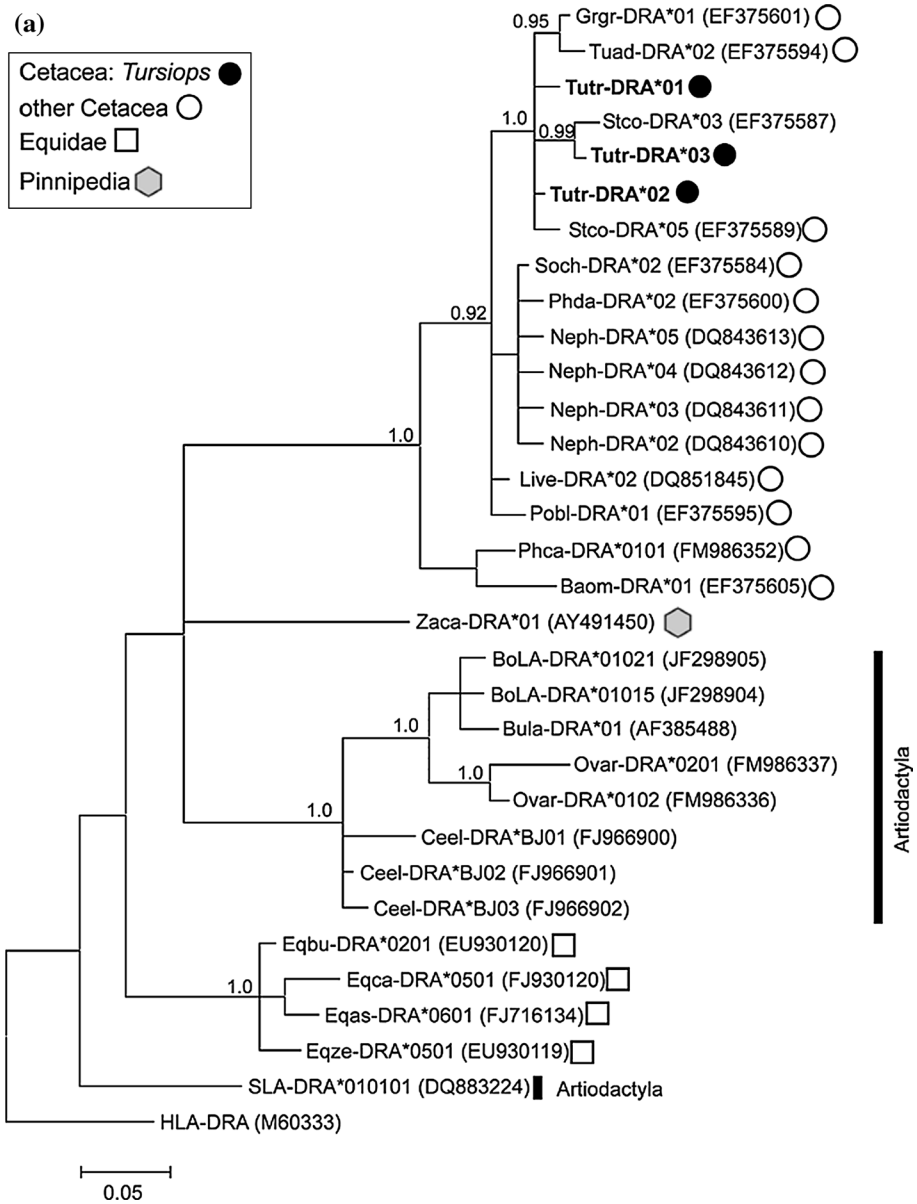


Fig. 2 The 50 % majority-rule consensus phylograms derived from Bayesian analysis of partial sequences of MHC class II DRA exon 2 (**a**) and partial sequences of cetacean MHC class II DQB exon 2 (**b**). Numbers at the nodes indicate Bayesian posterior probabilities (BPP); BPP values below 0.9 are not indicated. The human HLA-DRA allele and HLA-DQB1*201 allele were used as outgroups to root the trees. GenBank accession numbers are given in parentheses. Different *symbols* depict taxonomic designations according to the legend

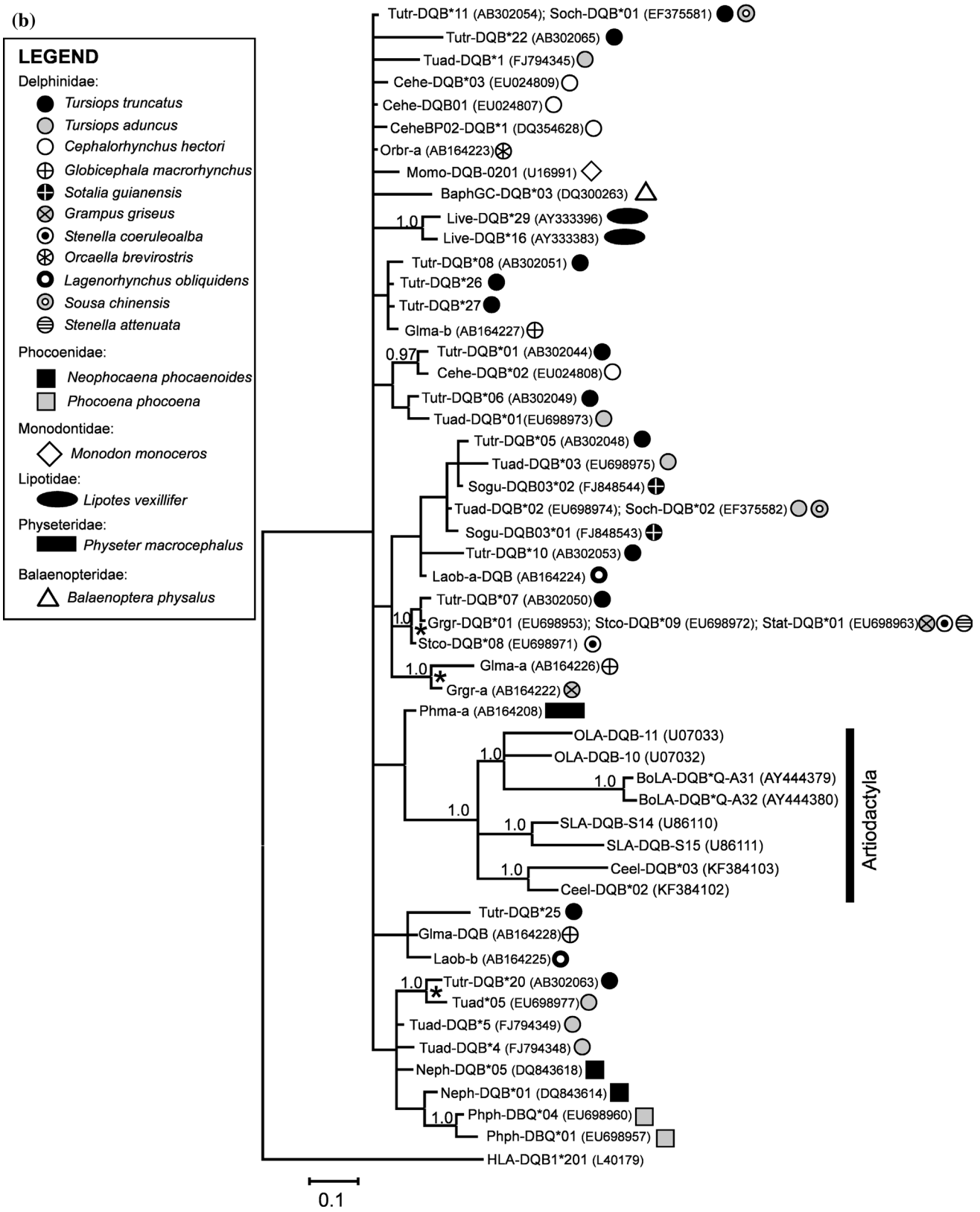


Fig. 2 continued

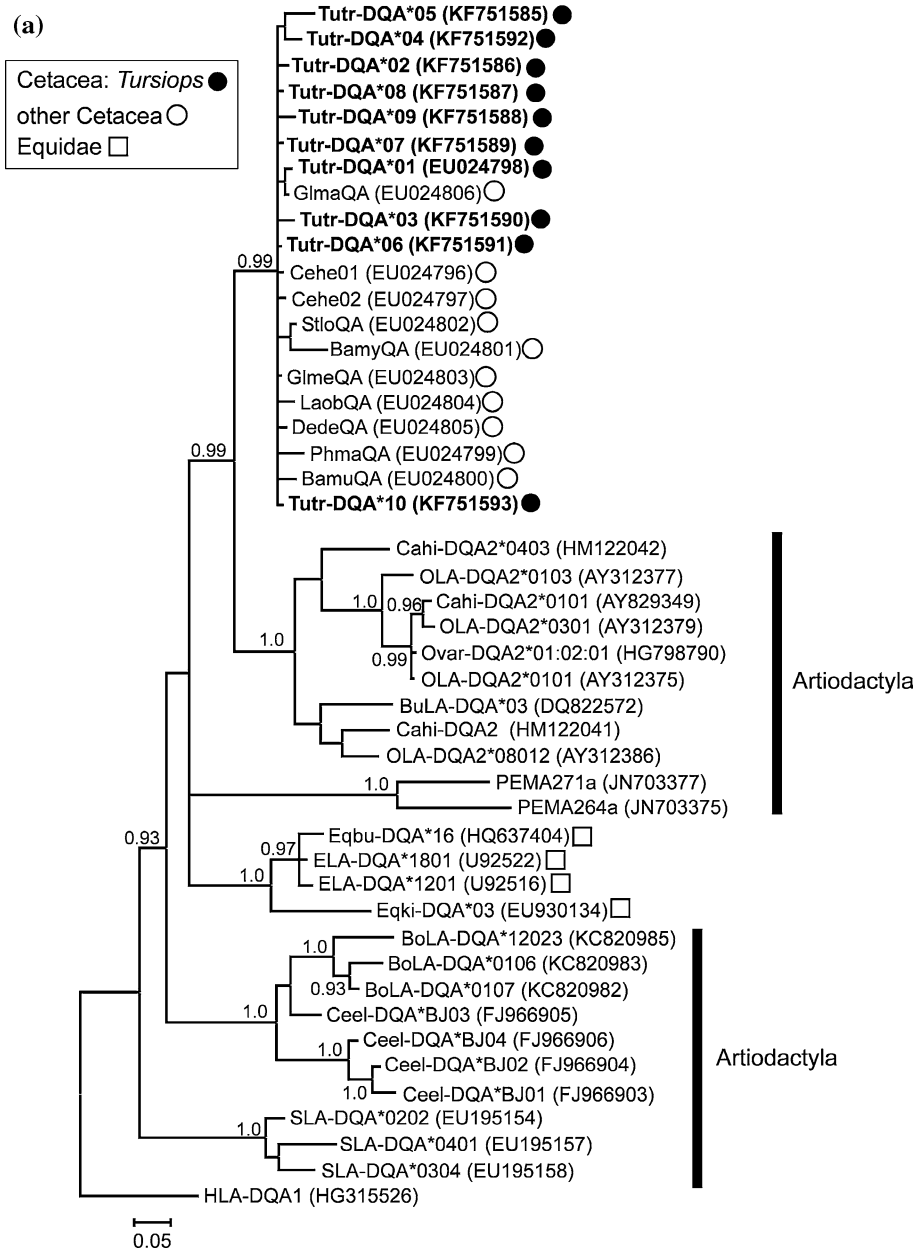


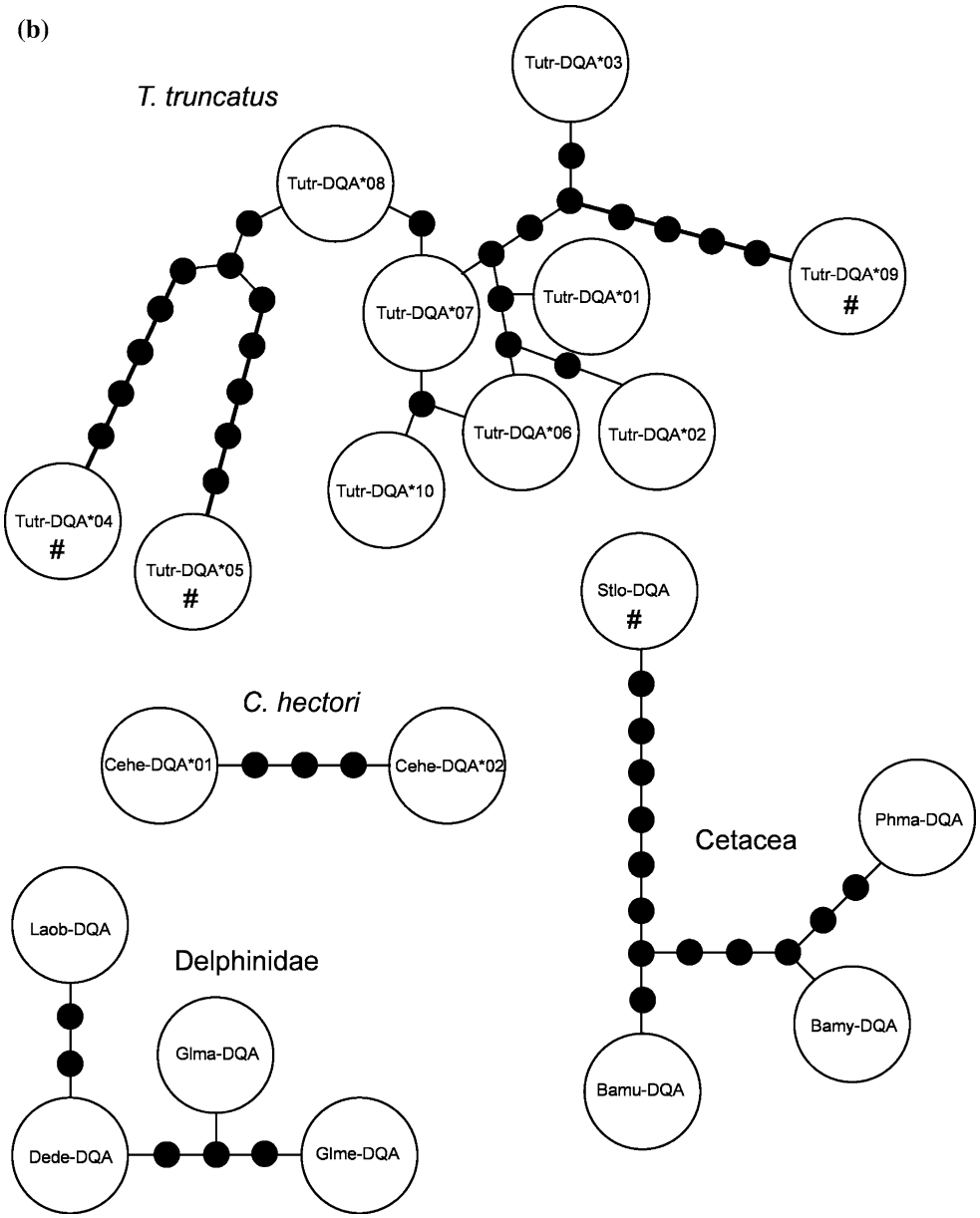
Fig. 3 **a** The 50 % majority-rule consensus phylogram derived from Bayesian analysis of MHC class II DQA exon 2. *Numbers* at the nodes indicate Bayesian posterior probabilities (BPP); BPP values below 0.9 are not indicated. The human HLA-DQA1*01 allele was used as an outgroup to root the tree. GenBank accession numbers are given in parentheses. Different *symbols* depict taxonomic designations according to the legend. **b** The statistical parsimony network of cetacean MHC class II DQA exon 2 sequences constructed with a 90 % parsimony connection limit. *Black circles* depict missing intermediates. Alleles not connected to any of the subnetworks with a 95 % parsimony limit are indicated by "#". Sequences belong to *T. truncatus* (Tutr, GenBank accession numbers EU024798, KF751585-KF751593), *Cephalorhynchus hectori* (Cehe), *Stenella longirostris* (Stlo), *Globicephala melas* (Glme), *Globiocephala macrorhinchos* (Glma), *Lagenorhinchus obscures* (Laob), *Delphinus delphis* (Dede), *Physeter macrocephalus* (Phma), *Balaenoptera musculus* (Bamu) and *Balaena mysticetus* (Bamy); GenBank accession numbers (EU024796, EU024797, EU024799-EU024806)

trans-genus clades: *Tursiops*, *Grampus* and *Stenella*; *Globicephala* and *Grampus*; *T. truncatus* and *T.aduncus.* These clades strongly suggest the presence of trans-species and trans-genus allelic polymorphism.

Alleles from the Yangtze River dolphin or baiji (*Lipotes vexillifer*) formed a distinct and well-supported clade, which was expected since it belongs to a family (*Lipotidae*). Many DQB alleles were assigned to unresolved basal polytomy probably as the result of a relatively recent diversification of the family *Delphinidae* and the shortness of the sequences used to construct the tree. In particular, three alleles from *Cephalorhynchus hectori* (Cehe) were included in polytomy, suggesting substantial intraspecies allele divergence. This is consistent with previous studies in leporids indicating higher intraspecific than interspecific allelic distances (e.g. Surridge et al. 2008).

Bayesian analysis of our MHC class II DQA sequence data revealed a monophyletic but completely unresolved Cetacea clade (Fig. 3a). Since network-building algorithms are superior to tree-building algorithms for analysing highly similar sequences (Posada and Crandall 2001), we further investigated the phylogenetic relationship of DQA alleles using a statistical parsimony network approach. The 90 % parsimony network is shown in Fig. 3b. Most alleles clustered according to species (*T. truncatus* and *C. hectori*) or family (*Delphinidae*). However, one cluster revealed close relationships among alleles belonging to the *Physeteridae* family (*Physeter macrocephalus*), the member of

Fig. 3 continued



suborder *Odontoceti*, and the members of suborder *Mysticeti* (*Balaena mysticetus* and *Balaenoptera musculus*). In the 95 % parsimony network, three *Tursiops* alleles and the *Stenella* allele (indicated by "#" in Fig. 3b) were not connected to any of the subnetworks. Unfortunately, we cannot draw firm conclusions about the evolutionary pattern underlying the phylogenetics of this gene because too few cetacean DQA allele sequences are available.

Discussion

In the present study, we describe the diversity of the MHC class II genes in bottlenose dolphins from the Mediterranean Sea. This is, to our knowledge, the first report of MHC class II haplotypes in the family *Delphinidae*. As expected, the DRA locus in dolphins showed negligible variability, comparable to the DRA loci not only in other cetacean species (Xu et al. 2007, 2008) but also in various other vertebrate species, such as canids (Wagner et al. 1999), cats (Yuhki and O'Brien 1997) and humans (Chu et al. 1994). In strong contrast to the DRA locus, the DQA and DQB loci in dolphins displayed considerable sequence variation. In the Adriatic Sea, we identified seven DQA sequences and nine DQB sequences (Table 2), with the sequences at each locus showing significant variation among themselves. In this way, bottlenose dolphins in the Adriatic Sea seem to have retained considerable MHC genetic variation, which is congruent with results obtained on neutral genetic loci that also indicated high level of genetic diversity (Galov et al.

2011). Diversity indicators such as the number of variable nucleotide sites and nucleotide and amino acid distances were higher for the DQB locus than for the DQA locus. This is consistent with the finding that most class II MHC polymorphism is concentrated in the beta genes (Reche and Reinherz 2003; Bondinas et al. 2007). In fact, our observed polymorphism likely underestimates the variation across the entire exon 2, given that our DQB sequences covered only 172 bp.

Lack of recombination between MHC loci causes alleles to be inherited together, leading to haplotype conservation (Degli-Esposti et al. 1992). Researchers have proposed that allele combinations that support efficient immune responses to pathogens persist due to selection operating over tens or hundreds of generations (Hedrick 2000; Garrigan and Hedrick 2003). In this way, haplotype analysis is often more valuable and revealing for population studies than is single-locus analysis since the persistence of particular MHC allele in a population considerably depends on functional relevance of particular haplotype it constitutes. However, we could not make detailed comparisons about haplotype distributions in dolphins at different locations because we are unaware of other reports of bottlenose dolphin haplotypes. Nevertheless, we did observe that the three DQA and three DQB alleles not found in the Adriatic Sea but found elsewhere in the Mediterranean formed distinct haplotypes. Although Natoli et al. (2005) identified only one eastern Mediterranean population, Sharir et al. (2011) found unique morphological characteristics of dolphins from the easternmost region of the Mediterranean (Levant) which have been attributed to particular environmental conditions. Unique Israeli haplotype and three haplotypes found exclusively in the Ligurian Sea could reflect habitat differences between regions and variation in pathogen pressures that lead to local MHC adaptation. Such geographic variation and MHC-based population differentiation have already been reported in wild populations (great snipe, Ekblom et al. 2007, the house sparrow; Loiseau et al. 2009, voles, Bryja et al. 2007; the grey seal; Cammen et al. 2011). However, to draw such conclusion, it is necessary to investigate variation at neutral loci to disentangle between genetic drift and diversifying selection. It is generally difficult to define the relative contributions that genetic drift and selection make to MHC diversity, particularly when selection and population bottlenecks act simultaneously (Alcaide 2010). Under these conditions, genetic drift can overcome selection (Radwan et al. 2007; Babik et al. 2008; Miller et al. 2010; Sutton et al. 2011).

We found convincing evidence that balancing selection has been acting historically on DQA and DQB loci over a long evolutionary period: d_N/d_S was high across DQA and DQB sequences; various approaches involving different statistical constraints identified particular codons affected by selection; and excess of sites segregating at high frequency was observed. Our d_N/d_S ratio for DQB sequences (6.44) corresponds well to those obtained from bottlenose dolphin from Taiwanese waters (7.7) (Yang et al. 2008).

However, several studies have reported low MHC polymorphism in cetaceans (Trowsdale et al. 1989; Murray et al. 1995; Hayashi et al. 2003; Nigenda-Morales et al. 2008), leading to the hypothesis that the balancing selection that acts on MHC is weaker in marine mammals than in terrestrial ones due to less exposure to pathogenic selection in marine environments (Slade 1992). Consistent with this hypothesis, Villanueva-Noriega et al. (2013) found that balancing selection on the DQB locus is weaker in cetaceans than in terrestrial mammals, such as artiodactyls and primates. In fact, the same study found the strength of balancing selection to vary even among cetacean species: the bottlenose dolphin and Hector's dolphin (*Cephalorhynchus hectori*) showed low DQB variation, while the short-finned pilot whale (*Globicephala macrorhynchus*) and the North Pacific whitesided dolphin (*Lagenorhynchus obliquidens*) showed high DQB variation. Our results contrasted with the findings of Villanueva-Noriega et al. (2013), whose analysis relied on the same 172-bp fragment of DQB exon 2 as in our study. We measured $d = 0.080$ and $d_N = 0.103$ at the DQB locus in bottlenose dolphins (Tables 1 and 3); these values are on the higher end of the cetacean range and are considerably higher than the average cetacean values ($d = 0.041$ and $d_N = 0.048$) reported by Villanueva-Noriega et al. (2013), but are similar to those obtained for bottlenose dolphin from Taiwanese waters ($d = 0.081$, $d_N = 0.085$) (Yang et al. 2008). In fact, the d values reported by Yang et al. (2008) and us (current study) are similar to those reported for various terrestrial mammals, including pig, 0.062 (Luetkemeier et al. 2009); brown bear, 0.064 (Kuduk et al. 2012); hare, 0.096 (Smith et al. 2011) and rat, 0.154 (Gouy de Bellocq and Leirs 2010). The d value associated with DQA alleles in this study (0.047) was also within the range reported for various terrestrial species: rodents, 0.061–0.088 (Gouy de Bellocq et al. 2005, 2009); leporids, 0.083 (Surridge et al. 2008); wolf, 0.018 (Arbanasić et al. 2013) and elephants, 0.039 (Archie et al. 2010). Similarly, d_N value obtained in this study (0.103) and in Yang et al. (2008) (0.085) for DQB sequences is similar to those of terrestrial mammals: brown bear, 0.065 (Kuduk et al. 2012); grey wolf, 0.084 (Arbanasić et al. 2013) and lemurs, 0.12 (Huchard et al. 2012). Only the dS values for DQB sequences obtained in our study (0.016) and in the study of Yang et al. (2008) (0.011) were consistent with the value reported by Villanueva-Noriega et al. (2013) for cetacean species (0.015). Both these values are lower than those in terrestrial species (Villanueva-Noriega et al. 2013). One explanation for the low d_S may be a slow substitution rate in cetaceans, as reported for baleen whales

(Jackson et al. 2009). Another explanation for low d_S, given that synonymous substitutions accumulate over time, may be that DQB polymorphism is relatively new. Whatever the explanation for low dS, the similarity between nucleotide distance (d) and d_N values obtained for bottlenose dolphins both from Mediterranean sea (this study) and Taiwanese waters (Yang et al. 2008) and those in terrestrial species imply that the balancing selection on MHC in the bottlenose dolphin is comparable to that in terrestrial mammal species. Another approach for assessing the strength of balancing selection is to measure the accumulation of variable sites. Of the 172 nucleotide sites in twelve bottlenose dolphin DQB alleles in this study, 40 were variable (Table 1). Following Villanueva-Noriega et al.' (2013) equation (which was based on the analysis of 92 alleles found in 17 cetacean species), the number of variable sites for 12 cetacean DQB alleles was expected to be 30.11 for the mean parameters ($v_{max} = 55.9$ and $k = 9.42$) and 44.03 for the highest values of parameters ($v_{max} = 67.4$ and $k = 5.84$). According to these expected values, the number of variable sites found in the bottlenose dolphin (40) places them in the high end of the range for cetaceans.

Balancing selection leads to allele retention over long evolutionary periods, often during speciation diversification events, giving rise to similar or even identical alleles in two descendent species. This so-called trans-species polymorphism is therefore considered convincing evidence that balancing selection has been operating over the long term (Garrigan and Hedrick 2003). Trans-species polymorphism was documented for MHC genes in numerous taxa (Landry and Bernatchez 2001), primarily between species of the same genus, but also between groups related at higher taxonomic levels, such as lagomorphs and rodents (Musolf et al. 2004; Gouy de Bellocq et al. 2009). We aimed to determine whether DQA and DQB loci in cetaceans show trans-species polymorphism based on phylogenetic analysis of sequences from the genus *Tursiops* and family *Delphinidae*. If such polymorphism is present, sequences should not cluster according to species. Villanueva-Noriega et al. (2013) already documented two trans-genus DQB polymorphism events between *T. truncatus* and *Globiocephala macrorhynchus* (family *Delphinidae*). We extended these results by showing clear phylogenetic evidence of trans-species and trans-genus DQB polymorphism based on the sharing of certain alleles among species and on the presence of well-supported clusters containing alleles intermingled from different species (Fig. 2b). These results suggest that some allelic lineages have been retained by balancing selection acting over evolutionary history and that allelic diversity predates the divergence of species or even genera. This interpretation is consistent with rapid radiation of the superfamily *Delphinoidea* proposed to have taken place approximately 12–16 million years ago (Rosel et al. 1995), followed more recently by divergence between subfamilies, genera and species (Ohland et al. 1995). This interpretation implies that alleles that are shared among the species of the superfamily *Delphinoidea* are older than their diversification time. Indeed, numerous studies have described MHC alleles tens of million years (my) old; for example, the oldest HLA lineages at the DRB1 locus have been estimated at 55 my old (Klein et al. 1998), while some DQA alleles in African mole rat are likely to be at least 48 my old (Kundu and Faulkes 2007). Further evidence for trans-species polymorphism lies in our observation of two subnetworks of DQA alleles that contain sequences from different species (Fig. 3b). Instead of invoking trans-species polymorphism, it may be possible to explain our findings as the result of convergent evolution, which explains genetic similarities as the result of similar adaptive solutions to similar environmental pressures (Xu et al. 2008). Another possible explanation for trans-species polymorphism is introgressive hybridisation. Cetaceans may have a potential to produce viable hybrid offspring (Amaral et al. 2007), and hybridisation events have been recorded in bottlenose dolphins in captivity (Zornetzer and DuYeld 2003). However, nearly two decades of monitoring dolphins in the Adriatic Sea and dissections of 191 bottlenose dolphins and 39 individuals of other dolphin species that occasionally occur in the Adriatic Sea (striped dolphin *Stenella coeruleoalba* and Risso's dolphin *Grampus griseus*) at the Faculty of Veterinary Medicine in Zagreb have failed to identify any potential hybrids (unpubl data). Therefore, it seems unlikely that bottlenose dolphins from the Adriatic Sea have been hybridising with other species.

Here, we have described notable diversity of MHC class II DQ alleles and three-locus haplotypes in bottlenose dolphins in the Adriatic Sea. Inferences drawn from selection tests and the presence of trans-species polymorphism suggest that balancing selection has strongly influenced the variability of MHC class II DQA and DQB loci in bottlenose dolphin. In fact, this balancing selection in bottlenose dolphin appears to be stronger than in most other cetaceans and, contrary to previous studies, comparable to that in terrestrial mammals. Detected differences in alleles and haplotypes among Adriatic and other Mediterranean locations open questions on the role of habitat distinctions in shaping MHC variability. Future studies should compare genetic variation at neutral and MHC loci in more populations from different locations in order to examine whether diversifying selection also contributes to MHC variability.

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