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Molecular characterization of expressed *DQA* and *DQB* genes in the California sea lion (*Zalophus californianus*)

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Abstract To date, there are no published MHC sequences from the California sea lion (*Zalophus californianus*), a thriving species that, by feeding high on the marine food web, could be a sentinel for disturbances in marine and coastal ecosystems. In this study, degenerate primers and RACE technology were used to amplify near-full-length (*MhcZaca-DQB*) and full-length (*MhcZaca-DQA*) expressed class II MHC gene products from the peripheral blood mononuclear cells of two California sea lions in rehabilitation. Five unique *Zaca-DQA* sequences and eight unique *Zaca-DQB* sequences, all encoding functional proteins, were identified in the two animals, indicating the presence of multiple *DQ*– loci in this species. An additional three *Zaca-DQB* sequences containing features compatible with pseudogenes or null alleles were also identified. Despite the identification of multiple *DQA* and *DQB* sequences, the degree of heterogeneity between them was extremely low. To confirm the limited degree of *Zaca-DQ* nucleotide variation between individuals, we used denaturing gradient gel electrophoresis to examine putative peptide binding region sequences from the peripheral blood leukocyte-derived RNAs of 19 wild-caught California sea lions from physically distinct populations. The pattern of *Zaca-DQ* sequence migration was identical between individuals and independent of geographical region. This apparent *Zaca-DQ* sequence identity between sea lions was confirmed by direct sequencing of individual bands. In combination, these findings raise important questions regarding immunogenetic diversity within this thriving species, and should prompt further research into the existence of a highly polymorphic sea lion class II MHC molecule with sequence features that support traditional peptide binding functions.

Keywords Mhc · Comparative immunology · Veterinary immunology · California sea lion

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Studies were performed jointly in the laboratories of Stott and Johnson

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the following accession numbers: AF502560 (*MhcZaca-DQA*01*), AF502561 (*MhcZaca-DQA*02*), AF502562 (*MhcZaca-DQA*03*), AF502563 (*MhcZaca-DQA*04*), AF502564 (*MhcZaca-DQA*05*), AF503397 (*MhcZaca-DQB*01*), AF503398 (*MhcZaca-DQB*02*), AF503399 (*MhcZaca-DQB*03*), AF503400 (*MhcZaca-DQB*04*), AF503401 (*MhcZaca-DQB*05*), AF503402 (*MhcZaca-DQB*06*), AF503403 (*MhcZaca-DQB*07*), AF503404 (*MhcZaca-DQB*08*), AF503405 (*MhcZaca-DQB*09*), AF503406 (*MhcZaca-DQB*10*), AF503407 (*MhcZaca-DQB*11*)

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Introduction

The major histocompatibility complex (MHC) is a family of highly polymorphic genes encoding a set of transmembrane proteins that are critical to the generation of immune responses (Klein and Sato 2000a, b; Paul 1999). These cell surface glycoproteins play a key role in the initiation of an immune response by binding foreign pep-

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tides and presenting them to T cells (Klein and Sato 2000a, b; Paul 1999). The polymorphism of these MHC-encoded proteins ultimately determines the repertoire of antigenic determinants to which an individual is capable of responding (Reizis 1998; Zinkernagel 1979). The high level of MHC class I and class II genetic variation found in most mammals is thought to be an adaptation to the large number of pathogens encountered by natural populations (Hughes and Hughes 1995; Hughes and Yeager 1998; Klein and Takahata 1990; Vogel et al. 1999). It has also been suggested that infectious disease epidemics of the past have played a central role in determining the MHC allele and haplotype frequencies observed in populations today (Yuhki and O'Brien 1990).

Genetic and antigenic diversity of the MHC could play an important role in a host's ability to accommodate rapidly evolving infectious agents that periodically afflict natural populations (Yuhki and O'Brien 1990). Conversely, a lack of variation at the MHC may increase the susceptibility of an isolated population to infectious disease epidemics, with potentially catastrophic consequences (Yuhki and O'Brien 1990). Because of the key role of MHC diversity in disease susceptibility, understanding polymorphism of these genes, and their products, is vital in studying infectious disease ecology at a population level. Furthermore, MHC genotyping can help identify individuals or populations with a low level of immunogenetic heterogeneity, and help us understand the microevolution of the host immune system to pathogenic and environmental influences (Hedrick 1994; Hughes and Hughes 1995). This is particularly important in marine species whose chemical and microbial environment is increasingly influenced by anthropogenic encroachment, increasing their risk of exposure to novel pathogens (De Swart 1996; Harvell et al. 1999). In this study, we examined MHC gene products from California sea lions (*Zalophus californianus*). This species is of particular interest because they, along with other marine mammals, feed high on the marine food web, and are excellent sentinels for disturbances in marine and coastal ecosystems globally (Gulland 1999). In addition, because of their migratory and breeding behaviors, there are a small number of California sea lion populations that are effectively geographically isolated from one another. This provides a natural model with which we can examine the immunogenetic consequences of different regional pathogenic and environmental influences on the MHC of a thriving species.

There are two broad classes of MHC molecules, MHC class I and II, involved in binding and presenting antigens to the cells of the immune system. MHC class I molecules predominantly bind peptides from proteins degraded in the cytosol, such as those produced during viral infections. Class II molecules primarily bind peptides from proteins that originate outside the cell, such as those produced by extracellular bacterial infections. Little is known about MHC organization and function in marine mammals. In fact, to date there are no published California sea lion MHC sequences. Since bacterial infections are an important cause of morbidity and mortality in this species (Dierauf et al. 1985), we examined the expressed products from two California sea lion MHC class II genes. This is the first study characterizing near-full- and full-length (*DQB* and *DQA*, respectively) MHC class II gene sequences in a marine mammal species. These genes were selected because trans-species conservation of class II MHC sequences has been shown in other mammals (Bontrop et al. 1999; Erlich and Gyllensten 1991; Fan 1989; Gustafsson et al. 1990; Kupfermann et al. 1992; Slierendregt et al. 1992; Yaeger and Hughes 1999). While there is limited information regarding the MHC of species closely related to the California sea lion (order/suborder Carnivora/Caniformia/Pinnipedia) (McKenna and Bell 1997), the class II MHC genes of a related terrestrial carnivore, the domestic dog (order/suborder Carnivora/Caniformia/Canidae); Schreiber et al. 1998), have been extensively studied (Polvi et al. 1997; Sarmiento et al. 1992, 1993; Wagner et al. 1996, 1998, 1999). These studies show that the canine *DQA* and *DQB* gene products are polymorphic, and may be important in generating peptide-binding diversity. In this study, a comparative analysis between California sea lion class II MHC genes and their counterparts in other marine and terrestrial mammals is described. The information gained from this study will be useful in designing future studies to examine California sea lion MHC immunogenetics at a population level.

Materials and methods

Animals and sample preparation

Caudal gluteal venous blood samples from two California sea lions (*Zalophus californianus*) in rehabilitation at a rescue facility on the central California coast (The Marine Mammal Center, Sausalito, Calif.) were collected into cell separation tubes (Vacutainer CPT, Becton Dickinson, Franklin, N.J.), and used for rapid isolation of peripheral blood mononuclear cells (PBMCs). These cells were cryopreserved in liquid nitrogen pending RNA isolation. Total cellular RNA was isolated by silica-based gel membranes combined with microspin technology (RNeasy; Qiagen, Valencia, Calif.). The isolated RNA was stored at –70 °C prior to RACE (rapid amplification of cDNA ends) cDNA synthesis.

RACE library construction

To facilitate the amplification of full-length gene transcripts, a cDNA population was constructed using SMART RACE cDNA amplification kits (Clontech, Palo Alto, Calif.) according to manufacturer's specifications. In brief, RNA from both animals was pooled into a single sample and used as a template for cDNA synthesis. Adaptor-like sequences were added to either the 5′ or 3′ end of cDNA fragments using RACE cDNA synthesis primers (5′- or 3′-CDS; Clontech) in two separate reactions. These modified cDNAs were generated from the pooled total cellular RNA by MMLV reverse transcriptase-driven first strand synthesis using lock-docking oligo(dT) primers and the SMART II oligo. The resulting 5′- and 3′-modified cDNA fragments were used as templates for subsequent PCR and RACE PCR reactions.

Primer design and RACE PCR amplification

Degenerate oligonucleotide primers recognizing conserved regions of each of two MHC class II genes, *DQA* and *DQB*, were

Table 1 MHC class II-specific primers

Name	Primer sequence	Specificity	Amplicon
DQA-U164	CATGAATTYGATGGMGATGARG	Mammalian *DQA*	249 bp: 3′ exon 1 to 5′ exon 3
DQA-L413	ARATGAGGGTGTTGGGCTG	Mammalian *DQA*	
DQB-U172	CYGAGGGCAGAGACTCKCC	Mammalian *DQB*	597 bp: 3′ exon 1 to 5′ exon 4
DQB-L769	CTCTGGGMAGATTCAGACTG	Mammalian *DQB*	
ZcDQAU72	CTTCAGAAACTGCACTTGAGAAGAG	Sea lion *DQA*	896 bp: 5′ exon 1 to 3′UTR
DQA 3′UTR	GAGGAGCGTTGGAAGAGAGAAGG	Mammalian *DQA*	
ZcDQBUEX1D	TGTTCCCAATGATGCCTGGGAAG	Sea lion *DQB*	1164 bp: 5′ UTR to 3′UTR
DQB3′UTR	GAGAACCAATAATCATGTTTAATTATG	Mammalian *DQB*	
ZcDQAU83	CTGACCACTTTGCTTCCTATGGC	Sea lion *DQA*	264 bp: 5′ exon 2 to 5′ exon 3
ZcDQALGC	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCG CTCACCTCAGGAACCTCATTGGTAGC	Sea lion *DQA*	
ZcDQBU96GC	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCG CCAGAGACTCTCCACAGGATTTCG	Sea lion *DQB*	284 bp: 3′ exon 1 to 3′ exon 2
ZcDQBL358	CTCGCCGCTGCAAGATCGTTCT	Sea lion *DQB*	

designed, based on nucleotide sequence alignments of equine, bovine, porcine, canine, and human RNA-derived MHC class II gene sequences (Table 1). To control for the inadvertent amplification of genomic DNA sequences, primers from different exons were paired. These primers were used to amplify short segments of sea lion *DQA* and *DQB* gene transcripts, from which sea lion *DQA*- and *DQB*-specific oligonucleotides could be designed.

PCR amplifications using these degenerate class II primers were performed on 20 ng of each RACE cDNA library in 50-µl volumes containing 20–60 pmol of each primer (either DQA-U164 and DQA-L413, or DQB-U172 and DQB-L769), 40 mM Tris-KOH (pH 8.3), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$ 3.75 µg/ml bovine serum albumin, 0.005% Tween-20, 0.005% Nonidet-P40, 200µM each dNTP, and 5 units of Advantage 2 *Taq* polymerase (Clontech, Palo Alto, Calif.). For 5′ gene transcript amplifications, the reactions contained 20 pmol of either DQA-L413 (for *DQA* gene products) or DQB-L769 (for *DQB* gene products), along with 1–5 pmol of the universal primer mix (UPM, SMART RACE cDNA amplification kit; Clontech). For 3′ gene transcript amplifications, the reactions contained 20 pmol of either DQA-U164 (for *DQA* gene products) or DQB-U172 (for *DQB* gene products), along with 1–5 pmol of the universal primer mix (SMART RACE cDNA amplification kit; Clontech). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, Mass.). To optimize reaction specificity a touchdown method was employed, starting with 5 cycles of 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min, followed by 5 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min, and ending with an extension step of 72 °C for 10 min. The products of these reactions were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Bands representing PCR products within the expected size range were excised from the gel, extracted and purified using a commercially available nucleic-acid-binding resin (Qiaex II Gel extraction kit; Qiagen). These isolated RACE fragments were then ligated into a T/A-type cloning vector (pGEM-T Easy vector systems; Promega, Madison, Wis.). Following transformation, growth, and blue-white selection in competent cells (SE DH5α competent cells; Life Technologies, Rockville, Md.), the DNA from positive clones was isolated. The nucleotide sequences of both strands were determined by dideoxy nucleotide methodology using an automated sequencer (Model 373; Applied Biosystems, Foster City, Calif.). The nucleotide sequences of these amplicons were compared using the Align and Contig sequence alignment software programs (Vector NTI; Informax, North Bethesda, Md.). For each of the *DQA* and *DQB* gene products, regions of sequence homology were identified and used to design sea lion MHC-specific oligonucleotides (Table 1), which were used to amplify near-full-length sequences in ensuing reactions.

These near-full- and full-length *DQB* and *DQA* gene transcripts were amplified from the pooled PBMC RNA-derived cDNA fragments. With the exception of the oligonucleotide composition and concentrations, the PCR conditions for these reactions were identical to those described above. Amplification reactions contained 20 pmol of both ZcDQAU72 and DQA 3′UTR (for *DQA* gene products) or ZcDQBUEX1D and DQB 3′UTR (for *DQB* gene products). Reaction specificity was optimized using a touchdown method that started with 5 cycles of 94 °C for 5 s, and 72 °C for 3 min, followed by 5 cycles of 94 °C for 5 s, 70 °C for 10 s, and 72 °C for 3 min, followed by 25 cycles of 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 3 min. The products of these reactions were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Bands representing PCR products of the predicted size were excised, extracted, cloned and sequenced using the procedures described above.

Analysis of RACE product sequences

The nucleotide sequences of the RACE products were analyzed using the Align and Contig sequence alignment software programs (Vector NTI; Informax) and compared to known sequences using the NCBI BLAST program (Altschul et al. 1990), and the IMGT/HLA database (Robinson et al. 2001). Non-synonymous and synonymous nucleotide substitution rates were calculated by the method of Nei and Gojobori (1986).

DGGE-based DQ-genotyping of 19 additional sea lions

An adapted denaturing gradient gel electrophoresis (DGGE) (Aldridge et al. 1998) was used to examine the degree of *Zaca-DQ* nucleotide variation between individuals from different geographical locations. Peripheral blood leukocytes were isolated by whole blood lysis of samples collected from wild-caught sea lions in the Sea of Cortez, Baja, Mexico (n=9), and from San Miguel Island, California, USA (n=10). The isolated leukocytes were stored in RNAlater (Ambion, Austin, Tex.) pending RNA isolation. Total cellular RNA was isolated by silica-based gel membranes combined with microspin technology (RNeasy, Qiagen, Valencia, Calif.), and stored at –70 °C prior to cDNA synthesis. A standard cDNA synthesis was performed on 2 µg of RNA template from each animal. Reaction conditions included 4 units reverse transcriptase (Omniscript; Qiagen, Valencia, Calif.), 1 µM random hexamers, 0.5 mM each dNTP, and 10 units RNase inhibitor, in RT buffer (Qiagen). Reactions were incubated for 60 min at 37 °C, followed by an enzyme inactivation step of 5 min at 93 °C and stored at –20 °C until further analysis.

New primer pairs recognizing the flanking regions of the putative peptide-binding site were designed to amplify a 264-bp fragment of *Zaca-DQA* exon 2 (ZcDQAU83 and ZcDQALGC, Table 1) and a 284-bp fragment of *Zaca-DQB* exons 1 and 2 (DQBU96GC and ZcDQBL358, Table 1). To enhance the sensitiv-

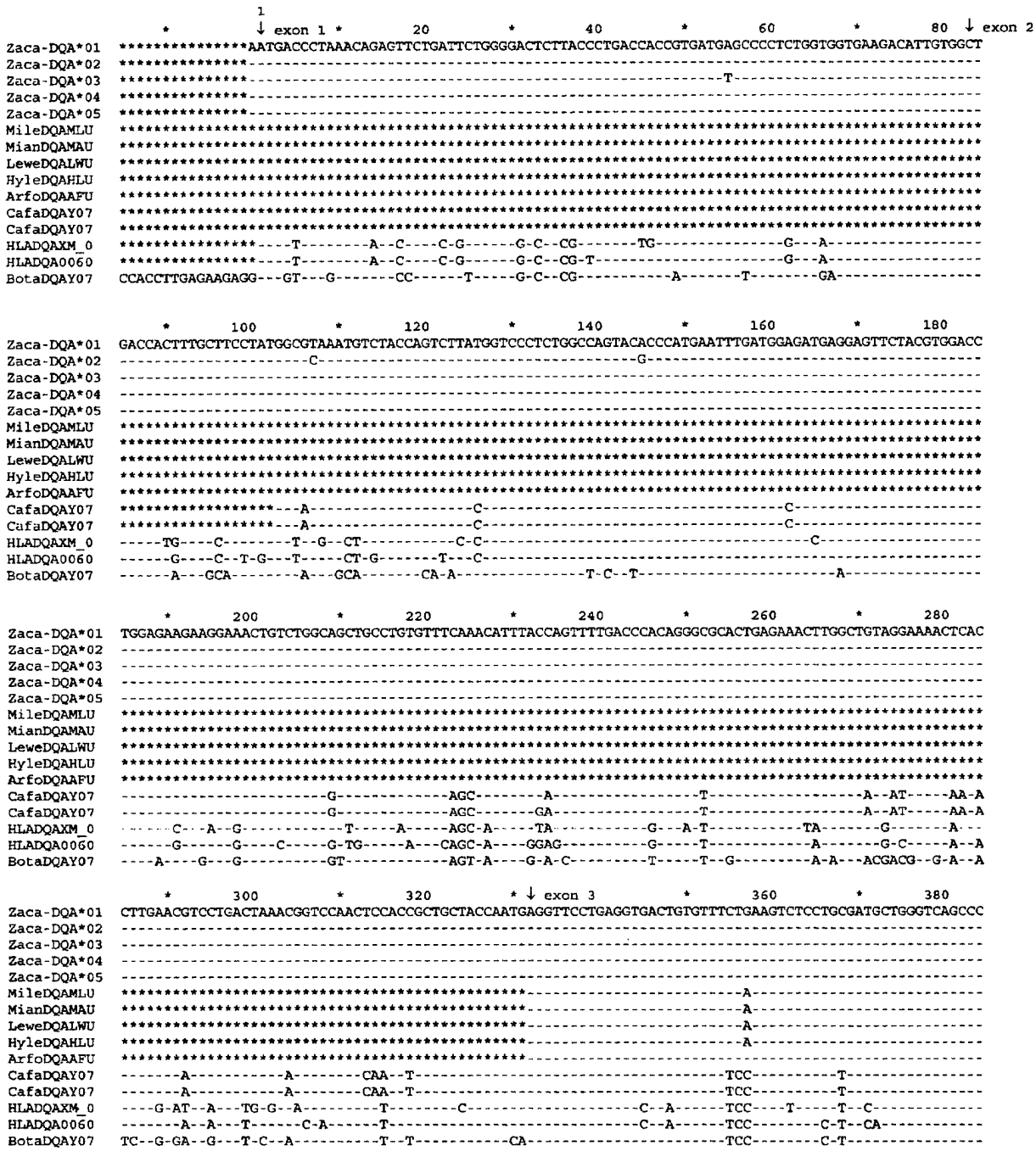

Fig. 1 Nucleotide sequence identity between CSL class II MHC clones (*Zaca-DQA*), human, canine, and other marine mammal *DQA* sequences. Abbreviations for individual species MHC molecules are as follows: *Zaca* California sea lion (*Zalophus californianus*); *Arfo* New Zealand Fur seal (*Arctocephalus forsteri*); *Hyle* Leopard seal (*Hydrurga leptonyx*); *Lewe* Weddell seal (*Leptonychotes weddelli*); *Mian* Northern Elephant seal (*Mirounga angustirostris*); *Mile* Southern Elephant seal (*Mirounga leonina*); *Phvi* Pacific harbor seal (*Phoca vitulina*); *Mosc* Hawaiian monk seal (*Monachus schauinslandi*); *Cafa* domestic dog (*Canis familiaris*); *HLA* human lymphocyte antigen; *Bota* domestic cow (*Bos taurus*). The MHC species label includes GenBank or IMGT accession numbers. The complete nucleotide sequence of *Zaca-DQA*01* is shown. *Single letters* and *dots* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *Zaca-DQA*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DQA*01* and HLA00601 (IMGT/HLA Database) (Robinson et al. 2001). The allelic numbers for the California sea lion sequences were assigned according to multispecies guidelines (Klein et al. 1990), with the assumption of a single DQA lineage based upon the close sequence homology between individual clones. Stop codon is *italicized* and marked with an *arrow* (▼)

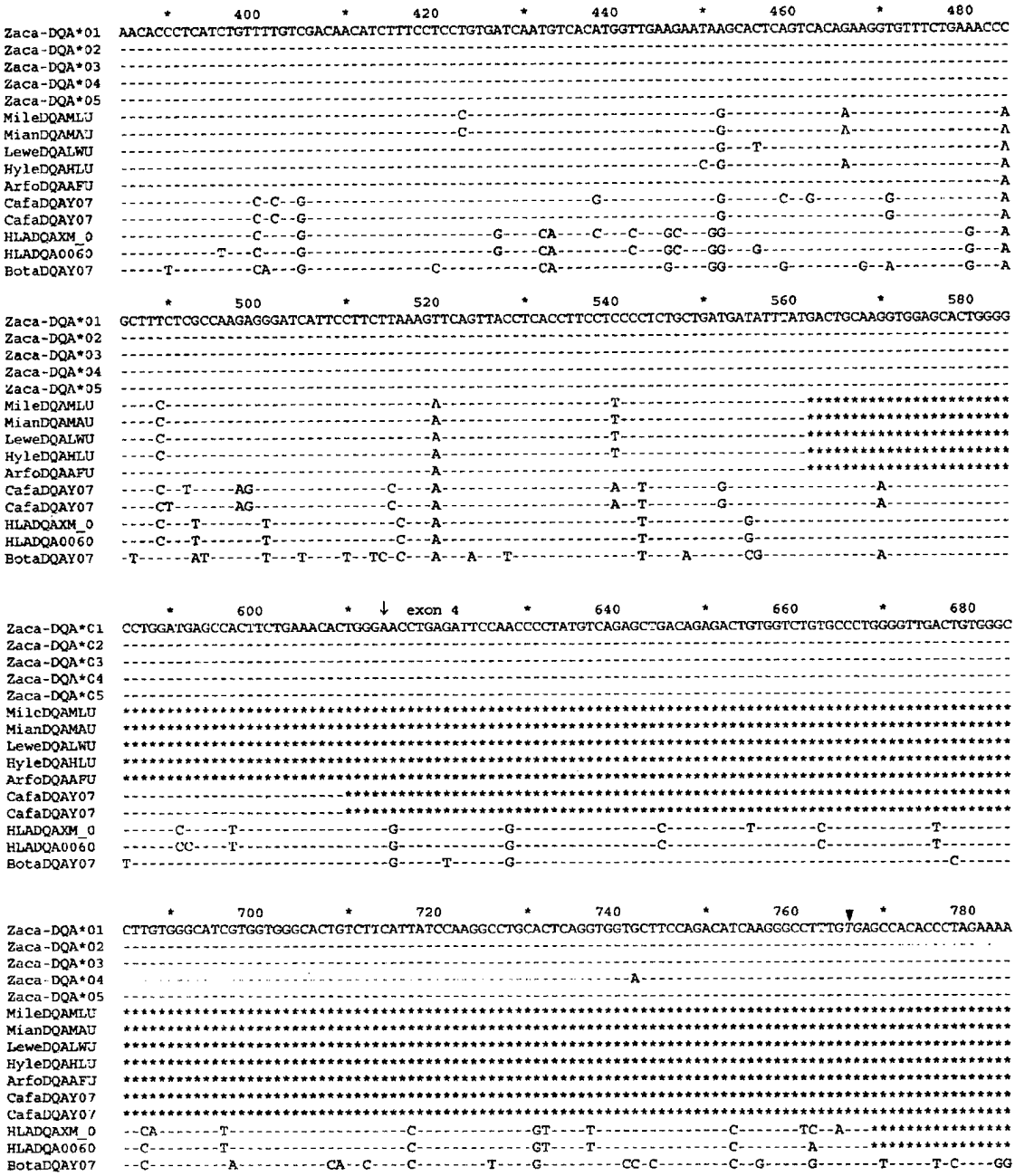

Fig. 1 (continued)

ity of the DGGE analysis, a GC-clamp was added to one of each primer pair as previously described (Sheffield et al. 1989). The optimal conditions for *Zaca-DQ* examination (25%–60% urea/formamide gradient in 12% acrylamide, 1×Tris/acetic acid/EDTA buffer, at 300 V and 60 °C for 3.5 h) were determined using perpendicular DGGE and a time-series analysis (Knapp et al, 1997). These conditions were used in parallel denaturing gels to compare the separation of *Zaca-DQ* sequences between individuals. The gel was visualized by staining with GelStar nucleic acid stain (BMA products, Rockland, Me.) according to manufacturer's specifications. The bands representing individual *Zaca-DQ* sequences were excised from the gels, re-amplified, and the nucleotide sequence determined as previously described (Knapp et al. 1997; Aldridge et al. 1998).

Results

Characterization of CSL DQA and DQB cDNA clones

Clones containing near-full- and full-length *Zaca* (*Zalophus californianus*) MHC class II-like sequences were obtained from the RACE cDNA products of two randomly selected California sea lions. The nucleotide and deduced amino acid sequence of the 896-bp (DQA-primer derived) and the 1164-bp (*DQB* primer derived) products were typical of transcripts from mammalian

```
                 *       800         *       820         *       840         *       860         *       880
Zaca-DQA*01   GGAGGTGCACCGCATGCCCACCGACCAGAGCAGAAGAGTGGACTTGATAGGTGACCTAGCACTGTTTCCTGGCCGAGTTTATCATATTCCTTCTCTCTTC
Zaca-DQA*02   ----------------------------------------------------------------------------------------------------
Zaca-DQA*03   ----------------------------------------------------------------------------------------------------
Zaca-DQA*04   ----------------------------------------------------------------------------------------------------
Zaca-DQA*05   ----------------------------------------------------------------------------------C-----------------
MileDQAMLU    ****************************************************************************************************
MianDQAMAU    ****************************************************************************************************
LeweDQALWU    ****************************************************************************************************
HyleDQAHLU    ****************************************************************************************************
ArfoDQAAFU    ****************************************************************************************************
CafaDQAY07    ****************************************************************************************************
CafaDQAY07    ****************************************************************************************************
HLADQAXM_0    ****************************************************************************************************
HLADQA0060    ****************************************************************************************************
BotaDQAY07    AAG-TGCA-TGT-CAT-TTTGA--A----****--A-A--------C---A--------A---A---T------A---CC-----G-A---------C--

                 *       900         *       920         *
Zaca-DQA*01   CAACGCTCCTC*****************************************
Zaca-DQA*02   -----------*****************************************
Zaca-DQA*03   -----------*****************************************
Zaca-DQA*04   -----------*****************************************
Zaca-DQA*05   -----------*****************************************
MileDQAMLU    ************************************************
MianDQAMAU    ************************************************
LeweDQALWU    ************************************************
HyleDQAHLU    ************************************************
ArfoDQAAFU    ************************************************
CafaDQAY07    ************************************************
CafaDQAY07    ************************************************
HLADQAXM_0    ************************************************
HLADQA0060    ************************************************
BotaDQAY07    -T--C------CTCATACTTCTCTCGGACTTAAGGTGCTGTATCATCTCA
```

Fig. 1 (continued)

class II genes. These transcripts were characterized as *MhcZaca-DQA* or *MhcZaca-DQB* based on alignments with human lymphocyte antigen (HLA), and canine lymphocyte antigen (DLA), *DQA* and *DQB* sequences (Figs. 1 and 2). The full-length (*DQA* primer-amplified) sequences (Table 1) showed high homology with previously described full-length human (87%), canine (88%) and bovine (79%) *DQA* sequences (Fig. 1). Similarly the near-full-length sequences amplified by the *DQB* primers showed a high homology with human (89%), canine (92%), and bovine (79%) *DQB* sequences (Fig. 2). Furthermore, the *DQB* primer-derived sequences contained several *DQ*-specific motifs, particularly within exon 3. In fact the nucleotides encoding amino acids 195, 198, and 200 (threonine, arginine, and aspartate, respectively) could be used to distinguish *DQB* from *DRB* products in several species (Fig. 2, Table 2). These *DQ*-specific motifs were identified by alignments with multiple published HLA-*DQB* and *DRB* sequences (Robinson et al. 2001). Further evidence for the identity of the *Zaca-DQ* primer-amplified sequences was provided by the significantly lower homologies observed in comparison with published *DRB* and *DRA* sequences from canine (65% *DRB*, 53% *DRA)*, and human (73% *DRB*, 55% *DRA*) genes (data not shown).

The largest *Zaca*-DQA and DQB gene products encoded deduced molecules of 255 and 265 amino acids (aa), respectively. All sequence-derived *Zaca*-DQα molecules were full-length and consisted of a 28-aa leader sequence, an 82-aa α1 domain, a 94-aa α2 domain, and a 51-aa connecting peptide/transmembrane/cytoplasmic tail domain (Fig. 3). Three forms of potentially functional class II MHC molecules were identified in the *Zaca-DQB* sequences (Fig. 4). These included near-full-length molecules (265 aa), β1 domain-deficient molecules (175 aa), and cytoplasmic domain-deficient molecules (257 aa). The near-full length *Zaca*-DQβ molecules consisted of a partial 32-aa leader sequence, a 90-aa β1 domain, a 94-aa β2 domain, a 37-aa transmembrane domain, and 8-aa and 4-aa cytoplasmic domains (Fig. 4). In addition, three *Zaca-DQB* sequences with features compatible with class II MHC pseudogenes were identified (Figs. 2, 4). These sequences contained variable-length deletions (range, 88–420 nucleotides) involving exons 2–6 and, in one instance, extending into the 3′UTR. These deletions created a frameshift and premature stop codon in each case. If these

Fig. 2 Nucleotide sequence identity between CSL class II MHC clones, human, canine, and other marine mammal *DQB* sequences. Abbreviations for individual species MHC molecules are as follows: *Zaca* California sea lion (*Zalophus californianus*); *Mosc* Hawaiian monk seal (*Monachus schauinslandi*); *Mile* Southern Elephant seal (*Mirounga leonina*); *Mian* Northern Elephant seal (*Mirounga angustirostris*); *Dele* Beluga whale (*Delphinapterus leucus*); *Momo* Narwhal (*Monodon monocerus*); *Cafa* domestic dog (*Canis familiaris*); *Arfo* New Zealand Fur seal (*Arctocephalus forsteri*); *HLA* human lymphocyte antigen. The MHC species label includes GenBank or IMGT accession numbers. The complete nucleotide sequence of *Zaca-DQB*01* is shown. *Single letters* and *dots* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *Zaca-DQB*01*. *Asterisks* indicate missing sequences. *Boxes* indicate codons used to distinguish *DQB* from *DRB*. Borders of each domain were assigned based upon sequence homology between *Zaca-DQB*01* and XM_011391 (human *DQB*, GenBank accession no. XM_011391). The allelic numbers for the California sea lion sequences were assigned according to multispecies guidelines (Klein et al. 1990), with the assumption of a single DQB lineage based upon the close sequence homology between individual clones. Stop codon is *italicized* and marked with an *arrow* (▼) ▶

```
                                                                 1
                    *       -40         *       -20         *    ↓ exon 1 *       20          *       40
Zaca-DQB*01  ************************************************************ATGGCTCTGCGGATCCCCAGAGGCCTCTGGA
Zaca-DQB*02  ************************************************************------------------------------
Zaca-DQB*03  ************************************************************------------------------------
Zaca-DQB*04  ************************************************************------------------------------
Zaca-DQB*05  ************************************************************------------------------------
Zaca-DQB*06  ************************************************************------------------------------
Zaca-DQB*07  ************************************************************------------------------------
Zaca-DQB*08  ************************************************************------------------------------
Zaca-DQB*09  ************************************************************------------------------------
Zaca-DQB*10  ************************************************************------------------------------
Zaca-DQB*11  ************************************************************------------------------------
MileDQBAF1   ******************************************************************************************
MianDQBAF1   ******************************************************************************************
MoscDQBAY0   ******************************************************************************************
MomoDQBU16   ******************************************************************************************
ArfoDQBAF1   ******************************************************************************************
CafaDQBM90   ******************************************************************************************
CafaDQBAF0   ***********************************************ATGTCTGGGAAG---A-----T-C------C-----T------
HLADQBXM_0   ***CAGATCCATCAGGTCCGAGCTGTGTTGACTACCACTTTTCCCTTCGTCTCAATTATGTCTTGGAAA-A----T-----------G-------TC--G
HLADQBXM_0   CAGATCCATCAGGTCCAAGCTGTGTTGACTACCACTACTTTTCCCTTCGTCTCAATTATGTCTTGGAAG-A----T----------TG-------TC--G

                    *       60          *       80          *       100         ↓ exon 2            *       140
Zaca-DQB*01  CAGCAGCTGAAATGATGATCCTGGTGGTGCTGAGCATCCCAGTGGCTGAGGGCAGAGACTCTCCACAGGATTTCGTGTTCCAGTTTAAGGGCGAGTGCTA
Zaca-DQB*02  ------------------------------------------------------------------------------------------------
Zaca-DQB*03  ------------------------------------------------------------------------------G-----------------
Zaca-DQB*04  ------------------------------------------G-----------------------------------------------------
Zaca-DQB*05  ------------------------------------------------------------------------------------------------
Zaca-DQB*06  ------------------------------------------------------------------------------------------------
Zaca-DQB*07  ------------------------------------------------------------------------------G-----------------
Zaca-DQB*08  ------------------------------------------------------------------**********************************
Zaca-DQB*09  ---------------------------------------T-------------------------**********************************
Zaca-DQB*10  ------------------------------------------------------------------------------------------------
Zaca-DQB*11  ------------------------------------------------------------------------------------------------
MileDQBAF1   ******************************************************************************************C-------
MianDQBAF1   ******************************************************************************************C-------
MoscDQBAY0   ******************************************************************************************-----TC-
MomoDQBU16   ******************************************************************************************
ArfoDQBAF1   ******************************************************************************************--------
CafaDQBM90   ******************************************************************************************--------
CafaDQBAF0   ---------TG-------------------------------------------------------------------A-----C---------
HLADQBXM_0   -----A---TG-CCT----G---TC-A---------C-------------------------CG------------A-------------AT------
HLADQBXM_0   T----A---TG-CCT----G----C-A---------C---G-----------------------CG-------------------------AT------

                    *       160         *       180         *       200         *       220         *       240
Zaca-DQB*01  CTTCACCAACGGGACGGAGCGGGTGCGGTCCCTGACCAGATACATCTATAACCGGGAGGAGTACGTGCGCTTCGACAGCGACGTGGGGGAGTACCGGCCG
Zaca-DQB*02  ------------------------------------------------------------------------------------------------
Zaca-DQB*03  ----------------------------CA----------C-------------------------------------------------------
Zaca-DQB*04  ------------------------------------------------------------------------------------------------
Zaca-DQB*05  ------------------------------------------------------------------------------------------------
Zaca-DQB*06  ------------------------------------------------------------------------------------------------
Zaca-DQB*07  ---------*********************************************************************************--
Zaca-DQB*08  ******************************************************************************************
Zaca-DQB*09  ******************************************************************************************
Zaca-DQB*10  ------------------------------------------------------------------------------------------------
Zaca-DQB*11  ------------------------------------------------------------------------------------------------
MileDQBAF1   ----------------------------GT----------------------------------------------------------------
MianDQBAF1   ----------------------------CT----------A---------------------T-----------------------------
MoscDQBAY0   T-ATT-----------------------CT----G------CA---------G--------G-----------------------------T-------
MomoDQBU16   ************C---------------CT-G---G-----------------------------------------------C---------G--
ArfoDQBAF1   ----------------------------CA-----------------------------A-----------------------------------
CafaDQBM90   T---------------------------CTT-----T-A----------------------T----------------------------T----G--
CafaDQBAF0   T---------------------------CTT-----T-A----------------------T----------------------------T----G--
HLADQBXM_0   ---------------A-----C-----TCTTG---G----AG------------A--A---AT----------------------------T----G--
HLADQBXM_0   ---------------C-----C------GGTG---------------------A----------C----------------------T---T---G--

                    *       260         *       280         *       300         *       320         *       340
Zaca-DQB*01  GTGACGGAGCTGGGGCGGCCGGACGCTGAGTACTGGAACAGCCAGAAGGACATCCTGGAGCGGACGCGGGCCGAGGCGGACACGGTGTGCAGACACAACT
Zaca-DQB*02  ------------------------------------------------------------------------------------------------
Zaca-DQB*03  ------------------------------------------------------------------------------------------------
Zaca-DQB*04  ------------------------------------------------------------------------------------------------
Zaca-DQB*05  ------------------------------------------------------------------------------------------------
Zaca-DQB*06  ------------------------------------------------------------------------------------------------
Zaca-DQB*07  ------------------------------------------------------------------------------------------------
Zaca-DQB*08  ******************************************************************************************
Zaca-DQB*09  ******************************************************************************************
Zaca-DQB*10  ------------------------------------------------------------------------------------------------
Zaca-DQB*11  ---------------------------------------T----------------------------------------------------
MileDQBAF1   --------------------CT----------**********************************************************
MianDQBAF1   --------------------CTT---------**********************************************************
MoscDQBAY0   --------------------CAC---------**********************************************************
MomoDQBU16   -----C--------C----G-AC---C-----------------------------------A---------CT-------****************
ArfoDQBAF1   ------------------------------**********************************************************
CafaDQBM90   --C-------C--------C-----------------C-A---------GAGA----C---GTA---------CT----------------------
CafaDQBAF0   --C--------C--------C-----------------C-A---------GAGA----C---GTA---------CT----------------------
HLADQBXM_0   ------CT-------T---T-C---C-------------------------------A---AA-----G-C--T-----G----------------
HLADQBXM_0   ------CC----------TT-----C----------T--------------------GA-GAC-----GTC--T------C--A-------------
```

Fig. 2 Legend see page 337

```
                    *       360         *         ↓ exon 3  *       400         *       420         *       440
Zaca-DQB*01   ATGGGATTGAGGAGAGAACGATCTTGCAGCGGCGAGTGGAACCTACAGTGACCATCTCCCCGTCCAGGACAGAGGTTCTGAACCACCACAACCTGCTGGT
Zaca-DQB*02   ----------------------------------------------------------------------------------------------------
Zaca-DQB*03   ----------------------------------------------------------------------------------------------------
Zaca-DQB*04   ----------------------------------------------------------------------------------------------------
Zaca-DQB*05   ----------------------------------------------------------------------------------------------------
Zaca-DQB*06   ----------------------------------------------------------------------------------------------------
Zaca-DQB*07   ----------------------------------------------------------------------------------------------------
Zaca-DQB*08   ****************************************************************************************************
Zaca-DQB*09   ************************************-------------------------------------------------------------
Zaca-DQB*10   ----------------------------------------------------------------------------------------------------
Zaca-DQB*11   ----------------------------------------------------------------------------------------------------
MileDQBAF1    ****************************************************************************************************
MianDQBAF1    ****************************************************************************************************
MoscDQBAY0    ****************************************************************************************************
MomoDQBU16    ****************************************************************************************************
ArfoDQBAF1    ****************************************************************************************************
CafaDQBM90    -C----GG--A---CTC--C-CG--------------------G-------------T--------A------------------------T--------
CafaDQBAF0    -C----GG--A---CTC--C-CG--------------------G-------------T--------A------------------------T--------
HLADQBXM_0    -CCA-T-G---CTCC-C----C------------------G--C-----------------A-------------CC--C--------------------
HLADQBXM_0    -CCA-T-G---CTCC-C----C------------------G--C-----------------A-------------CC--C--------------------

                    *       460         *       480         *       500         *       520         *       540
Zaca-DQB*01   CTGCTCAGTGACAGATTTCTACCCAGGCCAGATCAAAGTTCGGTGGTTTCGGAATGACCAGGAGGAGAGAGCTGGTGTCGTGTCCACTCCACTTATTAGG
Zaca-DQB*02   ----------------------------------------------------------------------------------------------------
Zaca-DQB*03   ----------------------------------------------------------------------------------A-----------------
Zaca-DQB*04   ----------------------------------------------------------------------------------------------------
Zaca-DQB*05   ----------------------------------------------------------------------------------------------------
Zaca-DQB*06   ----------------------------------------------------------------------------------------------------
Zaca-DQB*07   ------------------------------------------------------------------C-----------------------------C-
Zaca-DQB*08   ****************************************************************************************-----------
Zaca-DQB*09   ----------------------------------------------------------------------------------------------------
Zaca-DQB*10   ----------------------------------------------------------------------------------------------------
Zaca-DQB*11   ----------------------------------------------------------------------------------------------------
MileDQBAF1    ****************************************************************************************************
MianDQBAF1    ****************************************************************************************************
MoscDQBAY0    ****************************************************************************************************
MomoDQBU16    ****************************************************************************************************
ArfoDQBAF1    ****************************************************************************************************
CafaDQBM90    ---------------------T----------------C-----------------------C---C-----TG-----------------------
CafaDQBAF0    ---------------------T----------------C-----------------------C---C-----------------------------
HLADQBXM_0    ------G--------------T----C------------C---------------------------C------C--T--------C--C---------
HLADQBXM_0    ---------------------T----C------------C---------------------------C-A----C--T--------C--C---------

                    *       560         *       580         *       600         *       620         *       640
Zaca-DQB*01   AATGGGGACTGGACCTTCCAGATCCTGGTGATGCTGGAAATTACTCCCCAGCGAGGAGATGTCTACACCTGCCATGTGGAGCACCCCAGCCTCCAGGGCC
Zaca-DQB*02   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*03   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*04   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*05   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*06   ----------------------------------------------------------------------------------------********
Zaca-DQB*07   ----------------------C-----------------------------------------------------------------------A---
Zaca-DQB*08   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*09   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*10   ----------------------------------------------------------------------------------------------A---
Zaca-DQB*11   ----------------------------------------------------------------------------------------------A---
MileDQBAF1    ****************************************************************************************************
MianDQBAF1    ****************************************************************************************************
MoscDQBAY0    ****************************************************************************************************
MomoDQBU16    ****************************************************************************************************
ArfoDQBAF1    ****************************************************************************************************
CafaDQBM90    --------------------------T-----------G--G-------------------------T---------------G-----------A---
CafaDQBAF0    --------------------------T-----------G--G-------------------------T---------------G-----------A---
HLADQBXM_0    -----T-----------------------------------G-------------T-----C-------------C---------------------A---
HLADQBXM_0    --C--T-----------------------------------G-------------T-----C-------------C---------------------AA--

                    *       660 ↓ exon 4*       680         *       700         *       720         *       740
Zaca-DQB*01   CCATCACGGTGGAGTGGAGGGCACAGTCTGAATCTGCCCAGAGCAAGATGCTGAGTGGCATCGGAGGCTTTGTGCTGGGGCTGATCTTCCTCGGGCTGGG
Zaca-DQB*02   -------A--------------------------------------------------------------------------------------
Zaca-DQB*03   -------A--------------------------------------------------------------------------------------
Zaca-DQB*04   -------A--------------------------------------------------------------------------------C-------
Zaca-DQB*05   -------A--------------------------------------------------------------------------------------
Zaca-DQB*06   ****************************************************************************************************
Zaca-DQB*07   -------A--------------------C-------------------------------------------------------------------
Zaca-DQB*08   -------A--------------------------------------------------------------------------------------
Zaca-DQB*09   -------A--------------------------------------------------------------------------------------
Zaca-DQB*10   -------A--------------------------------------------------------------------------------------
Zaca-DQB*11   -------A--------------------------------------------------------------------------------------
MileDQBAF1    ****************************************************************************************************
MianDQBAF1    ****************************************************************************************************
MoscDQBAY0    ****************************************************************************************************
MomoDQBU16    ****************************************************************************************************
ArfoDQBAF1    ****************************************************************************************************
CafaDQBM90    -------A---C-----C-------------------------------------T--T-----C--------------------T-----T**
CafaDQBAF0    -------A---C-----C-------------------------------------T--T-----C--------------------T-----T--
HLADQBXM_0    -------C---------C----T--A----------------------------T--------C-----------------------------
HLADQBXM_0    ------TC---------C----T-------------------------------T--------C-----------------------------
```

Fig. 2 (continued)

```
                    *       760         * ↓ exon 5          *       ↓ exon 6   ▼ *       820         *       840
Zaca-DQB*01   CCTTATCATCCGCCACAGGAGCCAGAAAGGACCTCGTGGGTCTCTGCCAGCAGGGCTCCTGCACTGACGCCTGAGGGTAC*****CTGGGATT******C
Zaca-DQB*02   ------------T----------------------C-------------------------------------------*****--------******-
Zaca-DQB*03   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*04   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*05   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*06   ******************************************************************************************************
Zaca-DQB*07   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*08   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*09   ------------T-------------------------------------------------------------------*****--------******-
Zaca-DQB*10   ------------T----------------*************************-------------------------*****--------******-
Zaca-DQB*11   ------------T----------------*************************-------------------------*****--------******-
MileDQBAF1    ******************************************************************************************************
MianDQBAF1    ******************************************************************************************************
MoscDQBAY0    ******************************************************************************************************
MomoDQBU16    ******************************************************************************************************
ArfoDQBAF1    ******************************************************************************************************
CafaDQBM90    ******************************************************************************************************
CafaDQBAF0    ------------T-------------------- T   G      CA  G------------------T--------A---*****-G------******-
HLADQBXM_0    -----------AT--------T-------*************************---------------T------AC--TTTTAA--------GGTTAT-
HLADQBXM_0    ------T----AT--------T-------*************************---------------T------AC--TTTTAA--------GGTTAT-

                    *       860         *       880         *       900         *       920         *       940
Zaca-DQB*01   CGCCGTCTGTAATGCCTGCCTGTCCTTGCCCAGAATTCCCAGCTGCCTGAG**AGCCCATCCCCCACATGGATCCGAGCCCCACAGTGCCTCTGATACAG
Zaca-DQB*02   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*03   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*04   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*05   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*06   ******************************************************************************************************
Zaca-DQB*07   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*08   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*09   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*10   ------------------------------------------------**--------------------------------------------------
Zaca-DQB*11   ------------------------------------------------**--------------------------------------------------
MileDQBAF1    ******************************************************************************************************
MianDQBAF1    ******************************************************************************************************
MoscDQBAY0    ******************************************************************************************************
MomoDQBU16    ******************************************************************************************************
ArfoDQBAF1    ******************************************************************************************************
CafaDQBM90    ******************************************************************************************************
CafaDQBAF0    -AT-C-T----G--T-C-T------C-----------------------C**--A-TG----------A----A---T--T---*--A------*****
HLADQBXM_0    ACTTT-------C------T-----C-------------------T---T-TC----TG------TG-**----A---T--T------G--G-C-CG---
HLADQBXM_0    ACTTT-------C------T-----C-------------------T---T-TC----TG------TG-**----A---T--T------G--G-C-CG---

                    *       960         *       980         *       1000        *       1020        *       1040
Zaca-DQB*01   TCACCAGGTGCCCTCCTGGGACCCCTGCCT***GGCGGATCTGACTGC*GATGCTGCTCCCTGCACTGGTTTCAGAGCCTCTGCTGTGAGCTCCTTGCTA
Zaca-DQB*02   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*03   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*04   --------------------G---------***---------------*-----------------------------------------------
Zaca-DQB*05   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*06   ***********************-------***---------------*-----------------------------------------------
Zaca-DQB*07   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*08   --------C-----T--------------***---------------*-----------------------------------------------
Zaca-DQB*09   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*10   ------------------------------***---------------*-----------------------------------------------
Zaca-DQB*11   ------------------------------***---------------*-----------------------------------------------
MileDQBAF1    ******************************************************************************************************
MianDQBAF1    ******************************************************************************************************
MoscDQBAY0    ******************************************************************************************************
MomoDQBU16    ******************************************************************************************************
ArfoDQBAF1    ******************************************************************************************************
CafaDQBM90    ******************************************************************************************************
CafaDQBAF0    *******************----T-*****-***T-A-------GG--TT---T-----T------T--AC*************-----T-----C-----
HLADQBXM_0    C--------CAT-----TTC-T---CA---TGA-------**-G---T*--CC--A--T---------ACC*--C---------C*--T--A-G**--C-
HLADQBXM_0    C--------CAT-----TTC-T---CA---TGA-------**-G---T*--CC--A--T---------ACC*--C---------C*--T--A-G**--C-

                    *       1060        *       1080        *       1100        *       1120        *       1140
Zaca-DQB*01   GCAGCATCTCCTCCGTCCCCAAGGGCTTTTCC*GTTTCCATTCTTCCTCCTCAGATTGTGCAAGAGGAGCACACAGAAGCCCTTTAGCTGAATGTAGAGA
Zaca-DQB*02   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*03   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*04   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*05   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*06   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*07   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*08   ------------------------------*----------------------A------------------------------------------
Zaca-DQB*09   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*10   ------------------------------*-----------------------------------------------------------------
Zaca-DQB*11   ------------------------------*-----------------------------------------------------------------
MileDQBAF1    ******************************************************************************************************
MianDQBAF1    ******************************************************************************************************
MoscDQBAY0    ******************************************************************************************************
MomoDQBU16    ******************************************************************************************************
ArfoDQBAF1    ******************************************************************************************************
CafaDQBM90    ******************************************************************************************************
CafaDQBAF0    *---G----A---A-G---------A------T---------------T-A---------------A------TT------A----C-----GA------
HLADQBXM_0    --T------A---A-G---------G---*-T*-----T-----**---------C--CT------A------T*---AA--A---C----C-T-----C
HLADQBXM_0    --T------A---A-G---------G---*-T*-----T-----**---------C--CT------A------T*---AA--A---C----C-T-----C
```

Fig. 2 (continued)

```
                    *      1160         *      1180
Zaca-DQB*01   TTTTATCATAATTAAACATGATTATTGGTTCTC****
Zaca-DQB*02   ---------------------------------****
Zaca-DQB*03   ---------------------------------****
Zaca-DQB*04   ---------------------------------****
Zaca-DQB*05   ---------------------------------****
Zaca-DQB*06   ------------------------------*******
Zaca-DQB*07   ---------------------------------****
Zaca-DQB*08   ---------------------------------****
Zaca-DQB*09   ---------------------------------****
Zaca-DQB*10   ---------------------------------****
Zaca-DQB*11   ---------------------------------****
MileDQBAF1    ************************************
MianDQBAF1    ************************************
MoscDQBAY0    ************************************
MomoDQBU16    ************************************
ArfoDQBAF1    ************************************
CafaDQBM90    ************************************
CafaDQBAF0    ----------------TG-------GA------TGTA
HLADQBXM_0    ----T-ACAT-A-T--ACATGA-CC--AG-T******
HLADQBXM_0    ----T-ACAT-A-T--ACATGA-CC--AG-T******
```

Fig. 2 (continued)

sequences were translated, the final length of the truncated protein molecules would range from 46 to 219 aa, and contain a non-MHC-like carboxy-terminal peptide domain.

Variation between Zaca-DQA sequences

To maximize the identification of all *Zaca-DQA* sequences present in the two animals examined, multiple clones were sequenced. From these clones, five different *Zaca-DQA* sequences were found (Fig. 1). This suggested that the *DQA*-specific primers amplified products from at least two loci, if each sequence was considered a unique *Zaca-DQA* allele. None of the sequences exhibited features compatible with pseudogenes. The homology between these sequences was extremely high, with each containing only one or two unique nucleotides. These sequences were confirmed by performing two to four independent PCR reactions, by examining multiple clones, and by sequencing each clone in both directions, in compliance with HLA nomenclature rules (Bodmer et al. 1999). The deduced amino-acid sequences indicated that four out of five of these polymorphisms represented a non-synonymous nucleotide substitution (Table 3); the fifth substitution was in the 3′UTR (Fig. 3). Of the four polymorphic amino acid residues, two were found in the α1 domain, which encodes the putative MHC class II peptide-binding site (Brown et al. 1993; Stern et al. 1994). The other polymorphic residues were in the leader peptide and transmembrane domain (1/4 each). Heterogeneity of *Zaca*-DQα amino acid sequences between individuals was observed at residues –5, 13, 26, and 225 (Fig. 3). The relative positions of these polymorphic residues were compared with those described in other species. Although none of these regions coincided with polymorphic residues in dogs (Polvi et al. 1997), two coincided with polymorphic regions in humans (residues 13 and 26) (Brown et al. 1993; Stern et al. 1994). Even though the published marine mammal MHC sequences are limited in number and incomplete in length, a number of group-specific residue patterns were identified by inter-species comparison. *Zaca*-DQα contained sea-lion-specific motifs at residues 138 and 150, and a possible otariid-specific motif at residue 127. *Zaca*-DQα amino acids matched other marine and terrestrial carnivores at three positions (121, 125, and 141) and were conserved between available marine mammals at residues 95, 99, 110, and 143.

Variation between Zaca-*DQB* sequences

Eleven different *Zaca-DQB* sequences were identified from the clones containing *DQB*-specific primer amplified inserts. These results suggested the amplification of products from at least three loci. Of these 11 sequences, eight had features compatible with functional class II transcripts in other species, and the following comparisons were based on these sequences. The three remaining sequences had variable-length deletions, compatible with pseudogenes, as described above. As with *Zaca-DQA*, these sequences were confirmed by performing

Table 2 Sequence identity of California sea lion (*Zaca-DQB*) class II MHC clones, with human (HLA-*DQB*, HLA-*DRB*) and canine (DLA-*DQB* and DLA-*DRB*) sequences at the nucleotides encoding amino acids 194–201 of the class IIβ genes

Gene	*194*	*195*	*196*	*197*	*198*	*199*	*200*	*201*
Putative *DQB*-specific amino acid sequence	–	THR	–	–	ARG	–	ASP	–
HLA-*DQB*1*00629[a]	ATG	ACT	CCC	CAG	CGT	GGA	GAC	GTC
HLA-*DQB*2*00638[a]	ATG	ACT	CCC	CAG	CGT	GGA	GAT	GTC
HLA-*DQB*3*00639[a]	ATG	ACT	CCC	CAG	CGT	GGA	GAT	GTC
DLA-*DQB*[b]	ATG	ACT	CCC	CAG	CGA	GGA	GAT	GTC
*Mhc*Zaca-*DQB**01-*11	ATT	ACT	CCC	CAG	CGA	GGA	GAT	GTC
DLA-*DRB*[c]	ATA	GTT	CCT	CAG	AGC	GGA	GAG	GTC
HLA-*DRB*1*00719[a]	ACA	GTT	CCT	CGG	AGT	GGA	GAA	GTT
HLA-*DRB*1*00724[a]	ACA	GTT	CCT	CGG	AGT	GGA	GAG	GTT
HLA-*DRB*1*00749[a]	ACA	GTT	CCT	CGG	AGT	GGA	GAA	GTT
Putative *DRB*-specific amino acid sequence	–	VAL	–	–	SER	–	GLU	–

[a] IMGT/HLA numbers
[b] Dog lymphocyte antigen (DLA) GenBank accession number AF043908
[c] DLA GenBank accession number M29611

```
                 ↓leader     *         1    ↓ Alpha 1    20         *         40         *         60         *
Zaca-DQA*01   *****MTLNRVLILGTLTLTTVMSPSGGEDIVADHFASYGVNVYQSYGPSGQYTHEFDGDEEFYVDLEKKETVWQLPVFQTFTSFDPQGALRNLAVGKLT
Zaca-DQA*02   *****-----------------------------------A------------A-------------------------------------------
Zaca-DQA*03   *****-----------------C-------------------------------------------------------------------------
Zaca-DQA*04   *****-------------------------------------------------------------------------------------------
Zaca-DQA*05   *****-------------------------------------------------------------------------------------------
MileDQAMLU    ********************************************************************************************
MianDQAMAU    ********************************************************************************************
LeweDQALWU    ********************************************************************************************
HyleDQAHLU    ********************************************************************************************
ArfoDQAAFU    ********************************************************************************************
CafaDQAY07    ***************************************-I-------------------------------R----S---------------II-QN
CafaDQAY07    ***************************************-I-------------------------------R----S--R------------II-QN
HLADQAXM_0    *****-I--KA-L--A-A--A----C---------V------F---H---------------------T--------M-SK-I-----S----M----H-
HLADQA0060    *****-I--KA-L--A-A-------C---------V--C---L--F---------------------R---A-RW-E-SK-GG---------M--A-HN
BotaDQAY07    *****-V---A----A-A---M-----S-------IGT--ISI-HT-----Y----------------R----R----SK-AT---------I-TT-HN

                  80       ↓ Alpha 2 100          *         120         *         140         *         160         *
Zaca-DQA*01   LNVLTKRSNSTAATNEVPEVTVFLKSPAMLGQPNTLICFVDNIFPPVINVTWLKNKHSVTEGVSETRFLAKRDHSFLKFSYLTFLPSADDIYDCKVEHWG
Zaca-DQA*02   ------------------------------------------------------------------------------------------------
Zaca-DQA*03   ------------------------------------------------------------------------------------------------
Zaca-DQA*04   ------------------------------------------------------------------------------------------------
Zaca-DQA*05   ------------------------------------------------------------------------------------------------
MileDQAMLU    ****************-----------------------------------------R----K-----S-----------I-------------********
MianDQAMAU    ****************-----------------------------------------R----K-----S-----------I-------------********
LeweDQALWU    ****************-----------------------------------------R----------S-----------I-------------********
HyleDQAHLU    ****************-----------------------------------------R----K-----S-----------I-------------********
ArfoDQAAFU    ****************-----------------------------------------------S-----------I-------------********
CafaDQAY07    --I------Q-------------S---V----------L----------------R----------S----G----S-I---------E-----------
CafaDQAY07    --I------Q-------------S---V----------L----------------R----------S-F--G----S-I---------E-----------
HLADQAXM_0    -EFMMRQ----------------S-F-VT---------L--------V-I---S-G----------S--S-S----F-I----------E----------
HLADQA0060    --IMI--Y---------------S---VT---------L--------V-I---S-GQ---------S--S-S----F-I----------E----------
BotaDQAY07    -EIVIQ---------K-------S---V----------H----------I---R-G----------S--I-S-Y----IN-------D--V---------

                 180 ↓ transmembrane 200          *         220         *
Zaca-DQA*01   LDEPLLKHWEPEIPTPMSELTETVVCALGLTVGLVGIVVGTVFIIQGLHSGGASRHQGPL
Zaca-DQA*02   ------------------------------------------------------------
Zaca-DQA*03   ------------------------------------------------------------
Zaca-DQA*04   ------------------------------------------------T-------
Zaca-DQA*05   ------------------------------------------------------------
MileDQAMLU    ************************************************************
MianDQAMAU    ************************************************************
LeweDQALWU    ************************************************************
HyleDQAHLU    ************************************************************
ArfoDQAAFU    ************************************************************
CafaDQAY07    --------****************************************************
CafaDQAY07    --------****************************************************
HLADQAXM_0    --------------A--------L------S---M-------------R-V-------L-*
HLADQA0060    --Q-----------A---------------S----------------R-V---------*
BotaDQAY07    -----------D--A---------------------M---IL-----R---P-------*
```

Fig. 3 Amino-acid sequence identity between CSL class II MHC clones, human, canine, and other marine mammal DQα sequences. Abbreviations for individual species MHC molecules are as described for Fig. 1. The complete amino-acid sequence of *Zaca-DQA*01* is shown. *Single letters* and *dots* below the amino acid sequence represent amino acids that are, respectively, distinct from or identical to *Zaca-DQA*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DQA*01* and HLA00601 (human DQA, IMGT/HLA Database) (Robinson 2001)

Table 3 Sequence polymorphism of *MhcZaca-DQA* and *MhcZaca-DQB*, delineated by exon

Exons	1	2	3	4	5	6
DQA						
Nucleotide substitutions	1	2	0	1	n.a.	n.a.
Synonymous	0	0	0	0	n.a.	n.a.
Non-synonymous	1	2	0	1	n.a.	n.a.
DQB						
Nucleotide substitutions	2	5	3	2	1	0
Synonymous	0	2	1	1	1	0
Non-synonymous	2	3	2	1	0	0

two to four independent PCR reactions, by examining multiple clones, and by sequencing each clone in both directions, in compliance with HLA nomenclature rules (Bodmer et al. 1999).

As with *Zaca-DQA*, the sequence identity between the *Zaca-DQB* alleles was extremely high, with a total of only 14 nucleotide substitutions (Fig. 2). Of these 14 polymorphisms, the largest number were in exon 2 (5/14), with the remainder distributed between exon 1 (2/14), exon 3 (3/14), exon 4 (2/14), exon 5 (1/14), and 3′UTR (1/14) (Table 3). Exon deletions were not included in these nucleotide substitution calculations. The deduced amino acid sequences indicate that approximately two thirds of the nucleotide substitutions (8/13) were non-synonymous (Fig. 4, Table 3). The polymorphic *Zaca*-DQβ amino acid residues were equally distributed between the leader peptide (2/8), the β1 domain (3/8), the β2 domain (2/8), and the transmembrane region (1/8). The relative positions of these polymorphic residues were compared with those described in other species and examined using the *HLA-DR* model for class II peptide binding (Brown et al. 1993; Stern et al. 1994). *Zaca*-DQβ heterogeneity was observed at residues –5, –4, 9, 26, 30, 140, 182, and 212 (Fig. 4). While three of

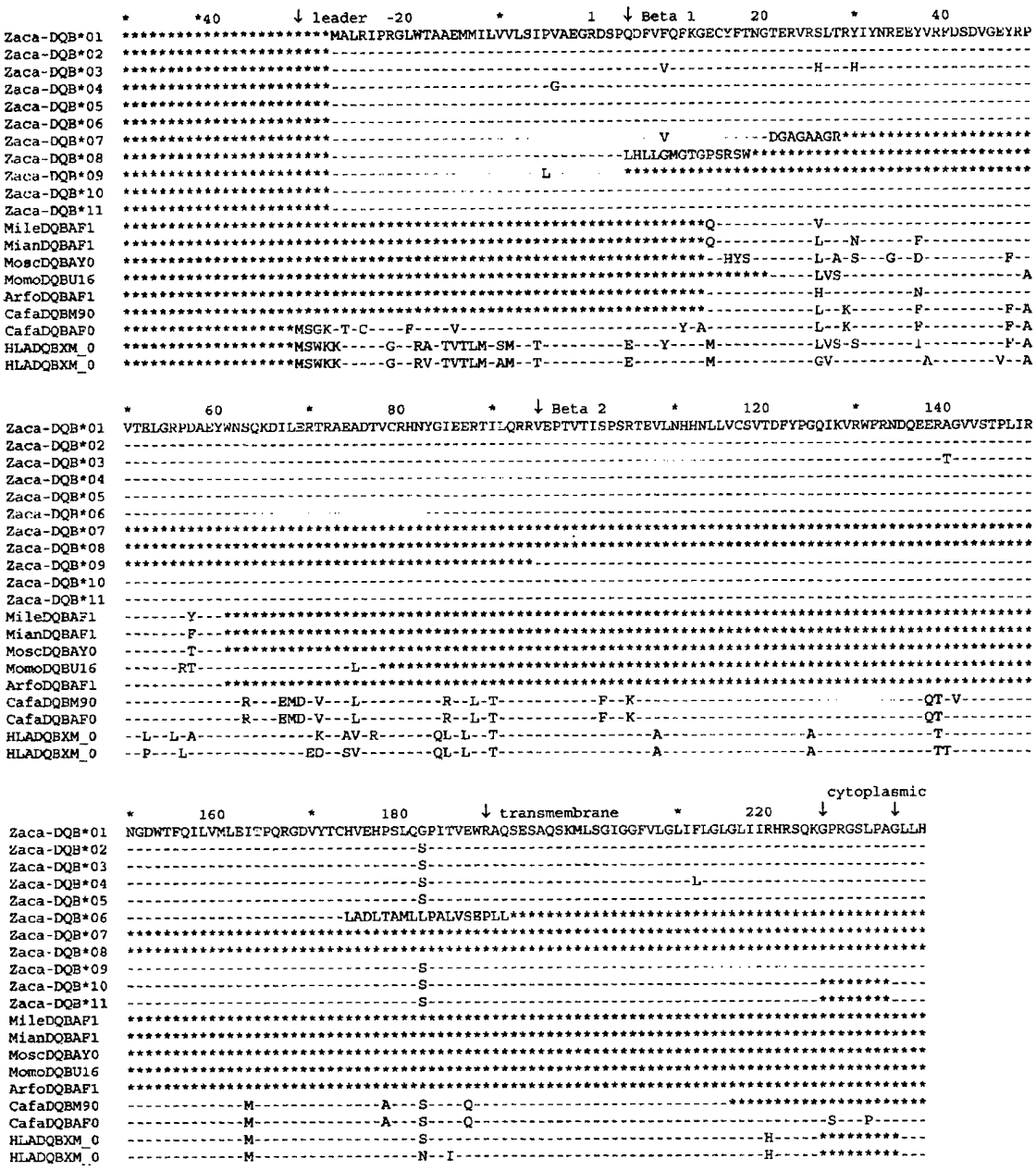

Fig. 4 Amino-acid sequence identity between CSL class II MHC clones, human, canine, and other marine mammal DQβ sequences. Abbreviations for individual species MHC molecules are as described for Fig. 2. The complete amino-acid sequence of *Zaca-DQB*01* is shown. *Single letters* and *dots* below the amino-acid sequence represent amino acids that are, respectively, distinct from or identical to *Zaca-DQB*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DQB*01* and XM_011391 (Human DQB, GenBank accession no. XM_011391)

these non-synonymous substitutions were located in the putative peptide-binding groove (Brown et al. 1993; Stern et al. 1994), only one of them coincided with polymorphic residues in dogs (residue 30) (Polvi et al. 1997) and two (residues 26 and 30) with those described in humans (Brown et al. 1993; Stern et al. 1994).

Three of the *Zaca-DQB* sequences were missing nucleotides representing exact and complete exons, either exon 2 (1/3) or exon 5 (2/3) (Fig. 2). In addition to their apparent exon deletions, each sequence also exhibited a unique nucleotide substitution, maintaining their discrete identity.

As with *Zaca*-DQα a number of group-specific residue patterns were identified by inter-species comparison. *Zaca*-DQβ contained an otariid-specific motif at residue 26, a shared motif with other marine and terrestrial carnivores at amino acid position 52, and showed homology with terrestrial carnivores only at positions 84, 87, 108,

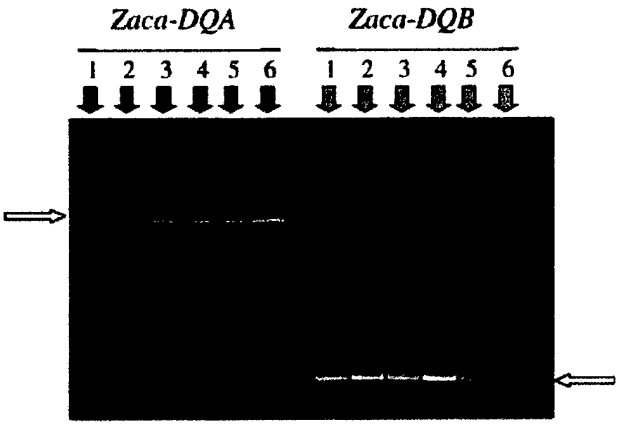

Fig. 5 Validation of limited *Zaca-DQ* sequence variability between individuals. Both *Zaca-DQA*- or *Zaca-DQB*-specific primers were used to amplify sequences containing the putative peptide-binding site from individuals from two different geographical locations. This figure shows products of these reactions from a representative subset of six animals compared by parallel denaturing gradient gel electrophoresis. For each analysis (*Zaca-DQA* and *Zaca-DQB)* an identical single band (*open arrows*) was observed in all animals examined, independent of geographical location. The sequence identity of the co-migrating bands was confirmed by direct sequencing of products from the extracted gel plugs

and 125. In contrast to *Zaca*-DQα, no sea-lion-specific motifs were identified in *Zaca*-DQβ. However, full-length sequences were not available for all species.

Validation of degree of *Zaca-DQ* sequence variation between individuals

To determine whether the limited *Zaca-DQ* sequence variability was consistent across individuals, the mobility patterns of partial length *Zaca-DQA* and *Zaca-DQB* sequences from 19 individuals were compared by DGGE. For both *Zaca-DQ* comparisons, a single band was observed from each individual (Fig. 5). Furthermore, within both the *-DQA* and *-DQB* comparisons, the single bands showed no differences in gel migration between individuals (Fig. 5). While this finding provided strong evidence for *Zaca-DQ* sequence homology between individuals, bands with similar migration patterns may have different sequences (Aldridge et al. 1998). Each band was therefore extracted, reamplified and directly sequenced. The results showed that, for the gene regions examined, the *Zaca-DQ* sequences were identical for all 19 individuals (data not shown).

Discussion

Infectious disease is the major cause of morbidity and mortality in many free-ranging seal populations. There is growing concern about the contribution of human pathogens as causes of disease in the marine environment (Harvell et al. 1999), and there have been numerous studies attempting to identify these pathogens. There are, however, fewer studies examining host susceptibility to emerging infectious diseases. Furthermore, conservation genetics in a number of marine species have expanded from their traditional focus on levels of genetic variation and inbreeding depression in small declining populations (Kretzmann et al. 1997), to include assessment of broader issues, such as the spatial and temporal aspects of population structure, demographics and the elucidations of systematics and phylogeny at any scale (Avise 1998). The variability and immunological importance of the genes in the MHC complex make them ideal for identifying genetic diversity, particularly in relation to pathogen exposure, both at an individual and population level.

The isolation and characterization of transcribed class II MHC gene products from California sea lion peripheral blood leukocytes revealed sequences with a similar composition to those classified as *DQA* and *DQB* in other species. Comparisons of the derived amino acid compositions supported the classification of these as functional molecules from at least two single *Zaca-DQA* gene loci and at least two *Zaca-DQB* gene loci. Three additional *Zaca-DQB* sequences with large nucleotide deletions causing a frameshift mutation and a premature stop codon were also identified. Since the sequences flanking the apparent deletion showed close homology with traditional *DQB* sequences it is unlikely that these were the result of PCR aberrations. They more likely represent nonsense transcripts of functional alleles (Voorter et al. 1997), or products of pseudogenes, arising from an earlier duplication of a traditional *DQB* locus (Groenen et al. 1990). Investigation of genomic DNA in additional sea lions would be necessary to accurately classify these apparent null alleles.

In this study, we used RACE technology to amplify full-length expressed class II gene sequences. The importance of this approach was demonstrated by the identification of sequence polymorphisms throughout the length of both *Zaca-DQA* and *Zaca-DQB* gene products. Previous studies of marine mammal MHC have only examined single exons from individual class II MHC genes (Hoelzel 1999; Murray and White 1998; Murray et al. 1999; Slade 1992). While these studies provide useful information, variation at one part of a gene, or one gene, is not a measure of variation for the entire MHC (Murray and White 1998). While multiple *DQ* sequences were derived from the two animals examined, the degree of heterogeneity between sequences was extremely low. In addition, the distribution of the polymorphic residues in sea lions was different from that reported in other species. This confirms findings in other pinniped species (Slade 1992), but contrasts sharply with the corresponding human and canine *DQ* genes (Robinson 2001; Polvi et al. 1997; Wagner et al. 1998, 1999). The disparity between the extent and distribution of sequence heterogeneity between the sea lion and the domestic dog, the closest relative with an extensively characterized MHC (Schreiber et al. 1998; Wagner et al. 1999), is particu-

larly significant because it contradicts the well-established concept of shared residues and motifs within and between species (Bontrop et al. 1999; Erlich and Gyllensten 1991; Fan et al. 1989; Gustafsson et al. 1990; Kupfermann et al. 1992; Slierendregt et al. 1992; Yaeger and Hughes 1999). In combination, these findings may indicate an independent (convergent) evolution associated with pathogen differences between the marine and terrestrial environments, or may bring into further question the concept of common ancestral lineages between marine and terrestrial carnivores.

Conclusions regarding *-DQ* variability in the sea lion population could not be drawn from the initial *Zaca-DQ* characterization studies because of the small number of animals included. To overcome this deficit, the putative *Zaca-DQ* peptide binding sites of 19 sea lions from two geographically distinct regions were examined by DGGE and direct sequencing. This confirmed that the lack of *Zaca-DQ* variability appears to be consistent across the sea lion population. While the characterization studies support the existence of multiple loci for both *Zaca-DQA* and *-DQB,* only a single *-DQA* and *-DQB* sequence was amplified from each of the 19 animals. This can be easily explained, since the DGGE-associated primers flanked a region that appears to be highly conserved between different loci.

It is difficult to make conclusions regarding the immunological importance of *Zaca*-DQ molecules, and their apparent lack of sequence variability without functional assays. The high sequence homology with *DQ* genes from other mammalian species and the existence of exon-5-deleted sequences in *Zaca-DQB* (Briata et al. 1989; Senju et al. 1992; Tsukamoto et al. 1987) support their characterization as classical class II MHC genes. Furthermore, the high proportion of non-synonymous nucleotide substitutions is suggestive of positive selection pressure on these gene loci (Hughes and Yeager 1998) and implies a functional role for these molecules in pathogen-specific immune responses. However, several features do not support a traditional role for the *Zaca*-DQ molecules in peptide binding. These include the near-equal numbers of non-synonymous and synonymous residue substitutions in the antigen recognition site, the amino acid replacements in conserved antigen recognition site positions, the high number of non-synonymous substitutions in non-peptide binding regions, the overall lack of sequence variation and the presence of exon-2-deficient sequences. In fact, the unusual pattern of non-synonymous and synonymous substitutions in the putative peptide-binding region closely resembles that used to describe non-classical class I genes, and as evidence for an evolutionary link to their classical counterparts (Hughes and Nei 1989). Clearly, there are insufficient data to attribute non-classical characteristics to the *Zaca-DQ* genes described here. In fact, accurate conclusions regarding the character of *Zaca*-DQ molecules are limited by the small number of individuals examined in this study. It will be important to conduct further studies, comparing more complete *Zaca-DQA* and *Zaca-DQB* genotypes between ecologically distinct sea lion populations, to further characterize these genes and their products, and to examine the dynamics of class II MHC variation in this species.

If *Zaca*-DQα and -DQβ molecules have secondary immunological roles, then, since sea lion populations continue to thrive, it is likely that the products of a different class II MHC gene are responsible for peptide-binding diversity in this species. While the MHC is polygenic in other species, a high degree of polymorphism is often confined to one or two genes (Escayg et al. 1996; Fabb et al. 1993; Klein et al. 1986; Mikko et al. 1997; Wagner et al. 1999). The identification of a polymorphic MHC gene in sea lions would be important, since there are several studies examining exon 2 of *DQA* and *DQB* genes in other seal species that are used as evidence for a lack of MHC diversity in pinnipeds (Hoelzel et al. 1999; Slade 1992). The increasing recognition of thriving species with apparent limited MHC polymorphism has also been used to question the importance of MHC diversity in the vulnerability of a population to disease (Mikko et al. 1999). If a yet undiscovered, more polymorphic class II MHC gene exists then these conclusions may be misleading. The most likely candidate genes are those encoding the DRα and DRβ molecules, which have been shown to be polymorphic in some terrestrial carnivores (Sarmiento et al. 1990; Wagner et al. 1996; Yuhki and O'Brien 1997).

In view of the increasing number of terrestrial pathogens encroaching on the marine environment (Bengston et al. 1991; Kennedy et al. 1988b; Osterhaus et al. 1988a), studies into marine mammal immunology are important in developing strategies to avert potentially catastrophic epidemics, particularly in declining or threatened populations (Domingo et al. 1990; Harvell et al. 1999; Kennedy et al. 1988a; Osterhaus et al. 1988; Osterhaus and De Vries 1988; Osterhaus 1989; Osterhaus et al. 1997). The sequence information from this study raises important questions regarding immunologic diversity in this thriving species. In addition they provide a framework from which further studies into the California sea lion MHC, and its role in infectious disease resistance, can be designed.

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