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Class II multiformity generated by variable *MHC-DRB* region configurations in the California sea lion (*Zalophus californianus*)

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Abstract In light of the immunological importance of molecules encoded within the major histocompatibility complex (*MHC*), there are numerous studies examining the variability of these genes in wildlife populations. An underlying assumption in many of these studies is that *MHC* diversity invariably arises from a high level of allelic variation at a single gene locus, leading to widespread descriptions of thriving species with apparently limited *MHC* polymorphism. Indeed, in a previous study we failed to find sequence features compatible with traditionally diverse peptide-binding functions in *MHC* class II (*DQA* and *DQB*) genes in California sea lions and therefore expanded the search for polymorphism to the *DRA* and *DRB* genes. Our results show that, in contrast to *Zaca-DQA*, *-DQB*, and *-DRA*, *Zaca-DRB* has sequence features compatible with antigen binding and presentation. In fact *Zaca-DRB* constitutes a gene family, comprising at least seven loci, each of which exhibits limited variability, and which are present in variable configurations between individuals. This unusual mechanism for generating *MHC DRB* diversity is similar to that observed in the rhesus macaque, but has not been reported in any other species. The identification of a novel system of class II *MHC* variability in the California sea lion justifies new studies into the organizational basis of immunogenetic diversity in other marine species, and its role in infectious disease susceptibility.

Keywords *MHC* · Comparative immunology · Veterinary immunology · California sea lion

The studies were performed jointly in the laboratories of J.L. Stott and M.L. Johnson

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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, and influence disease susceptibility and vaccine response (Paul 1999; Klein and Sato 2000a, 2000b; Kennedy et al. 2002). These cell surface glycoproteins play a key role in the initiation of an immune response by binding foreign peptides and presenting them to T cells (Paul 1999; Klein and Sato 2000a, 2000b). The polymorphism of these *MHC*-encoded proteins ultimately determines the repertoire of antigenic determinants to which an individual is capable of responding (Zinkernagel 1979; Reizis et al. 1998). Studies have indicated that antagonistic co-evolution between host and pathogen is evidenced by the high levels of genetic diversity observed at the *MHC* of many vertebrate hosts (Paterson et al. 1998). However, our understanding of *MHC* genetic variability is limited to a few, well-studied genes in a small number of species (Van Den Bussche et al. 1999). This is unfortunate considering the important role of infectious disease in the dynamics of many mammalian

populations, and the need of an individual to adapt to changing pathogen pressures.

In a previous study we characterized *MHC* class II gene sequences (*DQA* and *DQB*) in the California sea lion (*Zalophus californianus*) (CSL) (Bowen et al. 2002). Although multiple loci were identified for each *DQ* gene, the degree of sequence polymorphism was extremely low, and not compatible with traditional peptide-binding functions of classical *MHC* molecules. While the *MHC* is polygenic in other species, a high degree of polymorphism is often confined to one or two genes (Klein et al. 1986; Fabb et al. 1993; Escayg et al. 1996; Mikko et al. 1997; Wagner et al. 1999). As a result, we suspected the existence of an as yet unidentified polymorphic class II *MHC* gene in the California sea lion. Such a finding would be particularly significant in light of studies that have used *DQA* and *DQB* gene variation as evidence for a paucity in pinniped *MHC* diversity (Slade 1992; Hoelzel et al. 1999). 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 current study was directed at characterizing California sea lion DRα and DRβ molecules; the genes encoding these have been shown to be polymorphic in some terrestrial carnivores (Sarmiento et al. 1990; Wagner et al. 1996a, 1996b; Yuhki and O'Brien 1997). 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

Zaca-DRA and *-DRB* gene characterization using RACE technology

Blood from two California sea lions (*Z. californianus*) in rehabilitation (The Marine Mammal Center, Sausalito, Calif.) was collected from the caudal gluteal vein into cell separation tubes (Vacutainer CPT, Becton Dickinson, Franklin, N.J.), and processed to obtain peripheral blood mononuclear cells (PBMCs). 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 rapid amplification of cDNA ends (RACE) cDNA synthesis.

A cDNA population was constructed using SMART RACE cDNA amplification kits (Clontech, Palo Alto, Calif.) in order to facilitate the amplification of full-length gene transcripts. 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 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 oligonucleotide. The resulting 5′- and 3′-modified cDNA fragments were used as templates for subsequent polymerase chain reactions (PCRs) and RACE PCRs.

Degenerate oligonucleotide primers recognizing conserved regions of each of two MHC class II genes, *DRA* and *DRB,* were 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 *DRA* and *DRB* gene transcripts, from which sea lion *DRA*- and *DRB*-specific oligonucleotides could be designed. Polymerase chain reaction amplifications using these degenerate class II primers were performed on 20 ng of each RACE cDNA library in 50 μl volumes containing 20 to 60 pmol of each primer (either DRA-U1219 and DRA-L1512, or DRB-U182 and DRB-L729), 40 mM TRIS-KOH (pH 8.3), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$,

Table 1 Major histocompatibility complex (MHC) class II-specific primer pairs

Name	Primer sequence	Amplicon	Specificity
DRA-U1219	CCCGTGGAACTGGGAGAGC	235 bp; exon 3–exon 4	Mammalian *DRA*
DRA-L1512	CYRCATTCTCTGTKGTCTCTG		Mammalian *DRA*
ZCDRAU+Universal primer	CATCTGTTTCATCGACAAGTTCTCC	Exon 3, 3' end	CSL *DRA*
ZCDRAL+Universal primer	CCCAGTGCTCCACCTTGCAGTCATA	Exon 3, 5' end	CSL *DRA*
DRAU102	CATAAGTGGAGTCCCWGTGCTAG	741 bp; exon 1, 3′ UTR	CSL *DRA*
DRA3′UTR	GGAAACTGCATTGCCTGCAGGTG		CSL *DRA*
DRB-U182	CGGGACSGAGCGGGTKC	512 bp; exon 2–exon 4	Mammalian *DRB*
DRB-L729	CACTCAKCATCTTGCTCTG		Mammalian *DRB*
ZCDRB4U+Universal primer	GGAGAGGTCTACACCTGCCAAGTGG	Exon 3, 3′ end	CSL *DRB*
ZCDRB4L+Universal primer	CCACTTGGCAGGTGTAGACCTCTCC	Exon 3, 5′ end	CSL *DRB*
DRBU71	CCTRTCTTCTCTGYTCTCCAGC	821 bp; 5′ UTR, 3′ UTR	CSL *DRB*
DRBU116	GAGGCTCCTGGATGACAGCTYTG	776 bp; exon 1, 3′ UTR	CSL *DRB*
DRB3′UTR	AGCTGGGAAGGAAGGTTCTTCCT		CSL *DRB*
ZCDRBURN	CCCTCCCTTGGCTTGGGCTAG	270 bp; exon 1–exon 2/3	CSL *DRB*
ZCDRBLEX23	GTAGGCTCAACTCGCCGCTGC		CSL *DRB*
ZCDRB194A	TCATTTCTTGGAGCTGTTGAAGGG	249 bp; exon 2	CSL *DRB*
ZCDRB194B	TCATTTCTTGCTCCTGTTTAAGG	249 bp	CSL *DRB*
ZCDRB194C	TCATTTCTTGGAGCTGTTGAAGGC	249 bp	CSL *DRB*
ZCDRB194D	TCATTTCTTGCTCCTGTGTAAGTC	249 bp	CSL *DRB*
ZCDRB194E	TCATTTCTTGCACCTGTGTAAGGC	249 bp	CSL *DRB*
ZCDRB194F	TCATTTCTTGCACCTGTTTAAGGC	249 bp	CSL *DRB*
ZCDRB194G	TCATTTCTTGGAGCTGTTTAAGGC	249 bp	CSL *DRB*
Each with DRBlociR	CTCGCCGCTGCRCCRKGAAG		

3.75 μg/ml bovine serum albumin, 0.005% Tween-20, 0.005% Nonidet-P40, 200 μM each dNTP, and 5 U of Advantage 2 *Taq* polymerase (Clontech, Palo Alto, Calif.). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, Mass.) and consisted of 5 cycles at 94°C for 30 s, 30 cycles at 60°C for 30 s and 72°C for 2 min, 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 of the predicted size were excised from the gel, and 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 Align and Contig sequence alignment software programs (Vector NTI, Informax, North Bethesda, Md.). Regions of sequence homology were identified for each of the *DRA* and *DRB* gene products, and used to design sea lion MHC-specific oligonucleotides (Table 1), which were used to amplify 5′ and 3′ ends in ensuing RACE reactions.

The 5′ gene transcript amplification reactions contained 20 pmol of either ZcDRAL (for *DRA* gene products) or ZcDRB4L (for *DRB* gene products) along with 1–5 pmol of the Universal Primer Mix (UPM, SMART RACE cDNA amplification kit, Clontech). The 3′ gene transcript amplification reactions contained 20 pmol of either ZcDRAU (for *DRA* gene products) or ZcDRB4U (for *DRB* gene products) along with 1–5 pmol of the Universal primer mix (SMART RACE cDNA amplification kit, Clontech, Palo Alto, Calif.). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research). A touchdown method was employed to optimize reaction specificity, starting with 5 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min followed by 5 cycles at 94°C for 30 s, 64°C for 30 s, and 72°C for 3 min, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 3 min, ending with an extension step of 72°C for 10 min. Visualization, cloning and sequencing were performed as described above.

Full-length *DRB* and *DRA* 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 RACE PCR were identical to those described above. Gene product amplification reactions contained 20 pmol of both DRAU102 and DRA3′UTR (for *DRA* gene products) or DRBU71 or DRBU116 and DRB3′UTR (for *DRB* gene products). Reaction specificity was optimized using a touchdown method that started with 5 cycles at 94°C for 5 s, and 72°C for 3 min followed by 5 cycles at 94°C for 5 s, 70°C for 10 s, and 72°C for 3 min, followed by 25 cycles at 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min. Amplicon visualization, cloning and sequencing were performed using the procedures described above.

The nucleotide sequences of the RACE products were analyzed using Align and Contig sequence alignment software programs and compared with known sequences using the NCBI BLAST program (Altschul et al. 1990), and the IMGT/HLA database (Robinson et al. 2001). Phylogenetic analysis was performed on alignments (CLUSTAL) using the nucleotides of the individual *DRA* and *DRB* fragments with other related mammalian MHC sequences. Trees were constructed using the NEIGHBOR-joining program of PHYLIP (Felsenstein 1993) and were tested using the SEQBOOT and CONSENSE programs of PHYLIP to determine the degree of support for the particular tree nodes. Nonsynonymous and synonymous nucleotide substitution rates were calculated using MEGA version 2.1 (Kumar et al. 2001).

Table 2 Sequence polymorphism of *Zaca-DRA* and *Zaca-DRB*, delineated by exon**.** The nucleotides and derived amino acids of six *Zaca-DRA* and 12 *Zaca-DRB* sequences isolated from two California sea lions were compared and the number of synonymous (silent) and non-synonymous differences calculated. While numerous non-synonymous nucleotide substitutions reside in *Zaca-DRB* exon *2*, encoding the putative peptide binding region, they are infrequent and randomly distributed in *Zaca-DRA*. (n/a, not available)

	Exon					
	1	2	3	4	5	6
DRA						
Nucleotide substitutions	0	2	8	4	n/a	n/a
Synonymous	0	2	4	2	n/a	n/a
Non-synonymous	0	0	4	2	n/a	n/a
DRB						
Nucleotide substitutions	4	24	6	0	0	0
Synonymous	2	9	4	0	0	0
Non-synonymous	2	15	2	0	0	0

Examination of *Zaca-DRB* exon *2* variability

Based on data gained from full-length *DRB* sequences, a new primer pair recognizing the flanking regions of the putative peptide-binding site was designed to amplify *Zaca-DRB* exon *2* (Table 2). Peripheral blood leukocytes were isolated by whole blood lysis of samples collected from free-ranging sea lions in the Sea of Cortez, Baja, Mexico (n=4), and from San Miguel Island, Calif., USA (n=3). 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 U reverse transcriptase (Omniscript, Qiagen), 1 μM random hexamers, 0.5 mM each dNTP, and 10 U 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. The PCR and cloning were performed as described above but conditions were re-optimized for this new primer pair using a touchdown method that started with 5 cycles at 94°C for 5 s, and 72°C for 3 min followed by 5 cycles at 94°C for 5 s, 68°C for 10 s, and 72°C for 3 min, followed by 25 cycles at 94°C for 5 s, 64°C for 10 s, and 72°C for 3 min.

Examination of genomic *DRB* genotype using SSPs

Based on data gained from *DRB* exon *2* sequences, seven new primer pairs recognizing the flanking regions of the putative peptide-binding site were designed (Table 2). To validate our observation of multiple genotype configurations in cDNA, we examined genomic DNA from 58 San Miguel Island California sea lions. DNA was extracted from hind flipper skin punches using standard protocols (DNeasy, Qiagen) and the presence or absence of each single-strand polymorphism (SSP)-derived sequence type determined using PCR with an intercalating fluorescent dye. A series of seven SSP-based PCRs was performed on each individual. Each reaction contained 500 ng DNA in 25 μl volumes with 20 pmol SSP, TRIS-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ (pH 8.7), dNTPs, HotStar Taq DNA Polymerase (Quantitect SYBR Green PCR Master Mix, Qiagen), and 0.5 U uracil-N-glycosylase (Roche, Indianapolis, Ind.). Amplifications were performed in an iCycler (BioRad, Hercules, Calif.) under the following conditions: 2 min at 50°C, followed by 15 min at 95°C, and 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min. Reaction specificity was monitored by melting curve

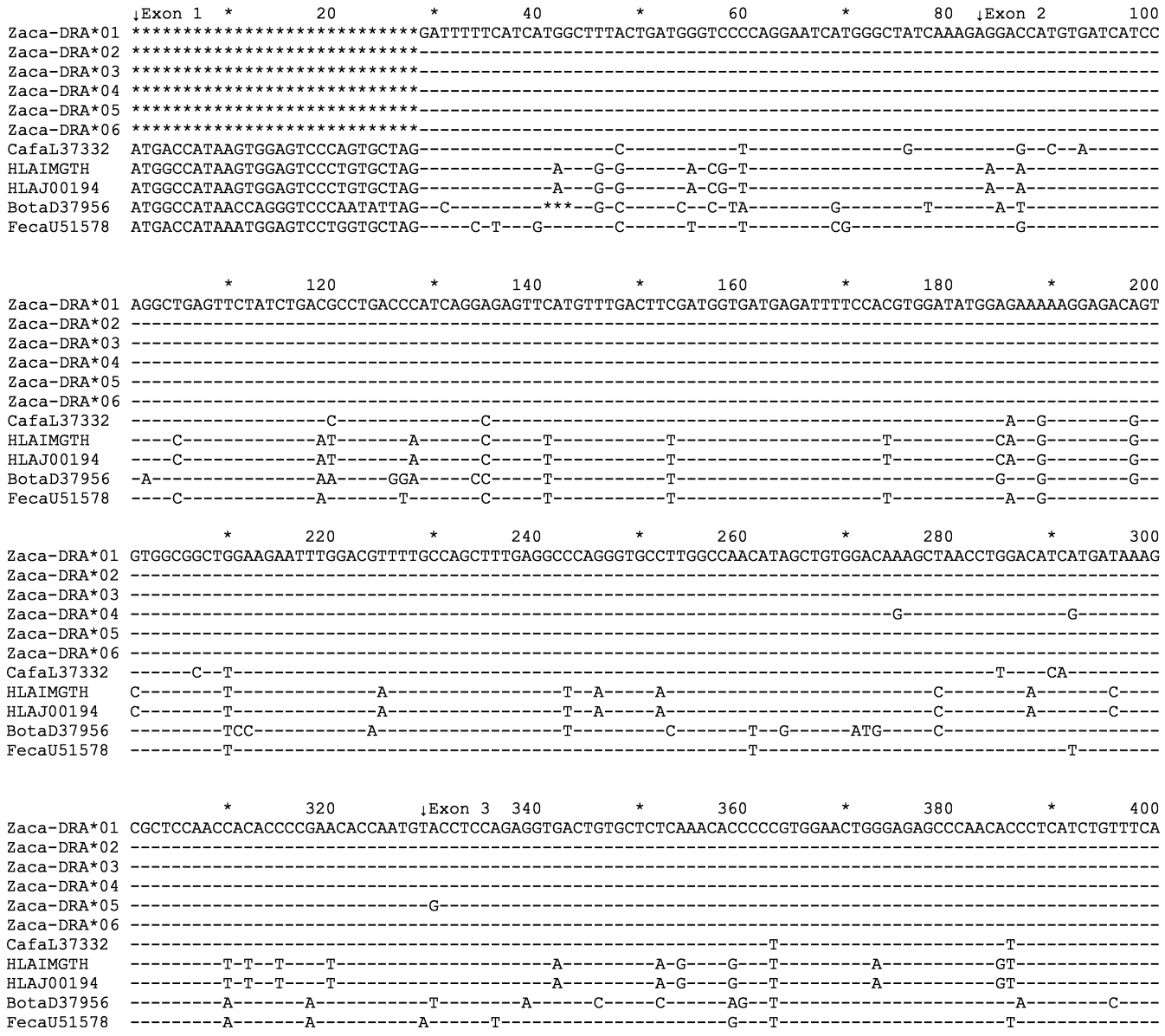

Fig. 1 Nucleotide sequence identity between California sea lion (CSL) class II major histocompatibility complex (MHC) clones (*Zaca-DRA*), human, canine, bovine, and feline *DRA* sequences. Abbreviations for individual species MHC molecules are as follows: *Bota*, domestic cow (*Bos taurus*); *Cafa*, domestic dog (*Canis familiaris*); *Feca*, domestic cat (*Felis catus*); HLA, human lymphocyte antigen; *Zaca*, California sea lion (*Zalophus californianus*). The MHC species label includes Genbank or IMGT Accession numbers. The complete nucleotide sequence of *Zaca-DRA*01* is shown. *Single letters* and *dots* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *Zaca-DRA*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DRA*01* and HLAJ00194 (IMGT/HLA Database) (Robinson et al. 2001). The allelic numbers for the CSL sequences were assigned according to multispecies guidelines (Klein and Takahata 1990), with the assumption of a single DRA lineage based upon the close sequence homology between individual clones. The stop codon is *italicized* and marked with an *arrowhead*

analysis using a final data acquisition phase of 60 cycles at 65°C for 30 s and verified by direct sequencing of randomly selected amplicons.

Results

Characterization of California sea lion *DRA* and *DRB* cDNA clones

Clones containing full length *Zaca* (*Z. californianus*) MHC class II-like sequences were obtained from the RACE cDNA products of two California sea lions. The

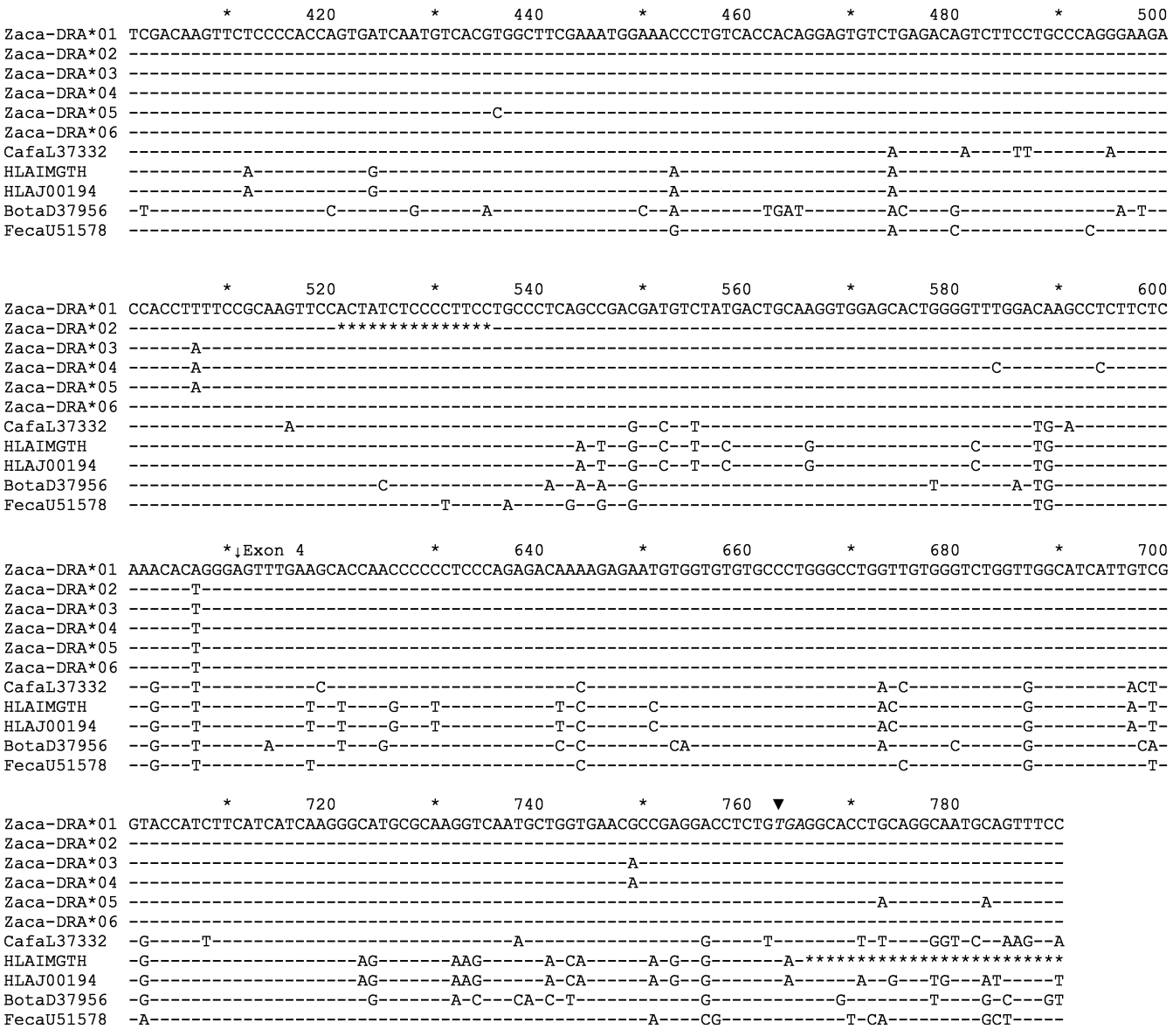

Fig. 1 (continued)

nucleotide and deduced amino acid sequence of the 734 bp (*DRA*-primer derived) and the 798 bp (*DRB*-primer derived) products were typical of transcripts from mammalian class II genes. These transcripts were characterized as *Zaca-DRA* or *Zaca-DRB* based on alignments with human lymphocyte antigen (HLA), and canine lymphocyte antigen (DLA), *DRA* and *DRB* sequences (Figs. 1, 2). The near full-length (*DRA* primer-amplified) sequences (Fig. 1) showed high homology with previously described full-length human (88.7%), canine (92.8%) and feline (92.7%) *DRA* sequences (Fig. 1). Similarly the full-length sequences amplified by the *DRB* primers showed a high homology with human (87.7%), canine (91.1%), and feline (88.4%) *DRB* sequences (Fig. 2).

The largest *Zaca-DRA* and *DRB* gene products encoded molecules of 244 and 266 amino acids, respectively (Figs. 3, 4). All *Zaca*-DRα molecules consisted of a partial 15 amino acid (aa) leader sequence, an 84 aa α_1 domain, a 107 aa α_2 domain, and a 38 aa connecting peptide/transmembrane/cytoplasmic tail domain (Fig. 3). One *Zaca-DRA* sequence showed a 15 nucleotide deletion in exon 3, which shortened the derived product by 5 aa (Fig. 3). *Zaca*-DRβ sequences consisted of a 29 aa leader sequence, a 95 aa β_1 domain, a 103 aa β_2 domain, a 23 aa transmembrane domain, and a 16 aa cytoplasmic tail (Fig. 4). In addition, one *Zaca-DRB* sequence had features compatible with a class II MHC pseudogene (Figs. 2, 4). This sequence contained a 23 nucleotide deletion in exon 2, which created a frame-shift and premature stop codon.

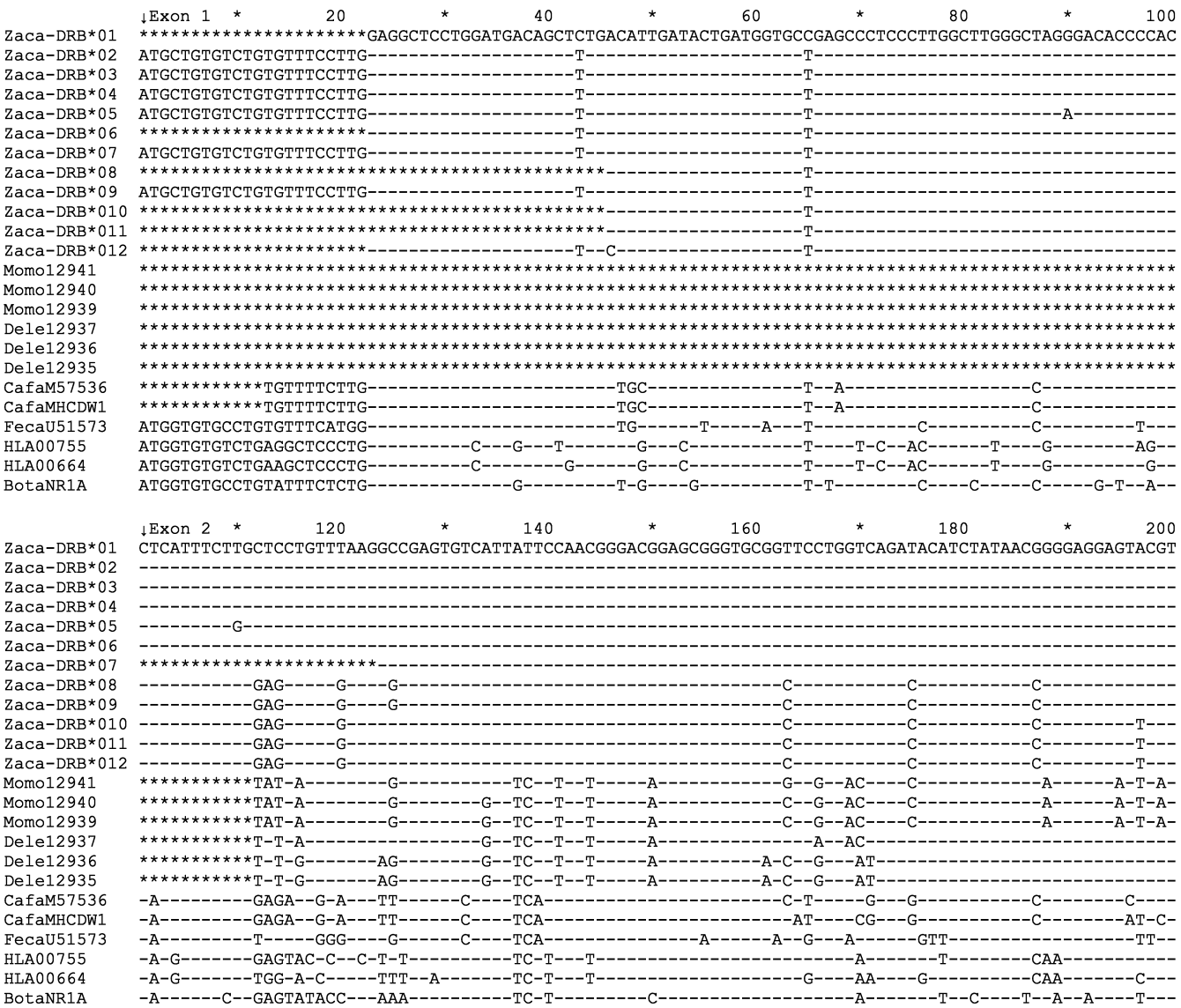

Fig. 2 Nucleotide sequence identity between California sea lion (CSL) class II major histocompatibility complex (MHC) clones, human, canine, bovine and feline *DRB* sequences. Abbreviations for individual species MHC molecules are as follows: *Bota*, domestic cow (*Bos taurus*); *Cafa*, domestic dog (*Canis familiaris*); *Dele*, beluga whale, (*Delphinapterus leucas*); *Feca*, domestic cat (*Felis catus*); HLA, human lymphocyte antigen; *Momo*, narwhal, (*Monodon monoceros*); *Zaca*, California sea lion (*Zalophus californianus*). The MHC species label includes Genbank or IMGT Accession numbers. The complete nucleotide sequence of *Zaca-DRB*01* is shown. *Single letters* and *dots* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *Zaca-DRB*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DRB*01* and HLA00664 (IMGT/HLA Database) (Robinson et al. 2001). The allelic numbers for the CSL sequences were assigned according to multispecies guidelines (Klein and Takahata 1990), with the assumption of multiple *DRB* lineages based upon the number of unique clones found in two CSLs. The stop codon is *italicized* and marked with an *arrow*

If translated, the length of the truncated protein molecule from this sequence would be 53 aa.

Variation in *Zaca-DRA* sequences

To maximize the identification of all *Zaca-DRA* sequences present in the two animals examined, multiple clones were sequenced. From these clones, six different *Zaca-DRA* sequences were identified (Fig. 1). This suggested that, if each sequence was considered a unique *Zaca-DRA* allele, the *DRA*-specific primers amplified products from at least two loci. None of the sequences exhibited features compatible with pseudogenes. The similarity 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 PCRs, by examining multiple clones, and by sequencing each clone in both directions, in compliance with HLA nomenclature rules (Bodmer et al.

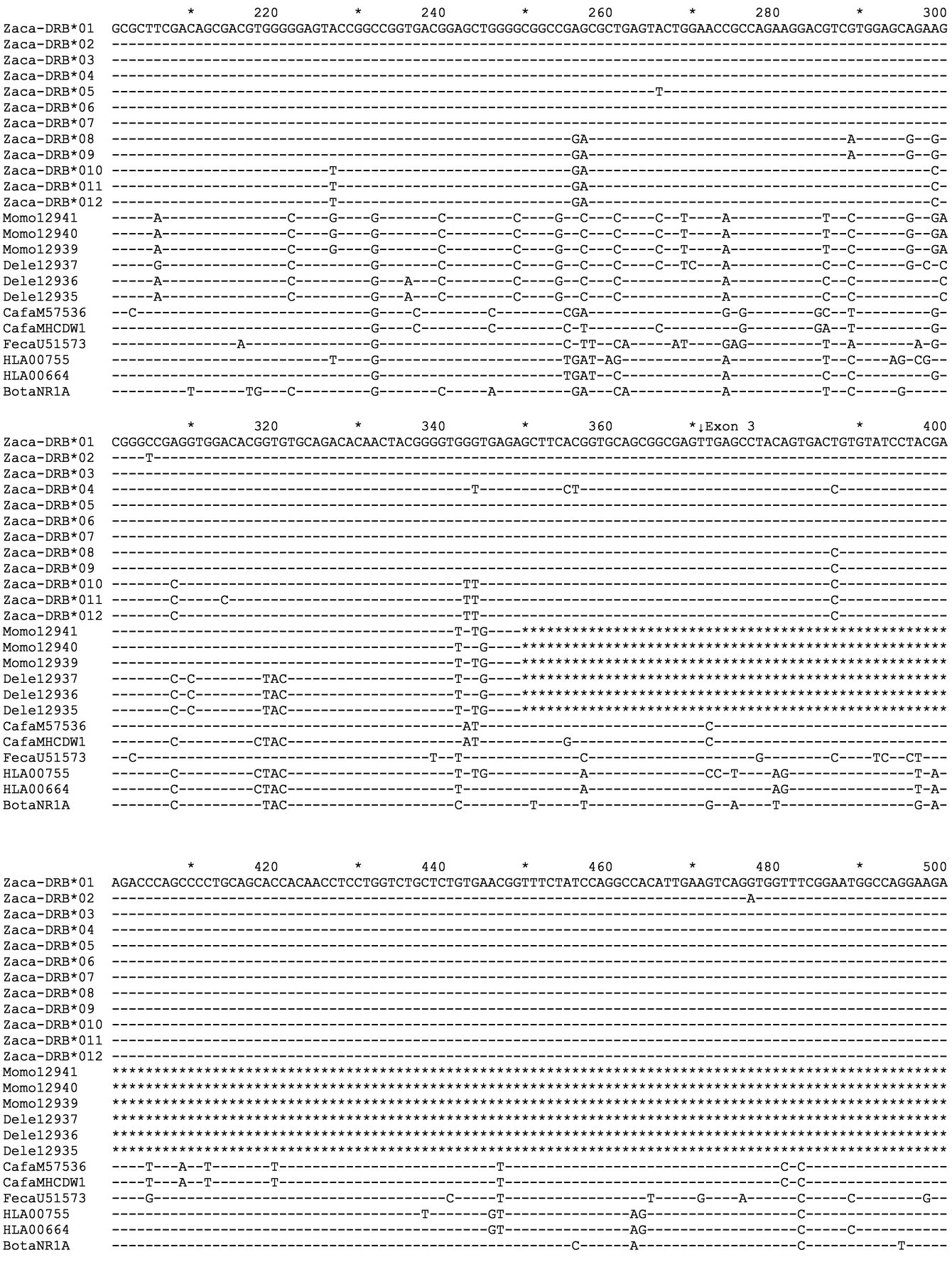

Fig. 2 (continued)

```
                    *       520         *       540         *       560         *       580         *       600
Zaca-DRB*01  GGAGGCTGGGGTCGTGTCCACAGGCCTGATCCGGAATGGAGACTGGACCTTCCAGACTCTAGTGATGCTGGAGACAGTTCCTCAGAGTGGAGAGGTCTAC
Zaca-DRB*02  ----------------------------------------------------------------------------------------T-----------------------
Zaca-DRB*03  ----------------------------------------------------------------------------------------T-----------------------
Zaca-DRB*04  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*05  ----------------------------------------------------------------------------------------T-----------------------
Zaca-DRB*06  ----------------------------------------------------------------------------------------T-----------------------
Zaca-DRB*07  ----------------------------------------------------------------------------------------T----------------------T
Zaca-DRB*08  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*09  -------------------------T----------------------------------------------------------------------------------------
Zaca-DRB*010 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*011 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*012 ------------------------------------------------------------------------------------------------------------------
Momo12941    ******************************************************************************************************************
Momo12940    ******************************************************************************************************************
Momo12939    ******************************************************************************************************************
Dele12937    ******************************************************************************************************************
Dele12936    ******************************************************************************************************************
Dele12935    ******************************************************************************************************************
CafaM57536   ---A--------T--------------A-----T---------------------TC--G-------------T------------C-------------------------
CafaMHCDW1   ---A--------T--------------A-----T---------------------TC--G-------------T------------C-------------------------
FecaU51573   ----A-----------------------------T---------------------C--G-----------A--------------------------------------
HLA00755     -A--A-------G-------------------AC---------------------C--G-----------A----------G------------T---------------
HLA00664     -A----------G-------------------A---------T-------------C--G-----------A----------G------------T---------------
BotaNR1A     ------------GA-C-----------------A-----------------------CA-G--------T--A--------------------------------------

                    *       620         *       640         *  ↓Exon 4          *       680         *       700
Zaca-DRB*01  ACCTGCCAAGTGGAGCACCCAAGCTTGATGAGTCCTGTCACCGTGGAATGGAGGGCACAGTCTGAGTCTGCGCAGAGCAAGATGCTGAGTGGAATTGGAG
Zaca-DRB*02  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*03  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*04  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*05  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*06  ----A-------------------------------------------------------------------------------------------------------------
Zaca-DRB*07  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*08  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*09  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*010 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*011 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*012 ------------------------------------------------------------------------------------------------------------------
Momo12941    ******************************************************************************************************************
Momo12940    ******************************************************************************************************************
Momo12939    ******************************************************************************************************************
Dele12937    ******************************************************************************************************************
Dele12936    ******************************************************************************************************************
Dele12935    ******************************************************************************************************************
CafaM57536   ----------------------T----CA--C-------------------------------T-----A----------------------C--G-
CafaMHCDW1   ----------------------T----CA--C-------------------------------T-----A----------------------C--G-
FecaU51573   --------C--------T-----TCGC-C---C---A----------G--------T--------A-----A----------------------C--G-
HLA00755     ------------------------G---CA--C---C----A----------A----G------A-----A--------------------G-C--G-
HLA00664     ----------------------TG---C---C---C----A----------A----G------A-----A--------------------G-C--G-
BotaNR1A     --------------------C---CA--CA--C---A----A--A------------G------C-----T------------A--------G-C--G-


                    *       720         *       740         *       760   ↓Exon 5       780       ↓Exon 6    ▼
Zaca-DRB*01  GCTTTGTTCTGGGTCTGCTCTTCCTCGTGGTGGGGCTGGTCATCTACTTCAGGAATCAGAAGGGACACTCTGGACTTCAGCCAACAGGACTCCTGAGC*TGA*
Zaca-DRB*02  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*03  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*04  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*05  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*06  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*07  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*08  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*09  ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*010 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*011 ------------------------------------------------------------------------------------------------------------------
Zaca-DRB*012 ------------------------------------------------------------------------------------------------------------------
Momo12941    ******************************************************************************************************************
Momo12940    ******************************************************************************************************************
Momo12939    ******************************************************************************************************************
Dele12937    ******************************************************************************************************************
Dele12936    ******************************************************************************************************************
Dele12935    ******************************************************************************************************************
CafaM57536   -------C-----------------T-CA---------T-------------A-------------------------------**********----------
CafaMHCDW1   -------C-----------------T-CA---------T-------------A-------------------------------**********----------
FecaU51573   ------------------------G-----------T-----------C----------A-------------------------------------------
HLA00755     -------G-----C-----------T-G--CC------T---------------------A----------------------G----T-----------
HLA00664     ----C--G-----C-----------T-G--CC------T---------------------A--------------------------T-----------
BotaNR1A     ----C------------T-------T-CC--------CT---------------------A----G-C--AC--------------G------------
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Fig. 2 (continued)

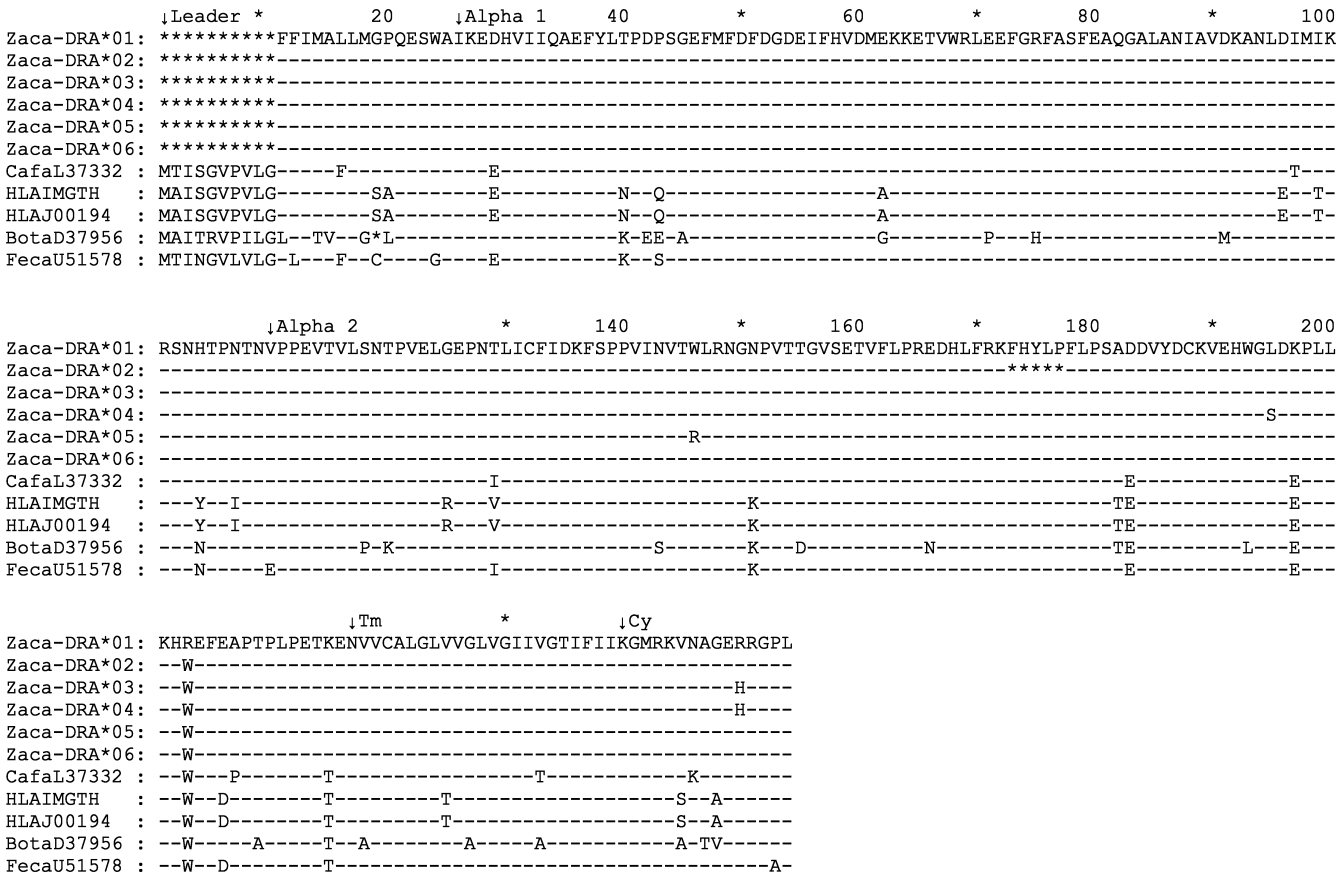

Fig. 3 Amino acid sequence identity between California sea lion (CSL) class II major histocompatibility complex (MHC) clones, human, canine, bovine, and feline DRα sequences. Abbreviations for individual species MHC molecules are as described for Fig. 1. The complete amino acid sequence of *Zaca-DRA*01* is shown. *Single letters* and *dots* below the amino acid sequence represent amino acids that are, respectively, distinct from or identical to *Zaca-DRA*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DRA*01* and HLAJ00194 (Human *DRA*, IMGT/HLA Database) (Robinson et al. 2001)

1999). The deduced amino acid sequences indicated that four of the 11 polymorphisms represented non-synonymous nucleotide substitutions (Fig. 3). Of the four polymorphic amino acid residues, none was found in the α_1 domain, which encodes the putative MHC class II peptide-binding site (Brown et al. 1993; Stern et al. 1994), three were found in exon 3, and one in the cytoplasmic tail. Polymorphism of *Zaca*-DRα amino acid sequences between individuals was observed at residues 146, 195, 203, and 250 (Fig. 3). None of these polymorphic residues coincided with variable regions in humans (Brown et al. 1993; Stern et al. 1994).

Variation in *Zaca-DRB* sequences

Twelve different *Zaca-DRB* sequences were identified from the clones containing *DRB*-specific primer-amplified inserts. These results suggest the amplification of products from a minimum of three loci in these two animals. Of these 12 sequences, 11 had features compatible with classical MHC class II transcripts in other species. All analyses of *DRB* employed only the complete 11 sequences; the apparent pseudogene with a 23 bp deletion was not included.

While variation between the *Zaca-DRB* sequences was relatively high (34 nucleotide positions; Fig. 2) as compared with *Zaca-DRA*, the apparent polymorphism was much less than described in most mammalian species. Of these 34 variable sites, the majority were in exon 2 (24/34), with the remainder distributed between exon 1 (4/34), and exon 3 (6/34). The deduced amino acid sequences indicated that the average number of non-

Fig. 4 Amino acid sequence identity between California sea lion (CSL) class II major histocompatibility complex (MHC) clones, human, canine, bovine, and feline DRβ sequences. Abbreviations for individual species MHC molecules are as described for Fig. 2. The complete amino acid sequence of *Zaca-DRB*01* is shown. *Single letters* and *dots* below the amino acid sequence represent amino acids that are, respectively, distinct from or identical to *Zaca-DRB*01*. *Asterisks* indicate missing sequences. Borders of each domain were assigned based upon sequence homology between *Zaca-DRB*01* and HLA00664 (Human *DRB*, IMGT/HLA Database) (Robinson et al. 2001)

```
                ↓Leader                    ↓Beta 1
                   *20         *             1         *       20         *        40         *        60         *
Zaca-DRB*01     ********GSWMTALTLILMVPSPPLAWARDTPPHFLLLFKAECHYSNGTERVRFLVRYIYNGEEYVRFDSDVGEYRPVTELGRPSAEYWNRQKDVVEQK
Zaca-DRB*02     MLCLCFLG-------------L-----------------------------------------------------------------------------
Zaca-DRB*03     MLCLCFLG-------------L-----------------------------------------------------------------------------
Zaca-DRB*04     MLCLCFLG-------------L-----------------------------------------------------------------------------
Zaca-DRB*05     MLCLCFLG-------------L--------------W--------------------------------------------------------------
Zaca-DRB*06     ********-------------L-----------------------------------------------------------------------------
Zaca-DRB*07     MLCLCFLG-------------L-----------RRVS-FQ-DGAGAVP-QIH-*****************************************
Zaca-DRB*08     ***************------L---------------E-L-G------------L---H---R----------------------D------------R-
Zaca-DRB*09     MLCLCFLG-------------L---------------E-L-G------------L---H---R----------------------D------------R-
Zaca-DRB*010    ***************------L---------------E-L--------------L---H---R----------------------D-------------T
Zaca-DRB*011    ***************------L---------------E-L--------------L---H---R----------------------D-------------T
Zaca-DRB*012    ********-------P-----L---------------E-L--------------L---H---R----------------------D-------------T
Momo12941       ************************************YQ--G------------V-T-H---E------------C-A------R---SL-S---F--R-
Momo12940       ************************************YQ--G--R---------L-T-H---E------------C-A------R---SL-S---F--R-
Momo12939       ************************************YQ--G--R---------L-T-H---E------------C-A------R---SL-S---F--R-
Dele12937       ************************************FQ-----R-----------T-----------C--------A------R---S--S------RH
Dele12936       ************************************FR--S--R--------QL-D--------------------AE-----R------S-------N
Dele12935       ************************************FR--S--R--------QL-D--------------------AE-----R------S-------N
CafaM57536      ****CFLG-------M-----LN--------------E-V-F-----------L---D---R--H-P---------A-------D-----G--E-----
CafaMHCDW1      ****CFLG-------M-----LN--------------E-V-F--------------A-D---R--I-----------A-------I--S-----E-----
FecaU51573      MVCLCFMG-------M-----L----------S----F-G-G---------Q--L---CF-----------E-----A-------I-K-M-E---F----
HLA00755        MVCLRLPG--C-AV---T---L-S---L-G--R-R--EYSTS----F---------D--F--Q--------------A-------DE----S---F--DE
HLA00664        MVCLKLPG--C------T---L-S---L-G--R-R--WQL-F----F-------L-E-C---Q--S-----------A-------D-----S--------
BotaNR1A        MVCLYFSG----A--I-----LC-------EIQ----EYT-K----F---------D--FH-----------W----A-------D-K---S---F----

                       80         *     ↓ Beta 2      *       120         *       140         *       160         *
Zaca-DRB*01     RAEVDTVCRHNYGVGESFTVQRRVEPTVTVYPTKTQPLQHHNLLVCSVNGFYPGHIEVRWFRNGQEEEAGVVSTGLIRNGDWTFQTLVMLETVPQSGEVY
Zaca-DRB*02     -V-------------------------------------------------------------------------------------------I--------
Zaca-DRB*03     ---------------------------------------------------------------------------------------------I--------
Zaca-DRB*04     --------------V---L-----------------------------------------------------------------------------------
Zaca-DRB*05     -----------------------------------------------------------------------------------------------I--------
Zaca-DRB*06     -----------------------------------------------------------------------------------------------I--------
Zaca-DRB*07     ************************************************************************************************************
Zaca-DRB*08     ------------------------------------------------------------------------------------------------------
Zaca-DRB*09     ------------------------------------------------------------------------------------------------------
Zaca-DRB*010    --A-----------F-------------------------------------------------------------------------------------
Zaca-DRB*011    --A-A---------F-------------------------------------------------------------------------------------
Zaca-DRB*012    --A-----------F-------------------------------------------------------------------------------------
Momo12941       --------------V-*********************************************************************************
Momo12940       ----------------*********************************************************************************
Momo12939       --------------V-*********************************************************************************
Dele12937       --A---Y---------*********************************************************************************
Dele12936       --A---Y---------*********************************************************************************
Dele12935       --A---Y-------V-*********************************************************************************
CafaM57536      --------------I----------------------T-----------------------L-----------------------I-----I--------
CafaMHCDW1      --A---Y-------I---A-----------------T-----------------------L-----------------------I-----I--------
FecaU51573      ------------------------------------------------------------------------T-------------------------
HLA00755        --A---Y-------V---------H-K--------------------S-----S------------KT--------H----------------R-----
HLA00664        --A---Y-------------------K--------------------S-----S------------K---------Q----------------R-----
BotaNR1A        --A---Y-------------------I-----A--------------------N---------H------------Q----------------------

                      180         *        ↓ Transmembrane    220 ↓ Cy    *
Zaca-DRB*01     TCQVEHPSLMSPVTVEWRAQSESAQSKMLSGIGGFVLGLLFLVVGLVIYFRNQKGHSGLQPTGLLS
Zaca-DRB*02     ------------------------------------------------------------------
Zaca-DRB*03     ------------------------------------------------------------------
Zaca-DRB*04     ------------------------------------------------------------------
Zaca-DRB*05     ------------------------------------------------------------------
Zaca-DRB*06     -Y----------------------------------------------------------------
Zaca-DRB*07     ******************************************************************
Zaca-DRB*08     ------------------------------------------------------------------
Zaca-DRB*09     ------------------------------------------------------------------
Zaca-DRB*010    ------------------------------------------------------------------
Zaca-DRB*011    ------------------------------------------------------------------
Zaca-DRB*012    ------------------------------------------------------------------
Momo12941       ******************************************************************
Momo12940       ******************************************************************
Momo12939       ******************************************************************
Dele12937       ******************************************************************
Dele12936       ******************************************************************
Dele12935       ******************************************************************
CafaM57536      ---------T-----------D--------------------A---F--------------*****
CafaMHCDW1      ---------T-----------D--------------------A---F--------------*****
FecaU51573      --H-----RT-------------------------------------F------------------
HLA00755        ---------T---------R----------------------GA--F--------------R-F--
HLA00664        ---------T---------R----------------------GA--F----------------F--
BotaNR1A        --------QT---------R-D------------------F--A---F--------RPT--------
```

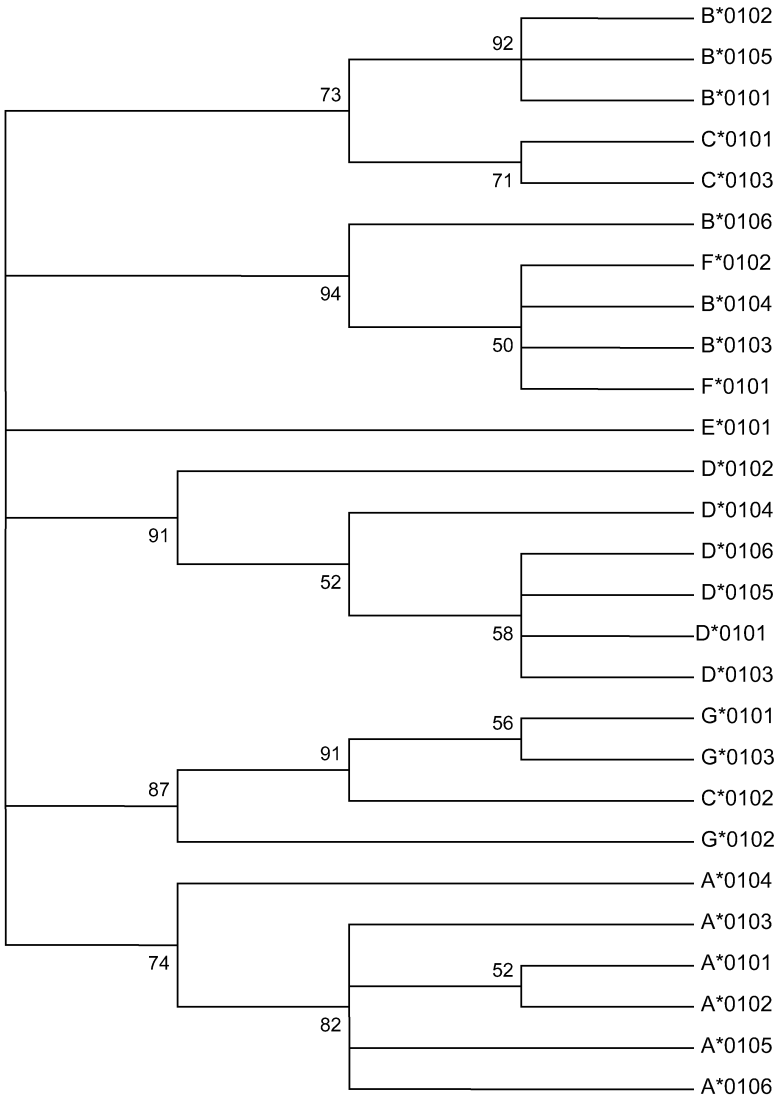

Fig. 5 Phylogenetic tree of putative *Zaca-DRB* peptide binding region. Phylogenetic analysis of a 270 bp *Zaca-DRB* exon 1-2 fragment from seven animals amplified by primers recognizing conserved regions in exon 1 and exon 2/3 showed several distinct clades. The prefix to each sequence label (*A–F*) refers to our current single-strand polymorphism (SSP)-based locus allocations, which rely on a specific exon 2 motif (nucleotides 102–125). The distribution of these different loci between clades shows the close structural relationship of the genes in this family, and supports the concept of a common evolutionary origin

synonymous sites per sequence was 6.9, and the average number of synonymous sites was 3.4 (Kumar et al. 2001; Fig. 4, Table 2). The polymorphic *Zaca*-DRβ amino acid residues (n=19) were located in the leader peptide (2/19), the β_1 domain (15/19), and the β_2 domain (2/19). The relative positions of these polymorphic residues were examined using the *HLA-DR* model for class II peptide binding (Brown et al. 1993; Stern et al. 1994). *Zaca*-DRβ polymorphism was observed at residues –14, –8, 8, 9, 11, 13, 26, 30, 34, 57, 70, 71, 73, 74, 76, 86, 90, 163, and 173 (Fig. 4). While 15 of these polymorphic sites were located in the putative peptide-binding groove (Brown et al. 1993; Stern et al. 1994), only eight (residues 9, 11, 13, 30, 70, 71, 74, and 86) coincided with those described in humans (Brown et al. 1993; Stern et al. 1994).

The information derived from these two sea lions was used to design a universal set of primers flanking exon 2 (ZCDRBuRN and ZcDRBLEx23, Table 1, Fig. 2), that would maximize amplification of class II *DRB* sequences in a particular individual. These flanking primers [5′, nucleotides (nts) 69–89; 3′, nts 360–380] encompassed the entire length of exon 2, and were used to characterize the putative peptide binding site of seven additional sea lions.

Exon 2 sequences from seven additional California sea lions

The analysis of clones obtained using the universal *Zaca-DRB* primers in seven additional California sea lions revealed 27 different sequences (Figs. 5, 6). The sequence differences were localized to 41 nucleotide positions, translated into 27 polymorphic sites in the derived 89 residue β_1 domain. The pattern of distribution of these polymorphic sites throughout the PBR differed from the hypervariable regions (*HVR*s) described in other species. In fact, 15 polymorphic sites fell outside the *HVR* boundaries defined in primates (Gaur and Nepom 1996), while the others were distributed between *HVR1* (n=3), HVR2 (n=5), and *HVR3* (n=4). Since the different sequences could be separated into seven broad categories

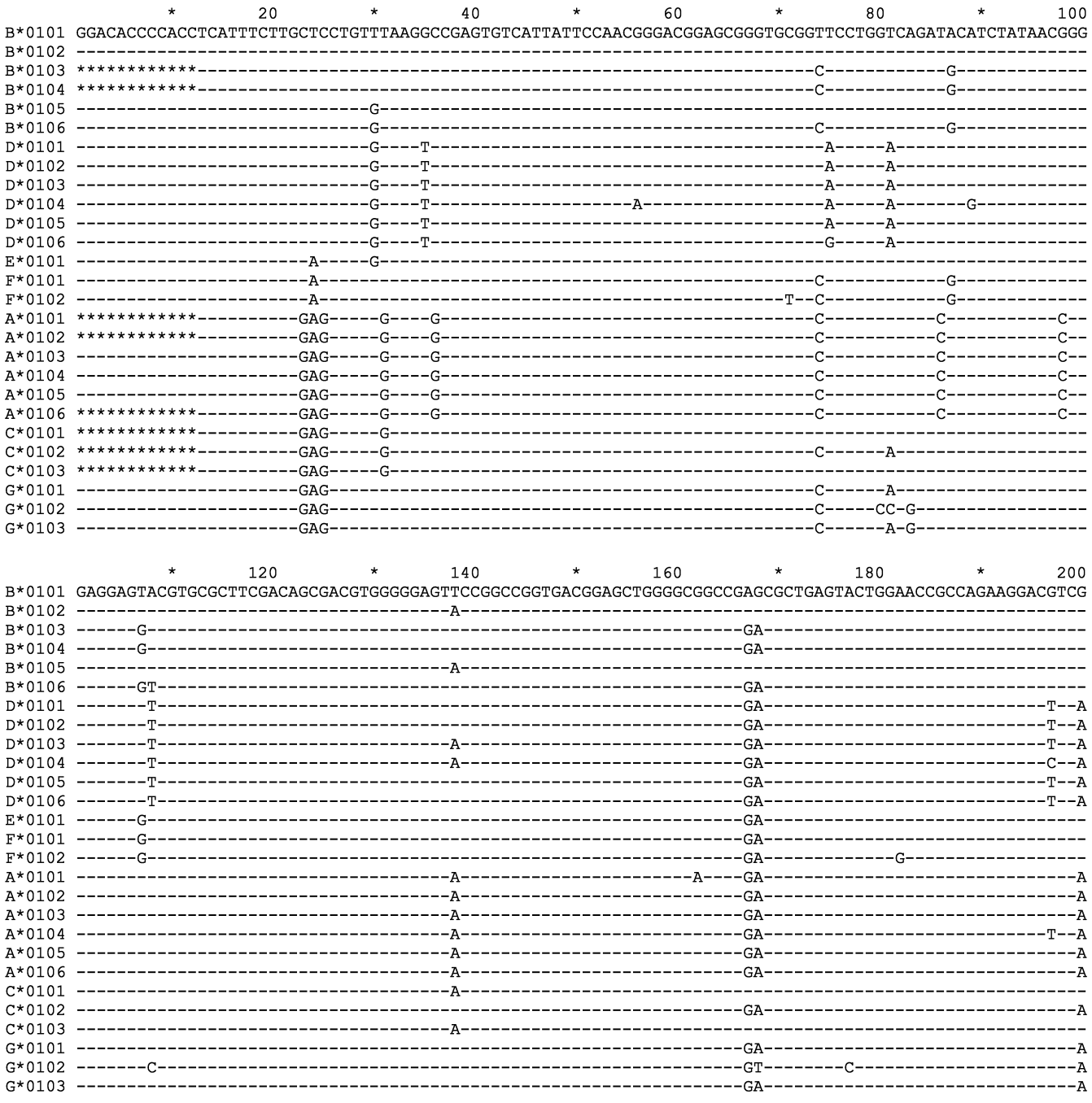

Fig. 6 Nucleotide sequence identity of the putative *Zaca-DRB* peptide binding region. Sequence analysis of a 270 bp *Zaca-DRB* exon 1-2 fragment from seven animals amplified by primers recognizing conserved regions in exon 1 and exon 2/3. The complete nucleotide sequence of *B*0101* is shown. *Single letters* and *dashes* below the nucleotide sequence represent nucleotides that are, respectively, distinct from or identical to *B*0101*. *Asterisks* indicate missing sequences

each clearly defined by a distinct nucleotide pattern in the 5′ region of exon 2 (nt 102–125) (Fig. 6), they were used to design seven sequence-specific 5′ primers that would amplify 246 bp segments of exon 2 for further *Zaca-DRB* analysis (Table 1). Each primer was 23 or 24 bp in length. Variable sites between primers were located at primer nucleotide positions 11, 12, 13, 18, 19, 23, and 24.

The specificity of the SSP *DRB* primers was confirmed by the fact that the amplicons from each were always consistent with the seven categories described above. Between three and seven different SSP-derived sequences were amplified from each sea lion, conferring a signature

Table 3 Single-strand polymorphism (SSP)-amplified haplotype configurations in seven sea lions The *Zaca-DRB* gene configurations of seven animals were determined using primers recognizing conserved regions in exon 1 and the exon 2/3 boundary to amplify a 312 bp fragment of exons 1–3. The wide range of configurations observed in this small group of animals, combined with the fact that these primer sets amplified two alleles in certain individuals, supports the existence of multiple *Zaca-DRB* loci

Animal ID						
LOMA7[a]	ELRA5[a]	ELGR5[a]	ELRA10[a]	CSL81[b]	CSL391[b]	CSL1784[b]
*A*0102*	*A*0101*	*A*0102*	*A*0104*	*A*0106*	*C*0101*	*A*0103*
*B*0101*	*B*0103*	*B*0102*	*A*0105*	*B*0103*	*C*0103*	*G*0102*
*D*0101*	*B*0104*	*B*0105*	*D*0102*	*B*0106*	*D*0103*	*G*0103*
*D*0106*	*C*0102*	*D*0101*		*D*0102*	*D*0104*	
	*F*0101*	*D*0105*		*F*0101*	*E*0101*	
	*F*0102*					
	*G*0101*					

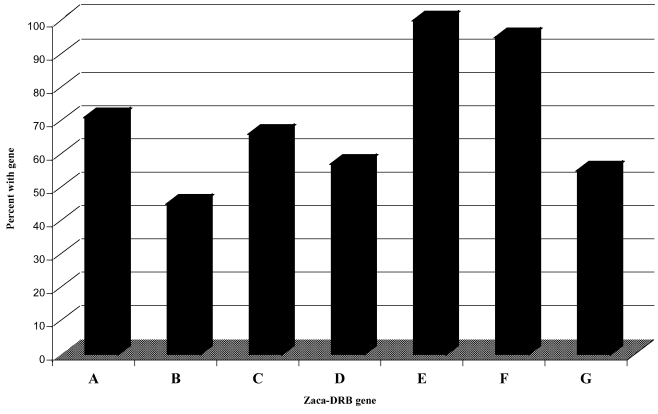

Fig. 7 Differential *Zaca-DRB* gene configurations in free-ranging sea lions. Single-strand polymorphism (SSP)-based genotyping was performed on 58 sea lions from San Miguel Island using a series of seven primer pairs. There was a wide range of gene configuration between animals, as evidenced by the variation in gene frequency within the population. For instance, *Zaca-DRB-E* and *-F* were present in almost all individuals, whereas *Zaca-DRB-B* was present in fewer than half of the animals examined

Zaca-DRB genotype on each individual (Table 3). The detection of seven sequences in ELRA-5 indicates the presence of at least four *Zaca-DRB* loci in this individual. However, examination of the sequence distribution of multiple sea lions supports the presence of at least six *DRB* loci, since SSP sets A, B, C, D, F, and G amplified two sequences in ELRA-10, ELRA-5, CSL391, ELGR-5, ELRA-5, and CSL1784, respectively. This conclusion is supported by the phylogenetic analyses of *Zaca-DRB* exon 2 sequences from these seven sea lions, which revealed at least six distinct clusters (Fig. 5). Since SSP-E failed to amplify more than a single sequence from any individual, it is uncertain whether this primer is locus or allele specific.

Examination of *Zaca-DRB* configuration polymorphisms using genomic DNA

Single strand polymorphism-based *Zaca-DRB* genotyping was performed on the DNA of a random sample of free-ranging sea lions from San Miguel Island (n=58) to examine whether variability in transcribed *Zaca-DRB* genes reflected genomic differences between individuals. The prevalence of each gene was not uniform in this population (Fig. 7). In fact, 23 different *Zaca-DRB* configurations were identified in the 58 animals, ranging in composition from two (1.7% of CSL) to seven loci (12% of CSL), with five loci being present most often (32.7% of CSL). The most frequent *Zaca-DRB* configurations in this population were *ACEFG* (8/58), *ACDEFG* (7/58) and *ABCDEFG* (7/58). A significant proportion of the configurations (14/23) were identified in single individuals only.

Discussion

A significant number of marine mammal populations are in decline or have been classified as threatened (Gilmartin et al. 1993; National Marine Fisheries Service 1995; Trites and Larkin 1996; Calkins et al. 1999). In efforts to aid recovery, and protect other species from similar events, these populations are under intense scientific scrutiny. A recurring theme in many of these investigations is the contribution of genetic diversity to both the initiation of the decline, and to the future survival of the population. Since infectious disease is an important cause of morbidity and mortality in many marine mammal species, it follows that epidemiological genetic investigations should include examinations of functionally important genes such as those in the MHC. To date, class II MHC gene investigations in marine mammal species have presumed that immunogenetic diversity is generated by polymorphism at one or two specific loci (Murray and White 1998), a reasonable assumption based on established knowledge in terrestrial species (Wagner et al. 1996a, 1996b, 1999; Mikko et al. 1999). However, in a previous study we examined sea lion MHC class II genes considered homologous to those important in generating peptide-binding diversity in related terrestrial carnivores but were unable to detect the expected sequence variability (Bowen et al. 2002). As a result of these findings we extended our search for a class II gene with sequence features that support traditional peptide

binding functions to include those encoding DRα and DRβ molecules.

Full-length *DRA* and *DRB* genes from the California sea lion were characterized. Convincing evidence was found that suggested sea lions have an unexpected and relatively unique method for generating class II MHC multiplicity. Our results support the existence of a *Zaca-DRB* gene family comprising at least seven loci, but with minimal sequence variation. Genes from these purported loci could be distinguished from one another by sequence-specific motifs located predominantly within the first three exons; not all loci were present in every individual. The number of *DRB* genes was highly variable between individuals (2–7), supporting a basis for class II MHC diversity arising from variation in *DRB* region configuration.

The appearance of multiple *DRB* loci with low degrees of sequence polymorphism, but with variable configurations between individuals described here is similar to that observed in the rhesus macaque (Doxiadis et al. 2001), but has not been reported in any other species. The multiplicity of *DRB* loci in other species ranges from one (cattle, white-tailed deer, dogs, narwhals) (Wagner et al. 1996a, 1996b; Murray and White 1998; Mikko et al. 1999), to two (sheep, red deer, beluga whales) (Murray and White 1998; Mikko et al. 1999), to three (cats) (Yuhki and O'Brien 1997) or more (humans). The MHC diversity in each of these latter species arises from a high level of allelic variation at a single locus, which, in certain species, may be paired with one or two relatively monomorphic loci (Van Eijk et al. 1992; Svensson et al. 1996; Robbins et al. 1997). The identification of multiple transcribed *DRB* genes in individual sea lions would be consistent with other mammals; however, the design for generating MHC diversity would appear to be different.

While ongoing studies are in progress to determine the true extent of class II variation in the California sea lion population, preliminary evidence from the limited number of animals included in this study (n=58), in which we were able to identify 23 different gene configurations, supports the existence of extensive multiplicity in this thriving population. This is particularly striking in view of the fact that fewer than 35 genotype configurations are currently recognized in the rhesus macaque (Doxiadis et al. 2001). It is difficult to speculate on the immunological advantages of such an unusual pattern of generating MHC diversity, particularly since it is shared by such disparate species. While it is possible that a similar mechanism of diversity exists in other marine carnivores, it is clear from extensive studies in the dog and cat that such a process is absent in these terrestrial carnivores (Schreiber et al. 1998; Wagner et al. 1999). The degree and distribution of sequence homogeneity between the sea lion and the domestic dog, the closest relative with an extensively characterized MHC, supports the well-established concept of shared residues and motifs within and between species (Fan et al. 1989; Gustafsson et al. 1990; Erlich and Gyllensten 1991; Slierendregt et al. 1992; Bontrop et al. 1999; Yaeger and Hughes 1999). The existence of an apparently dissimilar basis for generating diversity indicates either an independent (convergent) evolution associated with pathogen differences, or questions the concept of common ancestral lineages between marine and terrestrial carnivores (Ledje and Arnason 1996; Vassetzky and Kramerov 2002). Regardless, the ability of the California sea lion population to thrive in the face of a large number of microbial pathogens (Gulland et al. 1996; Lyons et al. 2001; King et al. 2002), suggests that this is an effective mechanism for maintaining immunologic vigor.

The findings of this study are particularly important in light of the ongoing debate regarding low MHC polymorphism in several marine mammal species. This debate has raised questions concerning the extent of general pathogen pressure in the marine environment [Slade 1992; Hoelzel et al. 1999; Marine Mammal Major Histocompatibility Complex (MHC) Workshop 2001, 14th Biennial Conference on the Biology of Marine Mammals, Vancouver, BC, personal communciation]. Unfortunately much of this speculation has arisen from an assumption that MHC diversity arises from one or more polymorphic loci. The identification of a novel system of class II MHC variability in the California sea lion MHC described here should stimulate new studies into the existence of immunogenetic diversity in other marine species. Furthermore, the results of this study will provide a framework from which future investigations can be directed at relating MHC polymorphism and population health.

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