Immunogenetics (2006) 58: 203–215
DOI 10.1007/s00251-005-0069-y

ORIGINAL PAPER

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Paucity of class I MHC gene heterogeneity between individuals in the endangered Hawaiian monk seal population

Received: 14 March 2005 / Accepted: 21 November 2005 / Published online: 10 March 2006
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Abstract The Hawaiian monk seal population has experienced precipitous declines in the last 50 years. In this study, we provide evidence that individuals from remaining endangered population exhibit alarming uniformity in class I major histocompatibility (MHC) genes. The peripheral blood leukocyte-derived mRNA of six captive animals rescued from a stranding incident on the French frigate shoals in the Hawaiian archipelago was used to characterize genes in the monk seal class I MHC gene family, from which techniques for genotyping the broader population were designed using degenerate primers designed for the three major established human MHC class I loci (HLA-A, HLA-B, and HLA-C), and by sequencing multiple clones, six unique full-length classical MHC class I gene transcripts were identified among the six animals, three of which were only found in single individuals. Since The low degree of sequence variation between these transcripts and the similarity of genotype between individuals provided preliminary evidence for low class I MHC variability in the population. The sequence information from the class I transcripts from these six animals was used to design several primer sets for examining the extent of MHC variability in the remaining population using a combination of polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE). Several DGGE assays, each one amplifying subtly different class I MHC gene combinations, were designed to compare exons encoding the highly polymorphic domains of the putative peptide-binding region of MHC class I. In combination, these assays failed to show interindividual variability at any of the class I MHC gene loci examined in either the six captive seals or in 80 free-ranging animals (~6.7% of the estimated population) representing all six major subpopulations of Hawaiian monk seal.

Keywords Major histocompatibility complex · Hawaiian monk seal · Endangered species · Immunogenetics · Immunology

Abbreviations MHC: Major histocompatibility complex · DGGE: Denaturing gradient gel electrophoresis · PCR: Polymerase chain reaction · HLA: Human leukocyte antigen · *Mosc-Mhc I*: *Monachus schauinslandi* MHC class I

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Introduction

Monk seal populations around the globe have suffered dramatic declines in recent years. Subsequent to the extinction of the Caribbean monk seal (*Monachus tropicalis*) (IUCN 1996), the Mediterranean monk seal (*Monachus monachus*) was classified as the world's most endangered phocid population with fewer than 400 individuals remaining in the two most viable groups on the Atlantic coast of North Africa and on the Aegean and Ionian Seas and other smaller populations having either declined dramatically or

disappeared completely (Aguilar 1999). While the situation with Hawaiian monk seal (*Monachus schauinslandi*) is less severe, the population is still listed as endangered, having shown an inability to rejuvenate much above a plateau of 1,400 individuals following the precipitous decline of approximately 60% between 1950 and 1993 (Carretta et al. 2001). The species is primarily located in the Northwestern Hawaiian Islands at six major reproductive sites including Kure and Midway Atolls, Pearl and Hermes Reef, Lisianski and Laysan Islands, and French Frigate Shoals. Smaller numbers of seals exist at Necker, Nihoa, and the Main Hawaiian Islands (Office of Protected Resources, NMFS). In recent years, the population has decreased by about 5% per year with the greatest loss on French Frigate Shoals (Ragen and Lavigne 1997). The causes of population decline probably differ by region but could include habitat disturbance, human intrusion, male-associated mortality to females and younger seals of both sexes, entanglement in nets, inability to forage successfully, shark predation and/or disease (Banish and Gilmartin 1992; Costas and Lopez-Rodas 1998; Harwood 1998; Osterhaus et al. 1997; Takei and Leong 1981; van de Bildt et al. 2000; Wirtz 1968).

The maintenance of genetic diversity in a species is one of the central concepts in conservation biology because it is believed to contribute to both short- and long-term survivabilities (Jimenez et al. 1994). It is hypothesized that a highly variable gene pool may act as a reservoir for adaptation during long-term environmental changes. While previous studies examining genetic diversity of monk seals indicate a dramatically low level of variability (Arnason et al. 1995; Stanley and Harwood 1997; Kretzmann et al. 1997; Pastor et al. 2004), the practical implications of such findings are unknown since they have largely focused on nonfunctional genetic markers. Given that infectious disease may have a role in the present or future decline of this fragile species, we chose to examine genes with immunobiological relevance (Hughes and Nei 1988; Hedrick and Kim 2000). Analysis of the major histocompatibility (MHC) gene complex was a logical approach, as the products are highly polymorphic and encode transmembrane glycoproteins that bind processed foreign peptides and present them to T lymphocytes. This binding is of such importance that it has been said that “all of protective immunity is built from and fuelled by these interactions” (Parham 2005).

The high level of MHC genetic variation found in most free-ranging populations probably reflects an adaptation to previous pathogen exposure (Hughes and Nei 1988; Hedrick and Kim 2000; Hughes and Yeager 1998). Moreover, since the polymorphism present in a population determines the chance that an individual is heterozygous, and heterozygous individuals can present more peptides, the level of polymorphism ultimately influences the repertoire of antigenic determinants to which an individual is capable of recognizing and responding (Hughes and Yeager 1998). It follows that a loss of MHC alleles arising from population decline will result in a net reduction of the range of peptides that can be presented to T lymphocytes, which is, in turn, likely to decrease immunologic vigor of the population (Dyall et al. 2000; Messaoudi et al. 2002). In view of the low numbers of surviving Hawaiian monk seals, the aim of this project was not to undertake an exhaustive characterization of class I MHC genes in this species, but rather to provide an estimate of MHC gene diversity and antigen-binding potential in this fragile species. This was achieved by characterizing monk seal MHC class I expressed gene sequences in a small number of animals and using this information as a basis for genotyping a significant proportion of the remaining Hawaiian population.

Materials and methods

Animals and sample preparation

Venous blood samples were collected from six captive Hawaiian monk seals originating from the French Frigate Shoals. Samples were collected into cell separation tubes (Vacutainer CPT, Becton Dickinson, Franklin, NJ) for rapid isolation of peripheral blood mononuclear cells (PBMCs). These cells were cryopreserved in liquid nitrogen pending RNA isolation. Total cellular RNA was isolated from each blood sample by silica-based gel membranes combined with microspin technology (RNeasy, QIAGEN, Valencia, CA). RNA was stored at −70°C prior to rapid amplification of cDNA ends (RACE) cDNA synthesis.

RACE library construction

cDNA populations were constructed from the PBMC-derived RNA of each animal using SMART RACE cDNA amplification kits (Clontech, Palo Alto, CA) to facilitate amplification of full-length gene transcripts. In brief, 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, and 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 polymerase chain reaction (PCR) and RACE PCR reactions.

Primer design and RACE PCR amplification

Degenerate oligonucleotide primers recognizing conserved flanking regions of each alpha exon of the three principal, established, classical human class I MHC genes (HLA-A, HLA-B, and HLA-C) were designed based on nucleotide sequence alignments of equine, bovine, porcine, canine, feline, and human RNA-derived MHC class I gene sequences (Table 1). These primers were used to amplify short- and medium-length 5' and 3' segments of monk seal class I MHC gene transcripts. PCR amplifications using these degenerate class I primer combinations were per-

Table 1 MHC-class-I-specific primers

Name	Primer sequence	Amplicon	Specificity
PpLA1a1U1	GGCTCCCACTCCCTGAGGT	250 bp; exon 2	Mammalian class I MHC
PpLAa1L250	GGCCTCGCTCTGGTTGTAG		
PpLAa2U270	GGGTCTCACACCYKCCAG	272 bp; exon 3	Mammalian class I MHC
Ppa2L542	GMGCWGCAGSGTCTCYTT		
PpLAU593	GGTCACCCTGAGGTGCTG	164 bp; exon 4	Mammalian class I MHC
PpLAL757	CAGCCCCTCATGCTGCAC		
ZcMS5u2	GGTATCAMCGCAGAGYACG	5' UTR	Pinniped class I MHC
Zc5uL1	GAASATGCAGGTCGTGMTG	5' UTR–exon 1 boundary	California sea lion class I MHC
Zc5ex12	TGGGCGGGCTCYCAACTC	Exons 1–2 boundary	California sea lion class I MHC
Msforuex1	YTCSTCCTGCTGCTGTCGG	Exon 1	Monk seal class I MHC
MSforex12	GAGACCTGRRCGGRCTCC	Exons 1–2 boundary	Monk seal class I MHC

MHC Major histocompatibility

formed on 20 ng of each RACE cDNA library in 50 μl volumes containing 20–60 pmol of each primer, 40 mM Tris–KOH (pH 8.3), 15 mM KOAc, 3.5 mM Mg(OAc)2, 3.75 μg/ml bovine serum albumin (BSA), 0.005% Tween 20, 0.005% Nonidet-P40, 200 μM each dNTP, and 5 U of Advantage 2 Taq polymerase (Clontech). 5' Gene transcript

```
                                                                                          ↓exon 2
                                                                                           1        *        20
Mosc1.1         **********************************************CGGCCCTGGCCGTGACCGAGACCTGGGCGGGCTCCCACTCCCTGAGGTATTTCT
Mosc1.2         **********************************************-----------------------------------------A------------
Mosc1.3         **********************************************-----------------------------------------A------------
Mosc1.4         **********************************************------------------------------------------------------
Mosc1.5         **********************************************-----------------------------------------A------------
Mosc1.6         **********************************************-----------------------------------------A------------
Phvi class I    ******************************************************************************----------------------
Feca FLA A      AGGATGCGGTTCGTGATGTCCCCAACTGTGCTCCTGCTGCTGTTGGG-----------C-C--C------------------------------------
Feca FLA B      *********************CGAACTGTGCTCCTGCTGCTGTTGGG-----------C-C--C------------------------------------
Feca FLA X      **AATGCGGTTCGTGATGTCCCCAACTGTGCTCCTGCTGCTGTTGGG-----------C-C--C------------------------------------
HLA-A           ***ATGGCCGTCATGGCGCCCCGAACCCTCCTCCTGCTACTCTTGGG----------C-----C-----------------------A------------
HLA-B           ***ATGGCCGTCATGGCGCCCCGAACCCTCCTCCTGCTACTCTTGGG----------C-----C-----------------------A------------
HLA-C           *****GCGGGTCATGGCGCCCCGAACCCTCATCCTGCTGCTCTCGGGA---------C----------------CT-----------A------------
HLA-G           AGGATGGTGGTCATGGCGCCCCGAACCCTCTTCCTGCTGCTCTCGGG-------A--C-----------------------------A-----------A
Cafa class I    ***ATGGAGGTGGTGATGCCGCGAGCCCTCCTCGTGCTGCTGTCGG-----------C-----CC----C------------------------------
Eqca class I    ************ATGATGCCCCGAACCTTCCTCCTGCTGCTGTCGGG-------A--C----A------------------------A-----------A

                    *        40         *        60         *        80         *        100        *        120
Mosc1.1         CCACCTCCGTGTCCCGGCCCGGCCGCGGGGAGCCCCGCTTCGTCATCGTCGGCTACGTGGACGACACGCAGTTCGTGCGCTTCGACAGCGACTCTGTCAG
Mosc1.2         A-------------------------------------------------------------------------------------------G-G-CG--
Mosc1.3         -------------------------------------G-A---GGAA-----------------------------------------------------
Mosc1.4         --------------------------------------------------------------------------------------------C-------
Mosc1.5         -------------------------------------G-A---GGAA-----------------------------------------------------
Mosc1.6         -------------------------------------G-A---GGAA-----------------------------------------------------
Phvi class I    A----G-----------------------------------A--GC-----------------------------C---G--------------------
Feca FLA A      A----G-G----------------T----------------A--TC---G-----------------------------G------------G-CCCG-A
Feca FLA B      A----G-G----------------T----------------A--GC---G-----------------------------G------------G-CCCG-A
Feca FLA X      A----G-G----------------T----------------A--TC---G-----------------------------G------------G-CCCG-A
HLA-A           T---A------------------------------------A--GC---G-----------------------------G--T---------G-C-CG--
HLA-B           T---A------------------------------------A--GC---G-----------------------------G--T---------G-C-CG--
HLA-C           A----G----------------------A------------A--GCA--G-----------------------------G------------G-C-CG--
HLA-G           G-G--G-----------------------------------A--GC-A-G-----------------------------G--------------G-CGT-
Cafa class I    A----------------------GCG-----C---------A--GC---------------------------------G------------G-G-C--C
Eqca class I    G----G-----------------------------------A--GC---------------------------------G------------G-C-CG--

                    *       140         *       160         *       180         *       200         *       220
Mosc1.1         TCTGAGGATGGAGCCGCGGGCGCCGTGGGTGGAGCAGGAGGGGCCGGAGTATTGGGACCAGGAGACGCGAAACGCCAAGAACAACGCACAGACTGACCGA
Mosc1.2         --C-------------------------A------------------------------T---------G-T--------G-----------G-TT----
Mosc1.3         ----------------------------A--------------------------------------------------G---G-----------C----
Mosc1.4         -------------------------------------------------------------------------------G---G-----------C----
Mosc1.5         ----------------------------A--------------------------------------------------G---G-----------C----
```

Fig. 1 Nucleotide sequence identity between Hawaiian monk seal class I major histocompatibility (MHC) clones and selected phocine, feline, human, canine, and equine class I sequences. Abbreviations for individual species MHC molecules are as follows: *Mosc* Hawaiian monk seal (*Monachus schauinslandi*), *Phvi* harbor seal (*Phoca vitulina*; GB: PVU88874), *Feca* domestic cat (*Felis catus*), FLA *feline lymphocyte antigen* (GB: UO7670, UO7672, FCUO7674), *HLA* human leukocyte antigen (GB: AF287959, HSUO3907, BCO10542, NM_002127), *Cafa* domestic dog (*Canis familiaris*; GB: DOGMHDL9A), *Eqca* horse (*Equus caballus*; GB: ECMHCB4). The complete sequence for the monk seal (*Mosc1.1*) molecule is illustrated. *Dashed lines* and a *single letter* indicate the amino acid residues identical to and different than those of *Mosc1.1*, respectively

```
Mosc1.6         ----------------------------A--------------------------------------------------G---G-----------C----
Phvi class I    --A-----C------------------------------------------C-------G---------G-GTCT-CG-GG-GC----------T-----
Feca FLA A      --CC---GAA------------------A------------------------------G-A-C-----G-TTTA-TT-G---C--------T-TC----
Feca FLA B      --CC---GAA----------------------------T--------------------G-A-C-----G-TTTA-TT-G---C--------T-TT----
Feca FLA X      --CC---GAA------------------A------------------------------G-A-C--C--G-TTTA-TT-G---C--------T-TT----
HLA-A           C-A-------------------------A-A-----------------------------------A--G--T-TG---GC-C--T--------------
HLA-B           C-A-------------------------A-A-----------------------------------A--G--T-TG---GC-C--T--------------
HLA-C           --CA--AGG--------------------------------------------------G------A-AG--GTA----CG-C-G------G--------
HLA-G           --C------------------------------------------------------AG-------A--G---A-----GC-C--------------A--
Cafa class I    -GG--------------------------------------------------------G-C-------G-C-AT----G-G-C-----G----TT----
Eqca class I    --C-------------------------A----------------------------AG----------G-T--T----GG-----------T-------

                    *       240         *       260         *↓exon 3            *       300         *       320
Mosc1.1         GTGGACCTGCAGACCCTGCGCGGCTACTACAACCAGAGCGAGGCCGGGTCTCACACCATCCAGTTGATGTACGGCTGTGACGTGGGGTCCGACGGGAACC
Mosc1.2         -G-AG----A-CGT------G------------------------------------------AAA----------------------------------
Mosc1.3         ---A-----------GCA-T-C-------------------------------------------A----------------------------------
Mosc1.4         ---A-----------GCA-T-C------------------------------------------------------------------------------
Mosc1.5         ---A-----------GCA-T-C------------------------------------------------------------------------------
Mosc1.6         ---A-----------GCA-T-C------------------------------------------------------------------------------
Phvi class I    ---AG--------A-------------------------------------------T-----AG----------------------C------------
Feca FLA A      ---A-----A-C--GT-T---C--------------------T-------G----A-------AGA-----T-------------ACC-A---C--CG-T
Feca FLA B      ---AG----G---A-AC------A-----T----------GTT----A--G----A-T-----ACA-----T---------A-C---C-TT-T--ACG--
Feca FLA X      T--A-----------GCC-T-C--------------------T----A--G----A-T-----GGA-----T-------------ACC-A---C--CG-T
HLA-A           -A-AG-----G--T-GC--T-C-------------------------T---------------A-------T-----C---------C-G------CG--
HLA-B           -A-AG-----G--T-GC--T-C-------------------------T---------------A-------T-----C---------C-G------CG--
HLA-C           ---AG-----G--A-------------------------------------------C------G------T-----C---C-----C--------CG--
HLA-G           A--A-----------------------------------------A-T---------C------G----ATT-----C---C-------------ACG--
Cafa class I    ---------G-C---------------------------------------------CG----ACC---------------C-----C--------CG--
Eqca class I    ---------A-C--------------------------------A-------------------AC-----T-----C---------C-A------CG--

                    *       340         *       360         *       380         *       400         *       420
Mosc1.1         TCCTCCGCGGGTACAGGCAGGACGCCTACGACGGCGCGGATTACATCGCCCTGAACGAGGACCTGCGCTCCTGGACCGCGGCGGACACGGCGGCGCAGAT
Mosc1.2         -T------------GA----TT---------------------------------------------------------------------------C--
Mosc1.3         -T------------GA----TT---------------------------------------------------------------------------C--
Mosc1.4         ----------------------------------------------------------------------------------------------------
Mosc1.5         ----------------------------------------------------------------------------------------------------
Mosc1.6         ----------------------------------------------------------------------------------------------------
Phvi class I    --------------G-----TGG-----------------------------------------------------------------------------
Feca FLA A      ----------------T------T----T------AA---------------------------------------------------C--C--------
Feca FLA B      ----------------T---ATG-----------------------------------------------------------------C-----------
Feca FLA X      ----------------T---TTG------------AA---------------------------------------------------C-----------
HLA-A           --------------CA-------------------AA-------------T-------------------T----------------T------T-----
HLA-B           --------------CA-------------------AA-------------T-------------------T----------------T------T-----
```

Fig. 1 (continued)

amplifications contained 20 pmol of a 3' primer along with 1–5 pmol of the Universal Primer Mix (UPM; SMART RACE cDNA amplification kit, Clontech). 3' Gene transcript amplifications contained 20 pmol of a 5' primer along with 1–5 pmol of the UPM (SMART RACE cDNA amplification kit, Clontech). PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA). Reaction specificity was optimized using a touchdown method 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, 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, 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, WI). Following transformation, growth, and blue-white selection in competent cells (SE DH5α competent cells, Life Technologies Inc, Rockville, MD), DNA from positive clones was isolated. Nucleotide sequences of both strands were determined by dideoxy nucleotide methodology using an automated sequencer (Model 373, Applied Biosystems, Foster City, CA). Nucleotide sequences of these amplicons were compared using the Align and Contig sequence alignment software programs (Vector NTI, Informax Inc., North Bethesda, MD).

For each of the class I gene products, regions of sequence homology were identified and used in a diverse primer design strategy directed at amplifying all near-full-length monk seal class I MHC gene transcripts present in the samples. This strategy entailed aligning Hawaiian-monk-seal-derived sequences with MHC class I sequences from other species and designing oligonucleotides for each of several conserved regions scattered throughout the length of the gene. This enabled primer design for sequences in all five recognized class I MHC exons as well as within the 5' and 3' untranslated regions (UTRs). These primers were then used in various combinations with each other to amplify as many near-full-length monk seal-specific MHC class I sequences as possible from each sample. To control for the inadvertent amplification of genomic DNA sequences, primers from different exons were paired.

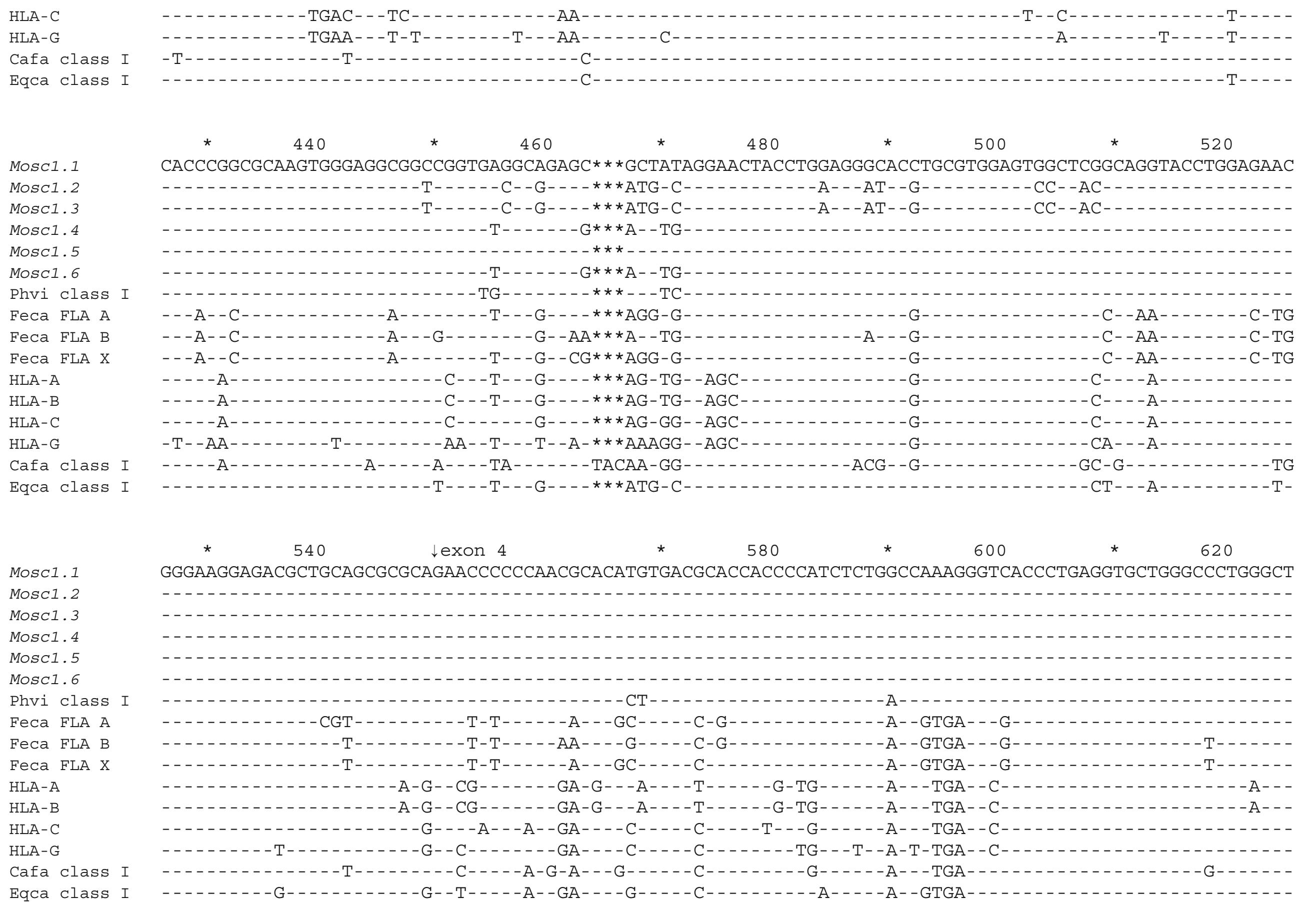

Fig. 1 (continued)

With the exception of oligonucleotide composition and concentrations, PCR conditions were identical to those described above. Amplification reactions contained 20 pmol of both 3′ and 5′ primers, and reaction specificity was optimized using a touchdown method that started with 5 cycles at 94°C for 5 s, and 68°C for 3 min, followed by 5 cycles at 94°C for 5 s, 62°C for 10 s, and 72°C for 3 min, followed by 25 cycles at 94°C for 5 s, 58°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

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

Validation of DGGE-based class I MHC genotyping by examining multiple pinniped species

An adapted denaturing gradient gel electrophoresis (DGGE; Aldridge et al. 1998) was used to examine the degree of class I MHC nucleotide variation between the six captive monk seals and other pinniped species. Peripheral blood leukocytes were isolated from venous blood samples collected from California sea lions (*Zalophus californianus*; n=3) and Pacific harbor seals (*Phoca vitulina*; n=3). Total cellular RNA was isolated as described above 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; samples were stored at −20°C until further analysis.

```
                    *        640        *        660        *        680        *        700        *        720    
Mosc1.1         TCTACCCTGCGGAGATCACACTGACCTGGCACCACGATGGGGAGGACCTGACCCAGGACACAGAGCTTGTGGAGACCAGGCCTGCAGGGGATGGGACCTT
Mosc1.2         -----------------------------------A----------------------------------------------------------------
Mosc1.3         -----------------------------------A----------------------------------------------------------------
Mosc1.4         -----------------------------------A----------------------------A-----------------------------------
Mosc1.5         ----------------------------------------------------------------------------------------------------
Mosc1.6         -----------------------------------A----------------------------A-----------------------------------
Phvi class I    -------------------C----T---------------------------------------------------------------------------
Feca FLA A      -------------------C-----------G-GT------C------AC-----------------------------------G--A-----------
Feca FLA B      -------------C-----C-----------G-GT-------------AC---------G-------------------------G--A-----------
Feca FLA X      ----------A--------C-----------G-GT------C------AC---------G-------------------------G--A-----------
HLA-A           -------------------------------G-GG-------------A------------G--------------------------------A-----
HLA-B           -------------------------------G-GG-------------A------------G--------------------------------A-----
HLA-C           -------------------------------G-GG-----C-------AA--T--------C--------------------A-----A-----A-----
HLA-G           ------------------T------------G-GG-------------A----------GTG-----C--------------------------A-----
Cafa class I    -------------------C-----------G-GG-------------A----------------G-------C--A-----------A-----------
Eqca class I    -------------------T-----------G-GT--C---------G-------------G---T-------------------------C-A------

                    *        740        *        760        *        780        *        800        *        820    
Mosc1.1         CCAGAAGTGGGCGGCTGTGGTGGTGCCTTCTGGACAGGAGCAGAGATACACGTGCCATGTGCAGCATGAGGGGCTGCCCGAGCCCCTCACCTTGAGATGG
Mosc1.2         ----------------------------C-----------------------------------------------------------------------
Mosc1.3         ----------------------------C-----------------------------------------------------------------------
Mosc1.4         ----------------------------------------------------------------------------------------------------
Mosc1.5         ----------------------------------------------------------------------------------------------------
Mosc1.6         ----------------------------------------------------------------------------------------------------
Phvi class I    -------------------------------------------------------------------------------A-----A---------T----
Feca FLA A      ----------------------------------G-------------------------------CA-----------------A---A----------
Feca FLA B      ----------------------------------G-------------------------------C------------------A-----C--------
Feca FLA X      ----------------------------------G-------------------------------C------------------A--------------
HLA-A           -------------T-------------------------------------C--------------------T------A-----------C--------
HLA-B           -------------T-------------------------------------C--------------------T------A-----------C--------
HLA-C           ------------A---------------------G-A-----------------------------C-----------A------------C--------
HLA-G           ------------A---------------------G-------------------------------------------G----------TGC--------
Cafa class I    ---------------C-----------------------------------------C--C-----------------G-----TA----GCG-------
Eqca class I    ----------------------------------G-------------------------------C-----------T------G-----C--------

                ↓exon 5             *        860        *        880        *        900        *        920    
Mosc1.1         CAGCCACCTCCTCAGCCCACCATCCCCATCATCTGGATCATTGCTGGTGTGGTTCTCCT***TG******CGGTCACTGTGGCCATGACTGGAGCTGTGA
Mosc1.2         -----------------------------------------------------------***--******------------------------------
Mosc1.3         -----------------------------------------------------------***--******------------------------------
Mosc1.4         -----------------------------------------------------------***--******------------------------------
Mosc1.5         -----------------------------------------------------------***--******------------------------------
Mosc1.6         -----------------------------------------------------------***--******------------------------------
Phvi class I    G----G----***------------------C-------------------A-------***-C******T---------------TTT-----------
```

Fig. 1 (continued)

New primer pairs, recognizing the flanking regions of the putative peptide-binding sites, were designed to amplify a 230-bp fragment of pinniped class I MHC exon 2 and a 234-bp fragment of pinniped class I MHC exon 3. Sensitivity of the DGGE analysis was enhanced with the addition of a GC-clamp to one of each primer pair as previously described (Sheffield et al. 1989). Optimal conditions for class I MHC gene 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 pinniped class I MHC sequences between individuals. The gel was visualized by staining with GelStar nucleic acid stain (BMA products, Rockland, ME) according to manufacturers' specifications. Bands representing individual class I MHC gene sequences were excised, reamplified, and the nucleotide sequence determined as previously described (Knapp et al. 1997; Aldridge et al. 1998).

DGGE-based class I MHC genotyping of the broader Hawaiian monk seal population

In view of the practical difficulties of performing a comprehensive examination of all class I genes in each individual, we decided to examine diversity in the broader monk seal population by performing multiple class I MHC gene comparisons between individuals using deviating primer sets. These additional primer pairs, recognizing variations in the flanking sequences of exons encoding the putative peptide-binding site, were used to in further PCR and DGGE-based genotyping techniques to compare different combinations of class I MHC genes between individuals from the broader monk seal population. Genomic DNA was isolated from the nasal swabs of 80 free-ranging animals representing the major subpopulations of Hawaiian monk seals (Kure and Midway Atolls, Pearl and Hermes Reef, Lisianski and Laysan Islands, and French Frigate Shoals). Exon-2- and exon-3-specific PCR was performed using the additional primer sets described

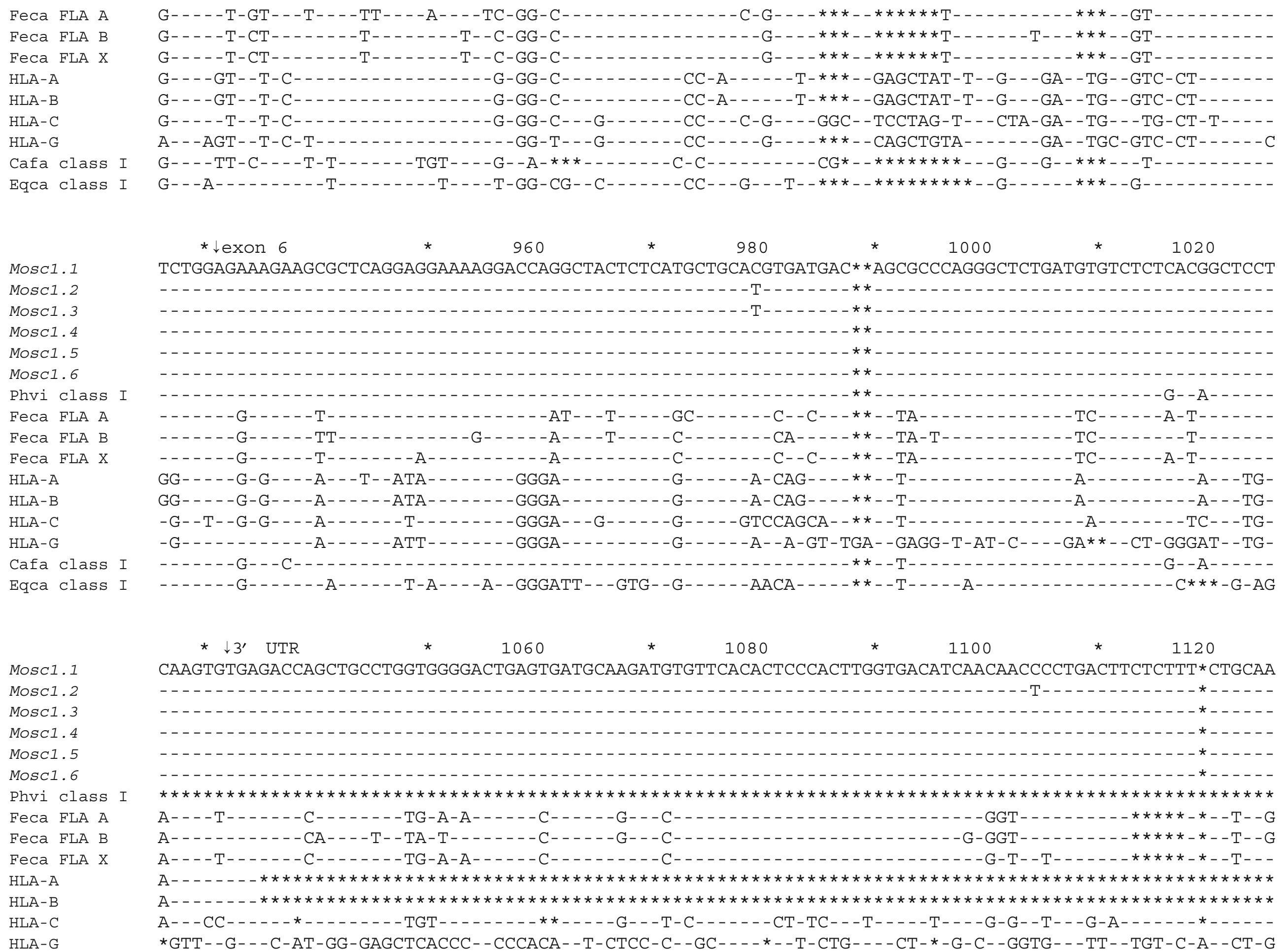

Fig. 1 (continued)

above, under newly optimized buffer conditions and thermal cycling parameters to those optimized and validated for the class I MHC genotyping of the captive monk seals, California sea lions, and Pacific harbor seals. Similarly, the procedures and conditions for DGGE band separation for these genomic samples were accomplished as described above for the cDNA-derived sequence genotyping.

Results

Characterization of Hawaiian monk seal class I MHC cDNA clones

Clones containing near-full- and full-length *Mosc* (*M. schaunslandi*) MHC class I (*Mosc-Mhc I*)-like sequences were obtained from the RACE cDNA products of each animal examined. The nucleotide and deduced amino acid sequence of the 1,019-bp products were typical of transcripts from mammalian major MHC class I genes. Five distinguishable full-length exons were identified spanning 270 (exon 2), 276 (exon3), 276, 96, and 99 bp, respectively. The transcripts were characterized as *Mosc-Mhc I* based on alignments with human, feline, equine and canine, and harbor seal MHC class I sequences (Figs. 1 and 2). The near-full-length transcripts showed high homology with previously described full-length sequences from these species harbor seal 90%, feline (FLA-A 82%, FLA-B 81.9%, FLA-C 82.6%), equine 83%, canine 84%, human (HLA-A 82%, HLA-B 82%, and HLA-C 81%; Figs. 1 and 2). However, in contrast to the HLA-based class I sequence classification system that has been adopted for species such as the domestic cat, there were no motifs identified in the monk seal sequences that could be used to subclassify them as HLA-A, HLA-B, or HLA-C orthologues.

The largest *Mosc-Mhc I* gene products encoded derived molecules of 339 amino acids but, due to the primer design, the 5' terminus of the molecules were incomplete, missing all but a 9-amino-acid fragment of the leader sequence. The remainder of the near-full-length molecules consisted of a 90-amino-acid $\alpha1$ domain, a 92-amino-acid $\alpha2$ domain, a 92-amino-acid $\alpha3$ domain, a 32-amino-acid transmembrane domain, and a 33-amino-acid cytoplasmic tail domain (Fig. 3).

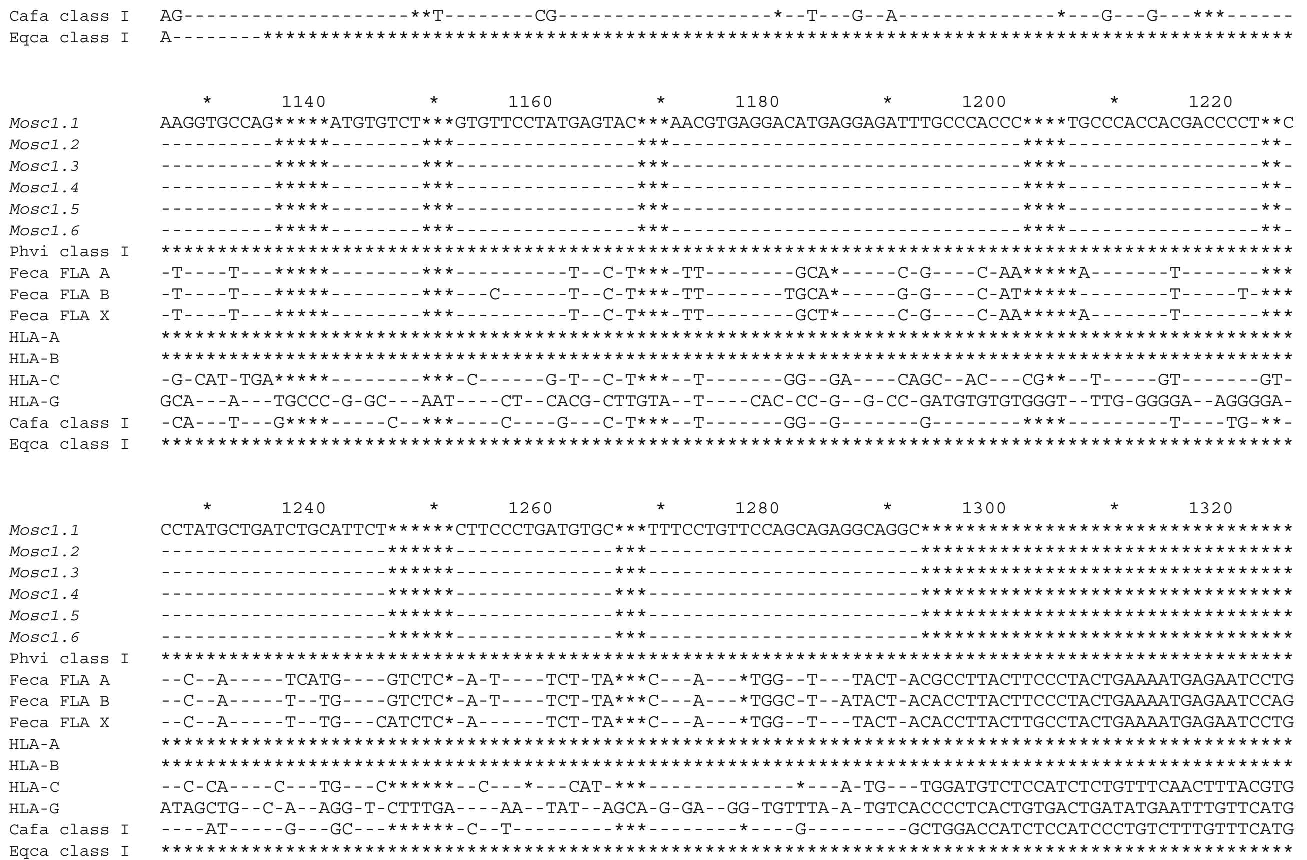

Fig. 1 (continued)

Variation between *Mosc-Mhc I* sequences

To maximize the identification of all *Mosc-Mhc I* sequences present in each of the animals, the products of multiple primer combinations were examined. From the six original individual monk seals, a total of six different *Mosc-Mhc I* sequences were identified (Fig. 1). The number of class I sequences identified per individual ranged from 3 to 4, supporting the likelihood that the combination of *Mosc-Mhc I* primers amplified products from at least two separate loci. None of the sequences exhibited features compatible with pseudogenes. Three of the amplified sequences (hereafter named *Mosc-Mhc I.1*, *Mosc-Mhc I.2*, and *Mosc-Mhc I.3*) were present in five of the six monk seals and were more different from each other than the three sequences (*Mosc-Mhc I.4*, *Mosc-Mhc I.5*, and *Mosc-Mhc I.6*) that were only amplified from one monk seal each (Figs. 1 and 2; Table 2). The variability between these three more common sequence patterns was significant with a total of 68 polymorphisms. Of these 68 polymorphisms, the majority were concentrated in exon 2 (36/68) and exon 3 (27/68), with the remainder distributed between exon 4 (3/68), exon 6 (1/68), and the 3'UTR (1/68; Fig. 1 and Table 2).

Mosc-Mhc I.1, *Mosc-Mhc I.2*, and *Mosc-Mhc I.3* sequences could be distinguished from one another by unique sequence-specific codon motifs at nt205-207, nt220-22, nt229-31, nt244-46, and nt289-91 (Fig. 1). Apart from these unique identifiers, *Mosc-Mhc I.1* showed more distinct motifs (n=20) than either *Mosc-Mhc I.2* (n=9) or *Mosc-Mhc I.3* (n=6). *Mosc-Mhc I.4*, *Mosc-Mhc I.5*, and *Mosc-Mhc I.6* were comprised largely of motifs from *Mosc-Mhc I.1*, *Mosc-Mhc I.2*, and *Mosc-Mhc I.3* and only contained unique motifs at nt118-120 (*Mosc-Mhc I.4*) and nt454-56, nt463-65, nt469-71, and nt688-690 (*Mosc-Mhc I.5 and Mosc-Mhc I.6*). In all cases, the sequences were confirmed by performing a minimum of ten independent PCR reactions for each primer combination in each animal, by examining multiple clones, and by sequencing each clone in both directions, in compliance with HLA nomenclature rules (Bodmer et al. 1999).

Despite the significant sequence differences between the three most common *Mosc-Mhc I* transcripts, no consistent pattern of sequence heterogeneity could be identified between individuals, suggesting the presence of three recurring alleles. In fact, apart from two nonsynonymous, single nucleotide polymorphisms (*Mosc-Mhc I.1* nt900 and nt989 in one individual), the *Mosc-Mhc I.1*, *Mosc-Mhc I.2*, and *Mosc-Mhc I.3* transcript sequences were identical for all five animals in which they were identified. For this reason and since exhaustive MHC class I sequencing was not performed in any of the animals, it was difficult to assign a specific *Mosc-Mhc I* haplotype to each individual, and therefore, a more rudimentary genotype classification scheme was employed (Table 2).

Comparison of the deduced amino acid sequences of the three most common *Mosc*-*Mhc I* transcripts with one another indicated that the majority of the polymorphisms (49/65) represented nonsynonymous nucleotide substitutions (Table 3), and that the great majority of these were concentrated in the α1 (27/49) and α2 (19/49) domains forming the putative MHC class I peptide-binding region (Yuhki and O'Brien 1988). The most significant heterogeneity of *Mosc*-*Mhc* I amino acid sequences within individuals was observed at two sites between residues 62 and 83 and 150 and 163. When the relative positions of these and other polymorphic residues were compared to those described in other species, a noticeable proportion (n=9) coincided with polymorphic regions (n=13) in humans (residues 9, 62, 66, 70, 77, 80, 114, 116, and 157; Yuhki and O'Brien 1988). Along with these patterns, phylogentic analysis distributed the different sequences in separate clades showing the close structural relationship of these genes to each other and supporting the concept of a common evolutionary origin. Furthermore, clear phylogenetic relationships between the marine and terrestrial carnivores examined were indicated by the high bootstrap values (Fig. 3).

Validation of degree of *Mosc*-class I MHC sequence variation between individuals

To further examine the *Mosc*-class I sequence variability between individuals, the mobility patterns of partial length *Mosc*-class I sequences from the six captive animals were examined by DGGE. To maximize the chances of identifying MHC sequence polymorphism between individuals, primer sets spanning each exon encoding the putative peptide-binding domains of the MHC class I molecule were used. As a result, this assay entailed two comparisons (exons 2 and 3, respectively) for each monk seal. The banding pattern was identical for each of the captive monk seals, both in number (exon 2, three distinct bands; exon 3, two distinct bands) and in migration properties (Fig. 4).

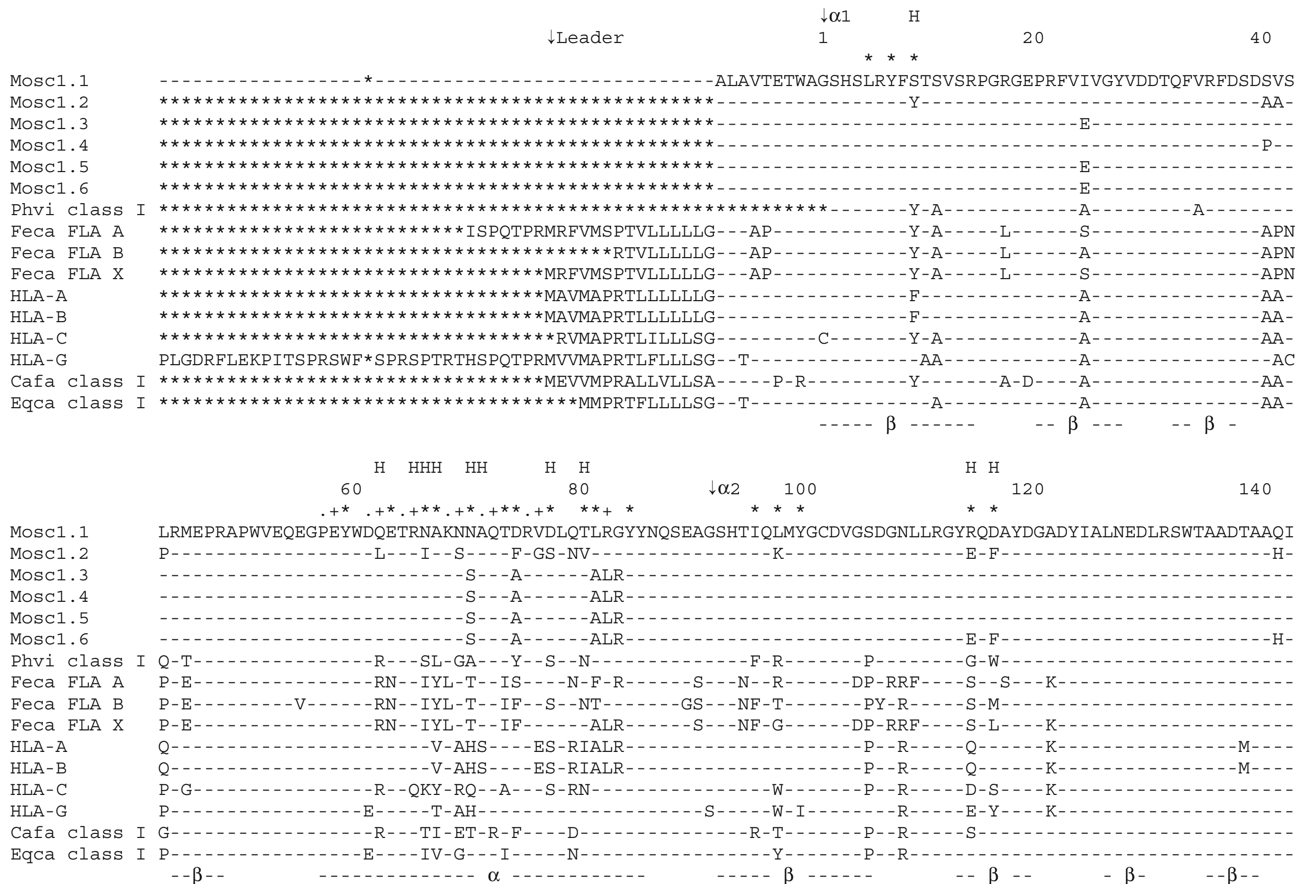

Fig. 2 Amino acid sequence identity between Hawaiian monk seal class I MHC clones and selected phocine, feline, human, canine, and equine class I sequences. Abbreviations for individual species MHC molecules are as in Fig. 1. The complete sequence for the monk seal (*Mosc1.1*) molecule is shown. *Dashed lines* and a *single letter* indicate the amino acid residues identical to and different than those of *Mosc1.1*, respectively. Regions representing two α-helix and eight β-sheet coding regions in the feline are indicated below the sequences (Yuhki and O'Brien 1988). *Asterisk* (*), *plus* (+), and *dot* (.) symbols above the sequence of *Mosc1.1* indicate amino acids toward the antigen-binding site, on an α-helix pointing up, and on an α-helix pointing away from the antigen-binding site and TCR recognition site, respectively, according to the homologous structure of the human HLA-A2 molecule. *H above the sequence* indicates polymorphic sites defined in human MHC class I molecules

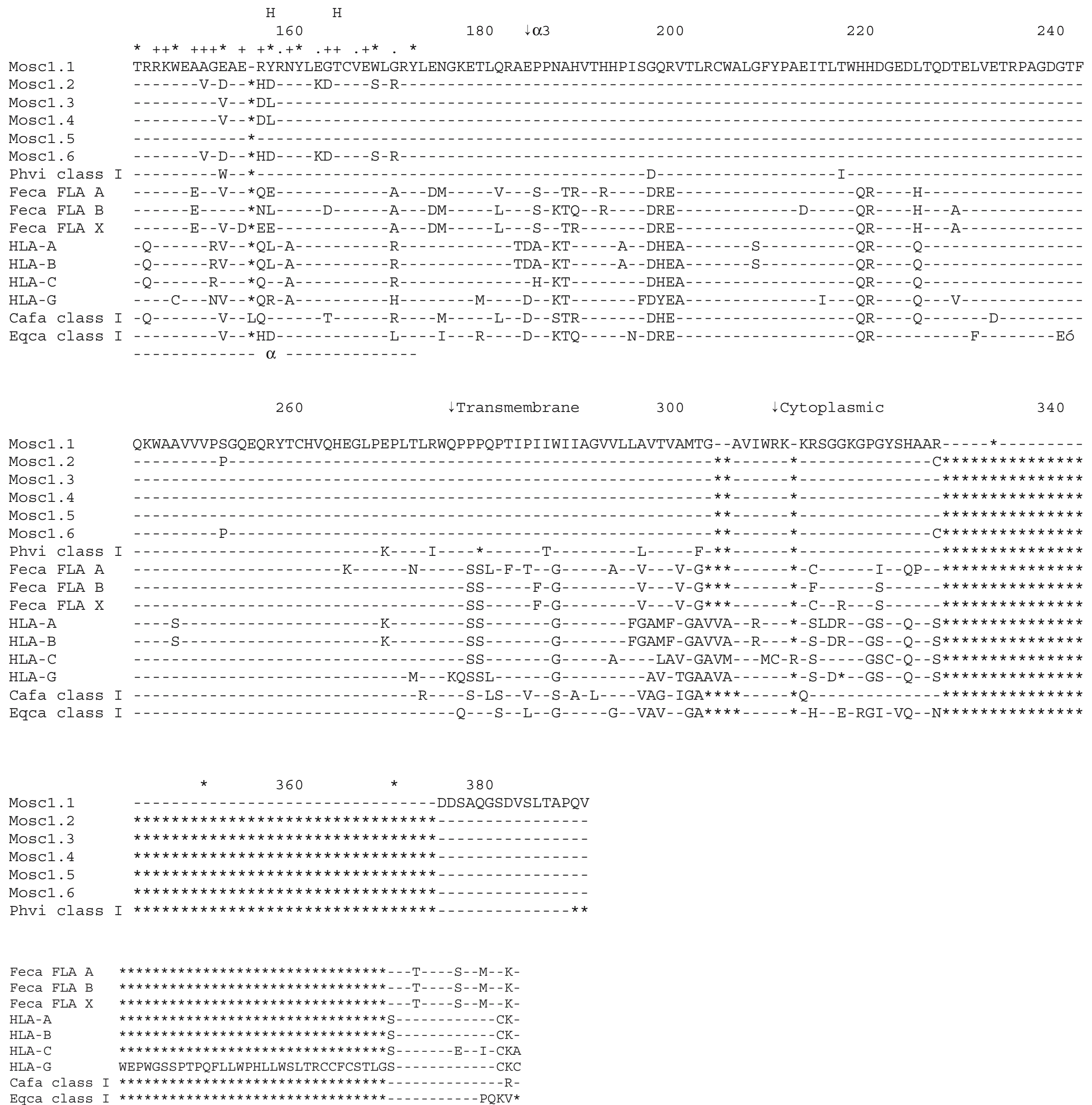

Fig. 2 (continued)

While this finding provided strong evidence for *Mosc*-class I 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 *Mosc*-class I sequences were identical between individuals and thereby substantiated the results of the RACE fragment sequencing. Furthermore, to ensure that the primers used were able to identify class I MHC gene sequence variability in closely related species, equivalent and parallel reactions were performed in each of three California sea lions and three Pacific harbor seals. The gel migration pattern observed in the monk seals was strikingly different from the other pinnipeds (Fig. 4). Not only were there several gel bands for each of the sea lions but, more importantly, each individual exhibited a specific fingerprint pattern of band migration as well. Comparable results were observed in the harbor seals (data not shown). By a similar process of gel extraction, reamplification, and direct sequencing, the variability in class I MHC transcript genotype revealed by this technique was confirmed in both the California sea lions and the Pacific harbor seals.

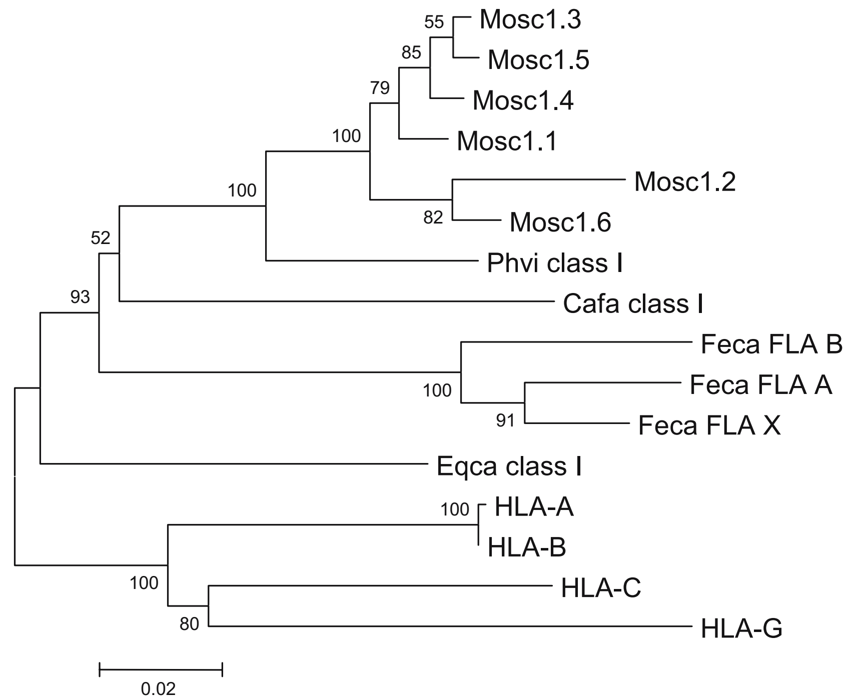

Fig. 3 Neighbor-joining tree analysis using 1,020-bp nucleotide sequences of $\alpha1$ domain, $\alpha2$ domain, $\alpha3$ domain, transmembrane region, and cytoplasmic tail in MHC class I genes based on Kimura's two-parameter distance. *Numbers* are bootstrap percentages (out of 500 iterations) in support of each node. Abbreviations are as in Fig. 1

Estimation of class I MHC gene sequence diversity in Hawaiian monk seal population

To examine *Mosc*-class I diversity in 80 additional monk seals, multiple primer combinations were used for additional DGGE-based genotyping. These primer combinations were selected to maximize the potential for amplifying as many representative class I MHC sequences as possible. Despite the extensive nature of these investigations, whatever primer pair combination was used, within each assay, the banding patterns from free-ranging animals were identical to each other and to the captive monk seals, both in number (exon 2, three distinct bands; exon 3, two distinct bands) and in migration properties (Fig. 4). However, the utility of using multiple primer pairs was

Table 2 *Mosc*-MHC class I transcription genotype of six captive Hawaiian monk seals (*Monachus schaunslandi*), determined from peripheral blood mononuclear cell-derived cDNA

	Mosc-Mhc I.1	*Mosc-Mhc I.2*	*Mosc-Mhc I.3*	*Mosc-Mhc I.4*	*Mosc-Mhc I.5*	*Mosc-Mhc I.6*
HMS79	X	X	X	X		
HMS81	X		X		X	
HMS83	X	X	X			
HMS85	X	X	X			X
HMS86	X	X	X			
HMS87	X	X	X			

Mosc Monachus schaunslandi

Table 3 Distribution of synonymous/nonsynonymous nucleotide substitutions between the three most common *Mosc*-MHC class I transcripts

Type of substitutions	Leader peptide	$\alpha1$ Domain	$\alpha2$ Domain	$\alpha3$ Domain	TM	CYT
Nonsynonymous	0	27	19	2	0	1
Synonymous	0	9	6	1	0	0

TM Transmembrane domain, *CYT* cytoplasmic tail domain

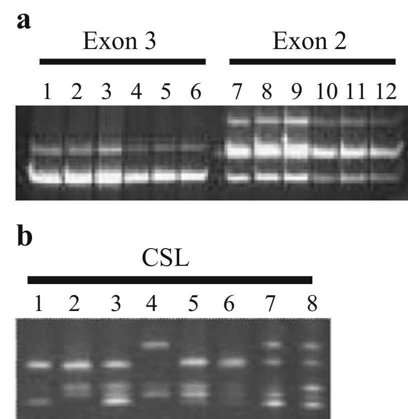

Fig. 4 Paucity of class I MHC variation in the Hawaiian monk seal demonstrated using polymerase chain reaction (PCR) coupled with denaturing gradient gel electrophoresis (DGGE). **a** Identical MHC class I genotype patterns for exon 2 (three bands) and exon 3 (two bands) in six different monk seals. **b** MHC class I exon 2 variation in individual California sea lions. Sample source: peripheral blood-derived cDNA (**a** and **b**)

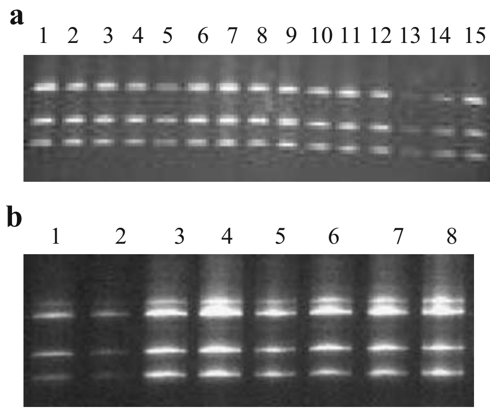

Fig. 5 Identical exon 2 banding pattern from randomly selected monk seals suing two different primer pairs (**a** n=15, **b** n=8). Sample source: nasal swab genomic DNA (**a** and **b**)

shown by the differences in migration patterns between assays (Fig. 5). The homogeneity of the *Mosc*-class I genes in the population was confirmed by gel extraction, re-amplification, and direct sequencing of the separated bands (data not shown).

Discussion

Traditionally, investigations on declining wildlife populations have focused on lack of genetic variability at neutral genetic markers (Kretzmann et al. 1997). However, in many species, conservation genetics is expanding to include analysis of biologically relevant gene systems, such as the MHC (Aguilar et al. 2004; Bowen et al. 2002, 2004, 2005). Such an approach is particularly relevant to marine species whose chemical and microbial environment is continually influenced by anthropogenic encroachment, increasing their risk of exposure to novel pathogens (De Swart et al. 1996; Harvell et al. 1999). The variability and immunological importance of the genes in the MHC complex make them ideal for identifying biologically relevant genetic diversity, particularly in relation to population viability.

This study was initiated by examining cDNA from six captive Hawaiian monk seals originating from the French Frigate Shoals. Characterization of MHC class I genes from these animals revealed a total of six unique full-length classical MHC class I gene transcripts showing high homology with MHC class I from other species; three sequences were common to most animals. Efficacy of the degenerate primers used to amplify these MHC class I transcripts was validated by analogous studies in California sea lions and harbor seals that identified substantial class I MHC variability in just the few animals examined. The extremely limited MHC class I sequence variation in the monk seals resembles the lack of diversity associated with nonclassical MHC genes in other species. However, the possibility that we inadvertently amplified nonclassical MHC genes is unlikely, as identical results were obtained using multiple primer pair combinations from both translated and untranslated gene regions. Furthermore, patterns of synonymous and nonsynonymous nucleotide substitutions indicated strong selection pressures focused on the putative peptide-binding regions.

Genetic analysis of the monk seal MHC was expanded using DGGE techniques in combination with primer sets spanning each exon encoding the putative peptide-binding domains of the MHC class I. The resultant DGGE banding pattern was identical for each of the captive monk seals. Sequencing of these bands confirmed this sequence homology among individuals. Eighty additional free-ranging monk seals, representing all major subpopulations (~6.7 % of the total population), were similarly analyzed and yielded identical results.

These data provide strong evidence that the surviving Hawaiian monk seal population has an unprecedented uniformity in MHC class I genes, the sequence variation of which is essential to maintaining immunological vigor and population health. This information is important, particularly in view of recent pathogen-associated mass mortalities in seals (van de Bildt et al. 2000) and given the recent extinction of the closely related Caribbean monk seal (IUCN 1996). While the origin(s) of this unprecedented lack of immunogenetic diversity is unknown, the congruence of these results with those from studies using neutral gene markers supports the view that demographic factors are primarily responsible. Whatever the inciting cause, in view of the fact that in most species, genetic factors impact the population prior to extinction (Spielman et al. 2004) and that recent studies have established a correlation between MHC polymorphism and the generation of a diverse and effective immune cell repertoire (Dyall et al. 2000; Messaoudi et al. 2002), these findings raise additional concern regarding the Hawaiian monk seal population. However, before strong conclusions regarding the immunogenetic status of these animals can be made, expansion of these studies to include an analysis of the companion MHC class II genes is required. The combination of information arising from examinations of these two functionally important gene families will help in future predictions regarding the immunological relevance of these findings.

Acknowledgements These studies were made possible by a generous grant from the Morris Animal Foundation. The authors thank Dr. Les Dalton, Dr. Jim McBain, and the staff of Sea World of San Antonio, TX, and Dr. Robert Braun (contract veterinarian) and staff of the Pacific Islands Fisheries Science Center, National Oceanic and Atmospheric Administration for the collection of samples from captive and wild seals, respectively. The collection of samples for this research was authorized by Marine Mammal Permit 848-1335 and by permit issued by Hawaiian Islands National Wildlife Refuge, U.S. Fish and Wildlife Service, U.S. Department of Interior. Experiments described in this paper conform to the current laws in the United States regarding the care and use of vertebrate animals for research.

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