

Characterization of major histocompatibility complex class I and class II genes from the Tasmanian devil (*Sarcophilus harrisii*)

Hannah V. Siddle · Claire Sanderson · Katherine Belov

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Abstract The Tasmanian devil (*Sarcophilus harrisii*) is currently threatened by an emerging wildlife disease, devil facial tumour disease. The disease is decreasing devil numbers dramatically and may lead to the extinction of the species. At present, nothing is known about the immune genes or basic immunology of the devil. In this study, we report the construction of the first genetic library for the Tasmanian devil, a spleen cDNA library, and the isolation of full-length MHC Class I and Class II genes. We describe six unique Class II β chain sequences from at least three loci, which belong to the marsupial Class II DA gene family. We have isolated 13 unique devil Class I sequences, representing at least seven Class I loci, two of which are most likely non-classical genes. The MHC Class I sequences from the devil have little heterogeneity, indicating recent divergence. The MHC genes described here are most likely involved in antigen presentation and are an important first step for studying MHC diversity and immune response in the devil.

Keywords Tasmanian devil · Marsupial · MHC · Transmissible Tumour · Conservation

The Tasmanian devil is the largest remaining marsupial carnivore. Its geographic range once extended across

mainland Australia, but it became confined to Tasmania at least 400 years ago because of the introduction of the dingo and human impacts (Archer and Baynes 1972; Jones et al. 2003). The Tasmanian devil population is believed to have undergone population crashes over the last 150 years, in the 1850s and at the beginning of the twentieth century (Bradshaw and Brook 2005), and has low diversity at microsatellite loci (Jones et al. 2004). The Eastern Tasmanian devil populations are suffering from a disease outbreak in the form of a contagious tumour, the devil facial tumour disease (DFTD; Pearse and Swift 2006). DFTD has been compared to the canine transmissible venereal sarcoma (CTVT), a naturally occurring tumour passed between dogs during coitus (Murgia et al. 2006). CTVT down-regulates the expression of major histocompatibility complex (MHC) genes that are responsible for the recognition of foreign antigens. Down-regulation of MHC expression allows CTVT to escape recognition by the host immune system. Given the low levels of genetic diversity in the Tasmanian devil population (Jones et al. 2004), it is also possible that devils lack diversity at MHC loci, allowing the tumour to pass between individuals undetected by the immune system. At present, nothing is known about the devil immune system, thus characterization of immune genes, including the MHC genes, is a crucial first step for understanding and controlling this unique disease.

The MHC is a large, multi-gene family found in all jawed vertebrates. MHC genes encode molecules that recognize foreign antigens and present them to T cells, resulting in an adaptive immune response (Doherty and Zinkernagel 1975). Antigen-presenting MHC genes are classified according to structure and function as Class I or Class II. Class I molecules consist of an α chain and an associated β 2-microglobulin and are expressed on the surface of all cells (Bjorkman and Parham 1990). Class II MHC molecules

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H. V. Siddle · C. Sanderson · K. Belov (✉)
Centre for Advanced Technologies in Animal Genetics
and Reproduction, Faculty of Veterinary Science,
University of Sydney,
RMC Gunn B19,
Sydney, NSW 2006, Australia
e-mail: kbelov@vetsci.usyd.edu.au

consist of an α and β chain, encoded by separate genes, and are expressed on the surface of Antigen Presenting Cells, such as monocytes and macrophages (Villadangos 2001). Class I molecules present endogenous antigens to cytotoxic T cells, while Class II molecules present exogenous antigens to T cells. MHC genes evolve rapidly and, in some cases, have taken on more divergent functions, often unrelated to antigen presentation (Stroynowski and Lindahl 1994). These genes are non-classical MHC and have low polymorphism and tissue-specific expression patterns compared to classical MHC genes.

MHC genes are among the most polymorphic in the vertebrate genome (Hedrick and Thomson 1983). This polymorphism is concentrated in the peptide binding region (PBR) of the Class I and Class II molecules and enables a population to respond to a broad range of pathogens (Hughes and Nei 1988). To date, the assessment of diversity at MHC loci in marsupials using allele sequencing has been restricted to the South American opossum (*Monodelphis domestica*, herein referred to as opossum; Gouin et al. 2006). Diversity has been assessed in Australian marsupials using only MLR and RFLP analysis because of the limited MHC sequence data available (McKenzie and Cooper 1994; Stone et al. 1996).

Class II β chain sequences have been isolated from Australian and American marsupials (Belov et al. 2006; Browning et al. 2004). These sequences, with the exception of the non-classical DM genes, belong to novel gene

families, DB, DA and DC, not found in eutherians (Belov et al. 2006, 2004; O'HUigin et al. 1998). The marsupial DA and DB Class II genes in marsupials are most likely responsible for antigen presentation (Belov et al. 2004). Class I genes have been isolated in a range of marsupials (reviewed in Siddle et al., submitted paper) and, like in eutherian mammals, form lineage specific clades because of rapid evolution (Houlden et al. 1996). The most extensive characterization of Class I genes has occurred in the opossum (Belov et al. 2006).

We have made the first genetic library for the Tasmanian devil, a spleen cDNA library, and have isolated and characterized full-length MHC Class I and Class II genes. Total RNA and DNA was extracted from spleen, blood, kidney and liver from a single, male Tasmanian devil (Individual I) using Trizol (Invitrogen). DNA was extracted from the blood of five additional devils using the DNeasy blood kit (Qiagen). A spleen cDNA library was constructed using the Clontech cDNA library construction kit (Clontech). The full-length cDNA was ligated into the λ Triplex2 vector. Ligations were packaged into lambda phage using the Gigapack III XL gold pack (Stratagene). After titration, 2×10^6 pfu were obtained, indicating a representative library.

A 242-bp fragment of the Class II DAB (exon 3) was amplified from devil genomic DNA, gel purified (GeneClean, Mobio), cloned into the pGEM-T easy vector system (Promega) and sequenced. The sequence was confirmed as a

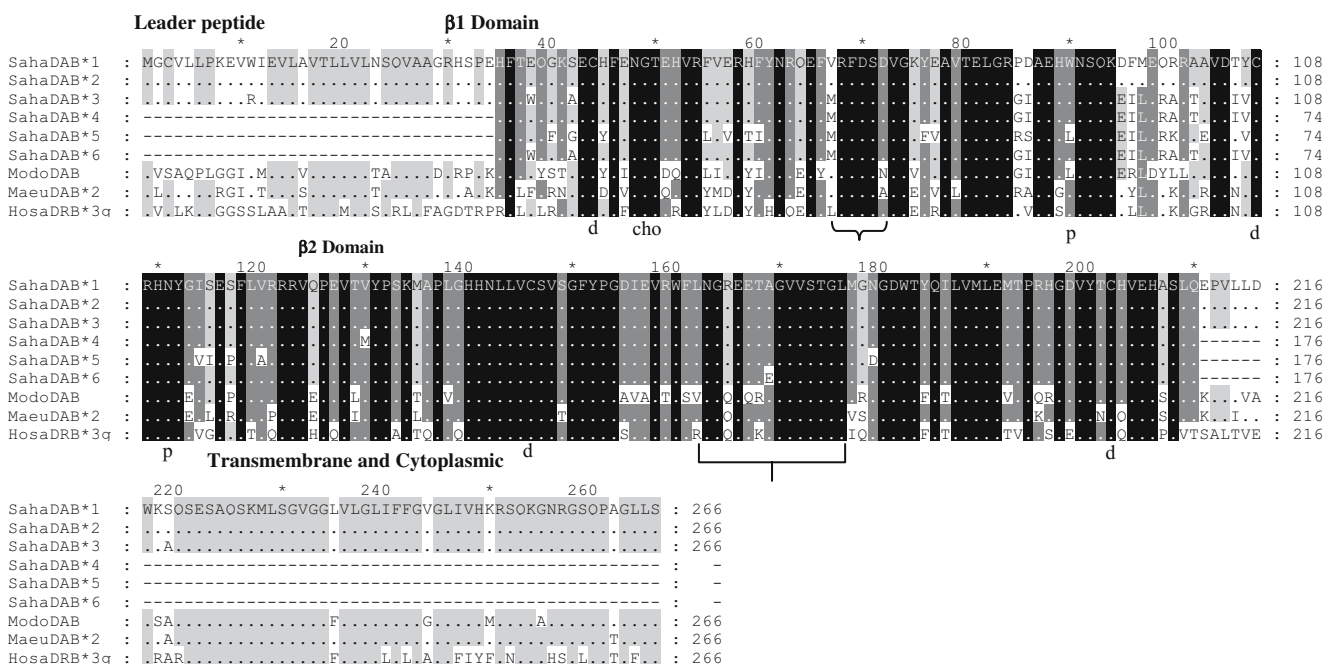


Fig. 1 Amino acid alignment of MHC Class II sequences from the Tasmanian devil, opossum, tamar wallaby and humans. Dots are conserved residues, and dashes are missing sequences. Residues of interest are indicated below the sequence as: *d*, conserved cysteine

residues; *p*, conserved sites for peptide binding; *cho*, glycosylation site; the conserved RFDS motif and putative CD4 binding site are indicated by brackets. Full-length nucleotide sequences shown in [supplementary materials](#)

Class II β 2 fragment using a tblastx against the Genbank database. The fragment was used as a probe to screen the spleen cDNA library as described previously (Belov et al. 2003), and three full length class II β chain MHC sequences were isolated (EF591102, EF591103, EF591104). Sequences were edited and quality checked using Sequencher 4.1.4 (Genecodes) and BioEdit (Hall 1999). Clustal W was used to align sequences, with some manual adjustments (Thompson et al. 1994). Each sequence had a leader peptide of 28 codons, a β 1 domain of 94 codons, a α 2 domain of 93 codons and a transmembrane and cytoplasmic region of 49 codons (Fig. 1; nucleotide sequence shown in Supplementary Figs. S1 and S2).

Gene-specific primers were designed from the full-length cDNAs (Supplementary Table S1, primer set 2) and used to amplify three additional unique class II sequences (EF591105, EF591106, EF591107) from spleen RNA of individual I, reverse transcribed using Superscript III Reverse Transcription kit (Invitrogen; for amplification conditions see Supplementary information). Positive gel bands were purified and cloned as described above, and 20 clones from each sample were sequenced in both directions. Two independent polymerase chain reactions (PCRs) were performed on the spleen sample to identify clones containing errors. We estimated that 10% of clones sequenced contained at least 2 bp of cloning error. Although this is a more conservative figure than previously reported in the literature, it was used as a guide in accessing sequences for errors (van Oosterhout et al. 2006). In total, six unique β chain class II sequences were isolated, belonging to at least three loci.

A neighbour-joining tree was constructed using Mega 3.1 with Class II β chain sequences from the Tasmanian devil, other marsupial species, eutherian mammals and non-mammals (Fig. 2). The devil class II sequences form a clade with marsupial MHC Class II DAB genes from the red-necked wallaby (*Macropus rufogriseus*), opossum, brushtail possum (*Trichosurus vulpecula*) and tammar wallaby (*Macropus eugenii*; bootstrap 100) and share 83.4% (average) nucleotide and 74.4% (average) amino acid identity with opossum and tammar wallaby Class II DAB alleles. We have classified these sequences as SahaDAB (*Sarcophilus harrisii*-DAB). Phylogenetic analysis distinguishes the marsupial DAB and DBB sequences as distinct clades, separate from the eutherian gene families; however, the exact phylogenetic relationship between the marsupial and eutherian class II gene families remains difficult to determine (Belov et al. 2004).

The devil DAB sequences show high sequence similarity (93.3–99.2% nucleotide identity and 86.9–98.2% amino acid identity), but form three phylogenetic clusters, possibly representing three loci that arose from gene duplication events (Fig. 2). Previous studies have reported the

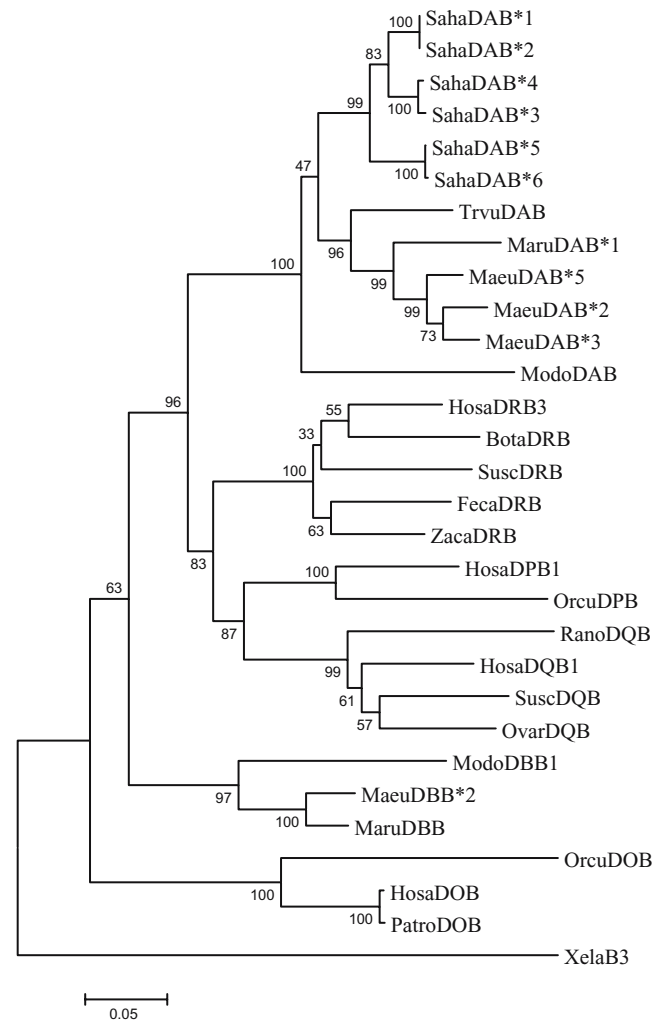


Fig. 2 Neighbour-joining tree of evolutionary relationships between mammalian and marsupial Class II sequences. NCBI accession numbers for sequences are as follows—Brushtail possum: TrvuDAB, AF312030; Red-necked wallaby: MaruDAB*1 M81624; Tammar wallaby: MaeuDAB*5 AY856414; MaeuDAB*2, AY856411; MaeuDAB*3, AY856412; MaeuDBB*1, AY438038; MaeuDBB*2, AY438039; Opossum: ModoDAB AF010497; ModoDBB1; Human: HosaDRB3, NM022555; HosaDOB, M26040; HosaDPB1, NM002121; Hosa DQB1, M20432; Cow: BotaDRB, D45357; Pig: SuscDRB, AY191776; Cat: FecaDRB, U51575; Sea lion: ZacaDRB, AY491464; Rabbit: OrcuDPB, AH001230; OrcuDOB, M96942; Rat: RanoDQB, X56596; Pig: SuscDQB, AY102478; Sheep: OvarDQB, L08792; Chimpanzee: PatroDOB, M24358; Xenopus: XelaB3, D13685

identification of a single DAB sequence from the opossum (Stone et al. 1999) and brushtail possum (Lam et al. 2001a), two DAB sequences from the red-necked wallaby (Schneider et al. 1991) and five sequences from the tammar wallaby (AY856411–414, AY438042), but the number of DAB loci has not been determined in any marsupial species to date.

Five of the six DAB sequences contain amino acid residues consistent with antigen-presenting Class II molecules (Fig. 1). There are cysteine bridges for stabilization of

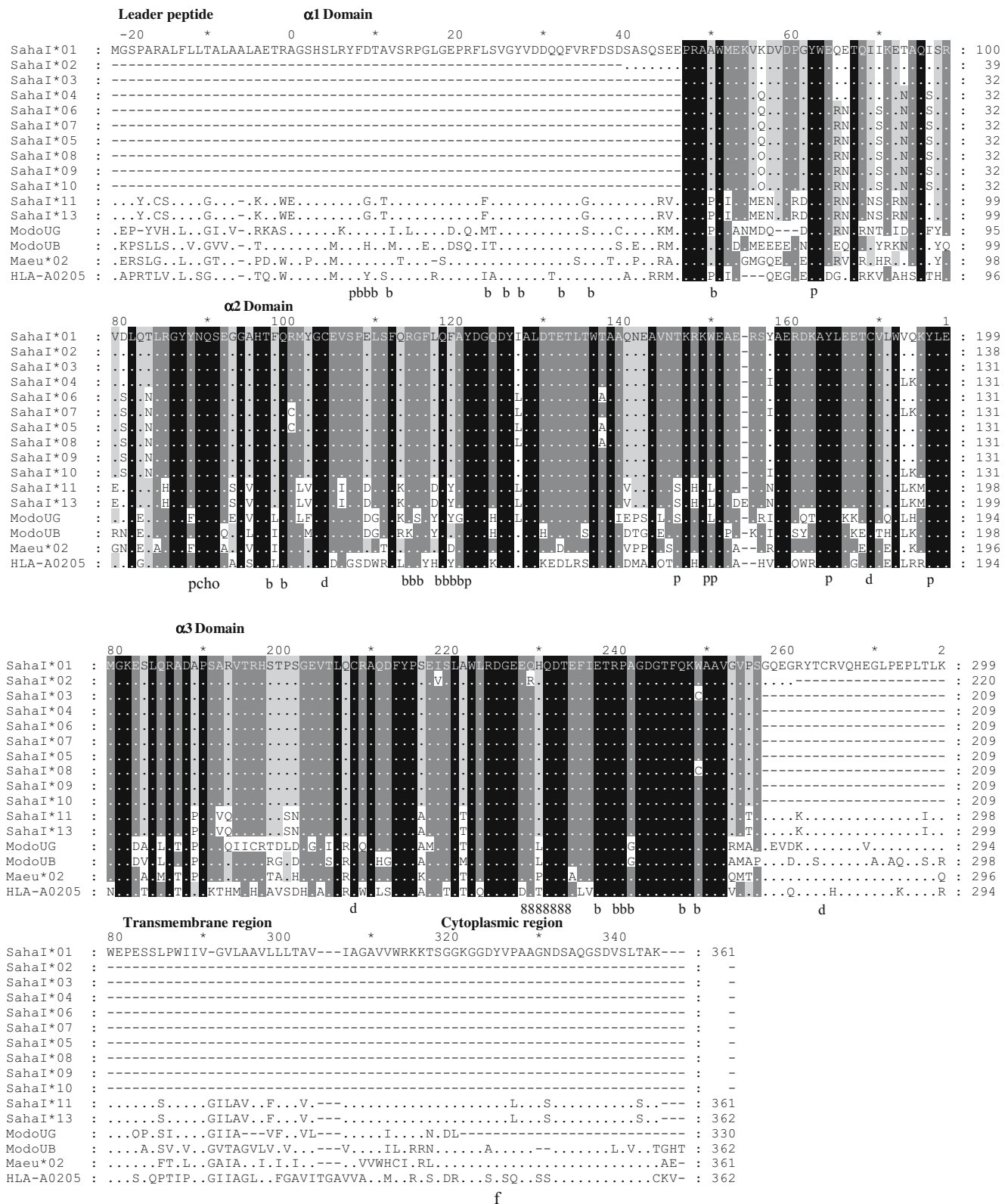


Fig. 3 Amino acid alignment of MHC Class I sequences from the Tasmanian devil, opossum, tammar wallaby and humans. Residues of interest are indicated below the sequence as: *p*, peptide binding sites;

b, β_2 -microglobulin interaction sites; *d*, conserved cysteines; 8, CD8 binding site; *cho*, glycosylation site; *f*, phosphorylation site. Full-length nucleotide sequences are shown in supplementary materials

the molecule in the $\beta 1$ and $\beta 2$ domains, an RFDS motive thought to be a CD4 binding site and the NGT glycosylation site in the $\beta 1$ domain (Auffray and Novotny 1986). A tryptophan at position 90 and an asparagine at position 111 have been identified as important for peptide binding (Brown et al. 1993). All four sequences have an asparagine at position 111; however, SahaDAB*05 has a lysine substituted for tryptophan at position 90. Although, a substitution at this position is rare in eutherian mammals, other marsupial DAB sequences also have this substitution, and it is not known whether it affects antigen binding (Belov et al. 2003).

The PBR of the devil DAB sequences has an excess of non-synonymous to synonymous substitutions (calculated using Mega 3.1). Tests for positive selection at PBR residues using the modified Nei–Gojobori method with Jukes–Cantor adjustment revealed that the PBR sites show evidence of evolving under the influence of positive selection (z statistic of 2.15 and p value of 0.017; Supplementary Table S2). Non-PBR residues, encompassing the $\beta 1$ and $\beta 2$ domains, show no evidence of positive selection (z statistic of 1.624 and a p value of 0.058). Although these sequences belong to multiple loci from a single individual, evidence of selection at the PBR suggests that these sequences are involved in antigen presentation in the devil. While Class II DAB genes are thought to play a role in antigen presentation, it has been proposed that marsupials (koala, opossum and tammar wallaby) have little MHC Class II diversity (McKenzie and Cooper 1994; Stone et al. 1996; Wilkinson et al. 1992). The sequences described here will be of use in assessing polymorphism in the devil.

A Class I exon 4 fragment was amplified from devil genomic DNA using previously described primers (for amplification conditions, see Supplementary information; Siddle et al. 2006). The sequence was confirmed as Class I exon 4 using a tblastx against the Genbank database. Four full-length (EF591089, EF591099, EF591100, EF591101) and one partial (EF591090) MHC Class I sequences were isolated from the devil spleen cDNA library. The Class I sequences have a leader peptide of 22 codons, an $\alpha 1$ domain of 92 codons, an $\alpha 2$ domain of 93 codons, an $\alpha 3$ domain of 91 codons and transmembrane and cytosolic domains of 60 codons (Fig. 3). There is an indel at position –6 in the leader peptide and position 291 in the transmembrane region, which distinguishes SahaI*01 and SahaI*02 from the remaining full-length sequences. In addition, there is an indel at position 154, which makes SahaI*13 unique. The full-length Class I sequences are 360 codons (SahaI*01, SahaI*02, SahaI*11, and SahaI*12) or 361 codons (SahaI*13). The devil Class I sequences share a three-codon indel in the transmembrane region and a three-codon indel in the $\alpha 1$ domain with other marsupial Class I sequences when compared to *HLA-A*.

Gene specific primers were used to amplify the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains of expressed Class I sequences from kidney, testis and liver tissue, from individual I using primer set 1 (Supplementary Table S1; for amplification conditions, see Supplementary information). PCR products were purified and sequenced as described above. Two

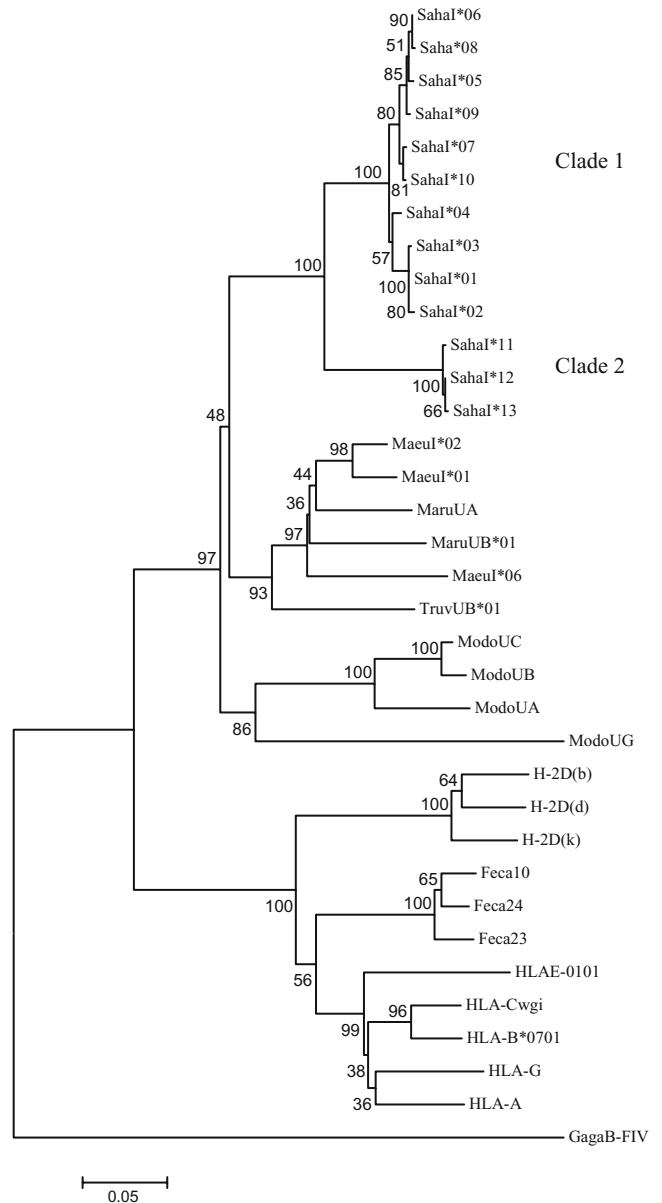


Fig. 4 Neighbour-joining tree of evolutionary relationships between non-mammalian, eutherian and marsupial Class I sequences. NCBI accession numbers for sequences are as follows—Tammar wallaby: Maeu*01, DQ304109; Maeu*02; Maeu*06, DQ304114; Red-necked wallaby: Maru UB*01, L04952; Maru UA*01, L04950; Brushtail possum: Trvu UB*01, AF359509; Opossum: Modo UB*01, AF522352; Modo UC*01, AF522352; Modo UA*01, AF1255540; Modo UG, DQ138606; Mouse: Mumu H-2D^b, U47325; Mumu H-2D^d U47326; Mumu H-2K^b U47328; Cat: Feca10, U07668; Feca23, U07669; Feca24, U07670; Human: HLA-Cw*1203, U06487; HLA-B*0701, U21052; HLA-G, M32800; HLA-E, XM04178; Chicken: Gaba B-FIV, AF013491

independent PCRs were performed on kidney tissue, and the rate of cloning error was estimated for this experiment. Eight additional unique Class I sequences were isolated (EF591091–98; total, 13 sequences). We predict there are at least seven Class I loci in the devil.

The devil Class I sequences were aligned with marsupial and eutherian Class I and analysed for residues important for antigen binding and presentation to T cells (Fig. 3). There are nine sites in the PBR of *HLA-A* that have been identified as important anchor points for peptide binding (Bjorkman et al. 1987) and are conserved in eutherian, marsupial and monotreme Class I (Lam et al. 2001b; Miska et al. 2002, 2004). All devil Class I sequences had these residues with the exception of SahaI*11, SahaI*12, and SahaI*13. These sequences had a serine rather than a threonine at position 146 and a lysine rather than a tryptophan at position 150. The residues, which interact with the β 2-microglobulin, were well conserved between the devil sequences and marsupial Class I, with the exception of position 119, which we have also found to be variable in tammar wallaby Class I sequences (Siddle et al. 2006). The residues in the α 3 domain, which form the CD8 binding site, are also well conserved between the devil sequences and other marsupial Class I.

To establish the phylogenetic position of the devil Class I sequences, we constructed a neighbour-joining tree with marsupial, eutherian and non-mammalian Class I genes (Fig. 4). The marsupial and eutherian Class I sequences formed two distinct clades with a chicken Class I sequence as an out group. Class I sequences from the Australian marsupials form a separate clade to the opossum Class I, which is not surprising as these groups diverged ~80 million years ago (mya; Kirsch et al. 1997). The devil Class I sequences form a sister group adjacent to the Class I sequences from diprotodont marsupials. This separation is unsurprising as the dasyuridae diverged from the diprotodontia ~60 mya (Springer 1997).

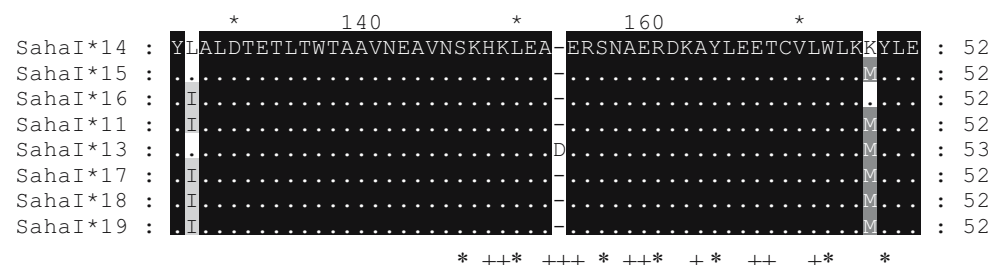
Interestingly, the devil Class I sequences formed two distinct clades (bootstrap 100; Fig. 4). Clade 1 contains the majority of the devil sequences (SahaI*01–*10), which are all closely related, sharing between 96.8 and 99.8% average nucleotide identity and between 93.3 and 99.5% average amino acid identity (Supplementary Table S3). The close relationship between these sequences makes it difficult to

assign alleles to loci. Clade 2 contains SahaI*11, SahaI*12, and SahaI*13. We predict that these sequences are distinct Class I loci. The sequences in the two clades share only 88.5% average nucleotide identity and 80.3% average amino acid identity. Overall, the 13 devil sequences show little sequence divergence compared to the 13 Class I genes of the opossum, which share between 49 and 83% nucleotide identity in the α 1, α 2 and α 3 domains (Belov et al. 2006). It is worth noting, however, that many of the more divergent opossum MHC Class I genes were only discovered after comprehensive annotation of the genome. Before the availability of full genome sequence, only closely related (>85% amino acid identity) Class I sequences had been identified (Miska and Miller 1999; Miska et al. 2004). While we propose that the Class I genes in a single Tasmanian devil may have low heterogeneity, it is possible that more divergent sequences are present but cannot be detected with these primers or are expressed at very low levels.

To investigate whether more evidence of sequence divergence could be found in the PBR sites of the Class I sequences compared to the non-PBR sites, the ratio of non-synonymous to synonymous substitutions was calculated, and the PBR sites were tested for evidence of positive selection in the same manner as for the devil Class II sequences. No evidence for positive selection was found in PBR residues or non-PBR residues (Supplementary Table S2). However, as alleles could not be assigned to loci, it is possible that selection may be detectable among alleles of a single locus.

We propose that SahaI*11, SahaI*12, and SahaI*13 (Fig. 4, clade 2) are distinct from the other devil Class I sequences. These sequences have substitutions at residues important for peptide binding and greater sequence divergence than the remaining devil Class I. To investigate whether these sequences represent non-classical Class I genes, we examined their expression patterns in a single devil and polymorphism in multiple devils ($n=6$). While the sequences found in clade 1 are expressed in a range of tissues, evidence of expression of SahaI*11, SahaI*12, and SahaI*13 was found only in the blood and spleen (Supplementary Table S4). This experiment was replicated in a second individual with identical results (data not shown).

Fig. 5 Amino acid alignment of the α 2 domain of putative non-classical Class I sequences from six devils. The PBR sites are marked as: *asterisks*, residue points towards the peptide binding site; *plus signs*, residue is on the α -helix pointing upwards towards the binding site



We amplified the $\alpha 2$ domain of devil Class I genes from genomic DNA of five additional devils using primer set 3 (for amplification conditions, see [Supplementary information](#); [Supplementary Table S1](#)). Positive bands were gel purified, and 20 clones from each sample were sequenced in both directions. Sequences corresponding to SahaI*11, SahaI*12, and SahaI*13 were identified based on sequence identity and unique substitutions. Nine unique sequences (based on nucleotide sequence) were amplified from two loci in six individuals (Fig. 5). The sequences had between 97.5 and 99.5% nucleotide identity and 94.3 and 100% amino acid identity. Only three sites in the $\alpha 2$ domain (52 of a possible 93 amino acids were analysed) were polymorphic, and all three sites were located outside the PBR. The marsupial non-classical locus, *Modo-UG*, and non-classical Class I sequences in eutherian mammals have low diversity at the PBR (Gouin et al. 2006; Stroynowski and Lindahl 1994). However, it is possible that all Class I loci in the devil lack significant polymorphism, and further investigation of Class I polymorphism in the devil is warranted to determine if these sequences are non-classical.

This study has provided the fundamental information required to study the MHC biology of Tasmanian devils in relation to DFTD. We have isolated Class I and Class II DAB sequences, which are likely to be involved in immune response and antigen presentation, and have developed markers to study MHC diversity in wild populations. Extensive polymorphism studies of the classical Class I and Class II MHC loci are now in progress in our lab.

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