Evolution of viviparity and the maternal immune system: Major histocompatibility complex (MHC) class I genes in skinks

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With the evolution of viviparity, the complex immune system of vertebrates has become a potential threat to the "semi-foreign" fetus. Mammalian embryos avoid immune rejection by altering placental expression of two types of major histocompatibility complex (MHC) genes, classical class I and non-classical class I genes. While non-classical class I genes are not orthologous across different mammalian species, their role in immune evasion is likely to have evolved in conjunction with viviparity deep in mammalian evolutionary history. It is difficult to investigate the transition to viviparity in mammals, because they display a single, ancient origin of live birth. Alternatively, oviparous and viviparous modes of reproduction in closely related species of squamates (lizards and snakes) make them excellent models for studying the evolution of immunological interactions between mother and embryo. We designed primers to amplify the α_2 and α_3 regions of MHC class I genes in scincid lizards and present the first class I sequences for skinks. Class I genes are expressed in the uterus of both oviparous and viviparous skinks both at non-reproduction and late gravidity and pregnancy. We found four putative classical and at least two putative non-classical class I genes expressed in the uterus of the viviparous skink *Pseudemoia entrecasteauxii*, indicating that this species encodes and expresses the genes that may be responsible for the mammalian phenomenon of "hiding" the embryo from the maternal immune system during pregnancy.

Keywords: Major histocompatibility complex (MHC), viviparity, skink, non-classical class I, *Pseudemoia*

Introduction

The shift from oviparity (egg-laying) to viviparity (live-bearing) is the most dramatic transition in the reproduction of amniotes. Viviparous animals retain the embryo *in utero* until it is completely developed. The evolution of viviparity requires thinning and eventual loss of the eggshell, facilitating transport of both respiratory gases and nutrients between mother and embryo via a placenta (Thompson et al. 2004). Immunological recognition of the embryo in oviparous animals is unlikely, since a calcified eggshell separates maternal and fetal tissues and there is no direct contact between the two. Evolutionary loss of the eggshell places maternal and fetal tissues in close apposition (Guillette 1993) and presents the following immunological quandary; while the maternal immune system is programmed to identify and destroy foreign pathogens, it must accommodate a "semi-foreign" fetus developing inside the female reproductive tract. Pregnancy defies the immunological rules that govern the rejection of foreign tissues, since the developing embryo is genetically distinct from its host (Medawar 1953; Bainbridge 2000). If the transition to viviparity involves a gradual thinning and eventual loss of the eggshell (reviewed in Blackburn 2006), then the co-evolution of immunological interactions between mother and embryo may be a major step in the evolution of live-birth.

Mammalian embryos evade the maternal immune system by regulating expression of genes in the major histocompatibility complex (MHC). The MHC is a multigene family found in all jawed vertebrates (Hedrick 1994) encoding both immune (Benacerraf 1981; Snell 1981) and non-immune related molecules (MHC Sequencing Consortium 1999). Classical class I MHC genes are highly polymorphic glycoproteins and are expressed on the surface of all cells, presenting antigens to T cells (Klein 1986). Non-classical class I genes have tissue-specific expression and display little to no polymorphism (Geraghty 1993; Stroynowski and Lindahl 1994; Gouin et al. 2006). Both types of class I genes have a similar structure, consisting of three alpha domains (α_1, α_2 and α_3), which interact with a β_2-microglobulin, and a transmembrane region that anchors the protein to the cell membrane. The α_3 domain, encoded by exon 4, is the least polymorphic domain. α_1 and α_2 domains, encoded by exons 2 and 3 respectively, are highly polymorphic and are responsible for binding to the antigenic peptide

(Bjorkman et al. 1987). The evolution of class I genes is a "birth and death" process, whereby frequent duplications of classical class I genes result in the evolution of species-specific non-classical class I genes (Hughes and Nei 1989). Species-specific evolution at the MHC means that non-classical class I genes in different species are not orthologous; non-classical class I genes display more sequence similarity with classical class I genes from the same species than with non-classical class I genes from other species (Hughes and Nei 1989). However, while non-classical class I genes are not orthologous, similarities at the amino acid level in the peptide-binding region (PBR) suggests they may evolve similar functions convergently (Yeager et al. 1997). The "birth and death" model of evolution at the MHC also produces lineage-specific expansions and contractions and results in great variation in the number of MHC class I loci among species (Table 1 (at end)).

The intermingling of uterine and embryonic trophoblast cells in the placenta provokes the maternal uterine immune response. MHC genes expressed by trophoblast cells are potential ligands for uterine natural killer (NK) cells, T-cells and lymphocytes (Moffett and Loke 2006), but the potential for contact between embryonic MHC genes and the maternal immune system depends on the extent to which trophoblast cells invade the uterine wall. Types of placentation in eutherian mammals have been simplified into three groups (Amoroso 1952; Moffett and Loke 2006). In epitheliochorial placentation, trophoblast cells contact the uterine epithelium but do not invade the uterine wall. In endotheliochorial placentation, trophoblast cells break down the uterine epithelium and come into contact with the endothelial cells of maternal blood vessels in the uterine wall. In haemochorial placentation, the trophoblast cells invade further, rupturing maternal blood vessels and bathing in a blood-filled intervillous space (Amoroso 1952).

Knowledge of the maternal-embryonic immune interaction is restricted mainly to humans and mammals with haemochorial placentae. In humans, the syncytiotrophoblast, which is a layer of binucleate cells that forms a barrier between the fetus and the mother, expresses no MHC antigens (Moffett-King 2002). Human extravillous trophoblast cells, exposed to NK cells in the decidua (remnants of the uterine wall), do not express the major classical class I molecules HLA-A and HLA-B that initiate allograft rejection (Faulk and Temple 1976; King et al. 2000). Instead, these cells express the non-classical class I molecule HLA-G at high levels (Kovats et al. 1990; Rouas-Friess et al. 1997). Recent work indicates that HLA-G suppresses T-lymphocyte function and dramatically inhibits cell lysis by NK cells (Lin et al. 2007; Selmani et al. 2007), and MHC molecules similar to HLA-G are expressed in the placenta of other primates, such as Rhesus monkeys (Boyson et al. 1997) and baboons (Stern et al. 1987). In mice, two non-classical class I genes (*Qa-2* and *blastocyst MHC*) are expressed on the surface of preimplantation mouse embryos and in mouse placenta (Warner et al. 1987; Sipes et al. 1996).

Epitheliochorial placentation involves a more simple apposition of maternal and embryonic tissues in comparison to the far more intimate association in haemochorial placentae. This simple apposition, however, is sufficient to provoke damaging immune responses by the mother. While the NK cells are absent from the uterine stroma in epitheliochorial placentae, the maternal immune systems of many ruminant mammals recognise the embryo via granulated lymphocytes in the uterine epithelium (Stewart 1998). In most mammals with epitheliochorial placentation, trophoblast cells lack MHC class I expression (Moffett and Loke 2006), but there are some exceptions, including horses (Baker et al. 1999) and cattle (Bainbridge et al. 2001; Davies et al. 2006).

Embryos of most mammals, regardless of placental type, decrease expression of classical MHC class I genes to evade the maternal immune system, and some species show increases in non-classical class I expression. Although non-classical class I genes are not orthologous across species, it is likely that the mechanism of regulating embryonic class I expression to avoid immune rejection is a result of the evolution of viviparity. As mammals display a single, ancient origin of viviparity, it is difficult to study the evolution of live birth in the mammalian lineage (Blackburn 2006). Live birth has evolved multiple times in squamate reptiles (lizards and snakes) (Packard et al. 1977; Shine 1983), both relatively recently (Camarillo 1990; Heulin et al. 1993) and at low taxonomic levels (Shine 1985; Blackburn 2000). Using squamates to study the evolution of viviparity allows comparisons of oviparous and viviparous modes of reproduction in closely related species (Blackburn 2006).

Viviparous squamates exhibit epitheliochorial placentation and the apposition of maternal and fetal tissues is sufficiently intimate to allow maternal immunorecognition of pregnancy. The thin acellular shell membrane, which separates mother and embryo in the early stages of pregnancy, is absorbed during the later stages of development in many viviparous species (Yaron 1985; Blackburn 1993), placing

maternal and fetal tissues in direct contact. The presence of interleukin-1α and -1β cytokines at the materno-fetal interface in the viviparous lizard *Chalcides chalcides* indicates immunological recognition of the embryo by its mother (Paulesu et al. 1995). Just as mammalian embryos avoid allograft rejection, it is likely that embryos in viviparous skinks also decrease classical class I expression and express non-classical class I genes to 'hide' from the maternal immune system. In comparison, down-regulation of classical class I expression would not be expected in oviparous embryos, as the eggshell prevents contact between maternal and embryonic tissues.

Knowledge of MHC organisation and variation in reptiles is limited by the lack of a reptilian model species in genome research (Wittzell et al. 1999; Miller et al. 2005). The current *Anolis carolinensis* (the green anole) sequencing project promises to provide a starting point for much of this work. In the meantime, class I sequences have been amplified from tuatara (Miller et al. 2005), the ameiva lizard and water snake (Grossberger and Parham 1992), geckos (Radtkey et al. 1996), and the chinese soft-shelled turtle using consensus primers. Predictions of classical and non-classical function of class I genes in reptiles have been made only for the tuatara, in which there appears to be two classical loci and one non-classical locus (Miller et al. 2007).

Before investigating the possibility of regulation of class I expression in the skink embryo, skink class I genes must first be characterised. Due to the rapid evolution of the MHC and the paucity of reptilian MHC sequences for comparison, class I genes in skinks may be very different from those in other vertebrates. In this study, we aimed to 1) isolate class I sequences from a range of Australian skinks; 2) investigate class I expression in oviparous and viviparous skinks using RT-PCR from uterine tissue; and 3) characterise expressed class I sequences in *Pseudemoia entrecasteauxii*, a viviparous skink with a complex placenta and significant placentotrophy (Stewart and Thompson 1993, 1996, Speake et al. 2004), by cloning sequences amplified from uterine cDNA.

Materials and methods

Tissue collection

Uterine tissue was collected from non-reproductive, vitellogenic and late pregnant/gravid females of each of six skink species, which display different parity modes (Table 2). Animals were euthanized by an intrathoracic injection of 0.1 mL of sodium pentobarbitone (6 mg/mL), and the viscera and oviducts were exposed with a ventral incision. We dissected and stored the incubation chambers of the uterus, which is the region that contacts the embryo to form the placenta in viviparous species, to investigate uterine gene expression during gestation resulting from interactions with the developing embryo. For pregnant and gravid females, an incision was made between the incubation chambers of the uterus and each incubation chamber was cut longitudinally and peeled away to expose the embryo. The embryo was gently removed from the incubation chamber of the uterus and a segment of the incubation chamber was excised. For each of the pregnant females, embryos were fixed in formalin and later dissected to determine the embryonic stage of development (according to Dufaure and Hubert, 1961). A segment of uterus from non-reproductive females was similarly excised, but the uterus was not cut longitudinally. Uterine tissue was immediately transferred into eppendorf tubes and snap-frozen in liquid nitrogen.

Table 2. Species of skinks used in this study. [a] *Saiphos equalis* is a bimodally reproductive species, oviparous in Sydney populations and viviparous in New England, NSW; [b] Placental type based on Weekes (1935) classification scheme.

Species	Skink lineage	Parity mode	Placental type
Ctenotus taeniolatus	*Sphenomorphus*	Oviparous	N/A
Eulamprus tympanum	*Sphenomorphus*	Viviparous	Simple (Type 1)[b]
Saiphos equalis[a]	*Sphenomorphus*	Oviparous	N/A
		Viviparous	Simple (Type 1)[b]
Lampropholis guichenoti	*Eugongylus*	Oviparous	N/A
Niveoscincus metallicus	*Eugongylus*	Viviparous	Simple (Type 2)[b]
Pseudemoia entrecasteauxii	*Eugongylus*	Viviparous	Complex (Type 3)[b]

DNA and RNA extractions

Total genomic DNA and total RNA were isolated using a mono-phasic solution of phenol and guanidine thiocyanate (TRI Reagent, Molecular Research Center, Inc. Cincinnati, OH) (Chomczynski, 1993). Concentrations of DNA and RNA samples were quantified using a NanoDrop ND-1000 Spectrophotometer, and RNA quality was analysed using an Agilent 2100 Bioanalyser. Total RNA was treated with RNAse-free DNase (Promega) and then reverse transcribed into cDNA using SuperScript III First Strand Synthesis System and random hexamers (Invitrogen) following manufacturer's instructions.

MHC class I in scincid genomes and isolation of MHC class I α_3 sequences

Sequences from the α_3 domain of the class I alpha chain were amplified from genomic DNA from a single female from six skink species. A fragment spanning 140 bp (excluding primers) of exon 4 of class I genes was amplified from genomic DNA using consensus primers (MHCIF 5'-GCCGAGTTCACGGCTTCTACCCC -3' and MHC1R 5'- TCACAGCCATACATCTGCTG -3') designed from aligned reptile, bird, amphibian, fish and mammalian MHC class I sequences (Table 3). MHC class I sequences were obtained from GenBank and aligned using BioEdit 7.0.1 (Hall 1999) with manual adjustments. Since very few reptilian sequences are available, class I sequences in mouse (*Mus musculus*) and chicken (*Gallus gallus*) were used to search for conserved regions of Class I sequences in the unannotated genome of a polychrotid lizard, the green anole (*Anolis carolinensis*). BLAST searches were carried out using the blastall interface on the WEHI website provided by Dr. A.T. Papenfuss. Anole BLAST results, and two anole Class I predictions (provided by Ricardo Godinez, Harvard University) were included in alignments.

PCR amplification (Invitrogen) was performed under the following conditions in 10 µL volumes: 0.2 units of *Taq* DNA polymerase, 1.0 µL of 10x buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 1.5 mM $MgCl_2$, and ~100 ng of skink genomic DNA or 50 ng of skink uterine cDNA. The following cycle conditions were used for PCR amplification: 94 ºC for 3 min; 40 cycles of 94 ºC for 30 s, 72 ºC for 1 min 20 s and 72 ºC for 1 min 30 s; 72 ºC for 10 min; 4 ºC hold. Amplified PCR products were separated using agarose gel electrophoresis, and the amplified bands were excised and cleaned (UltraClean PCR Clean-up Kit, MoBio). PCR products from four species (*S. equalis*, *E. tympanum*, *C. taeniolatus* and *P. entrecasteauxii*) were cloned using pGEM-T Easy vector (Promega, Madison, Wisconsin) and plasmid DNA was extracted from four positive clones from each species (UltraClean 6 Minute Mini Plasmid Prep Kit, MoBio). Fragments were sequenced at the Australian Genome Research Facility using vector primer Sp6.

Table 3. Oligonucleotides used in this study. [a] 3' nucleotide position in tuatara MHC Class 1 sequence (DQ145788).

Primer name	Sequence (5'-3')	Position	T_m (°C)
2MHCF	CAGCAGATGTATGGCTGTGA	410[a]	60
2MHCR	GCAGATCTCCTCCAGGTAG	578[a]	60
IMHCF	CTACCTGGAGGAGATCTGCA	598[a]	62
IMHCR	CCTTGGGGTAGAAGCCGTG	720[a]	62
MHCIF	GCCGAGTTCACGGCTTCTACCCC	736[a]	72
MHCIR	CATGCTCCACGTGGCACTGGTA	873[a]	72

MHC class I expression in skink uterus

Consensus primers IMHCF (5' -CTACCTGGAGGAGATCTGCA -3') and IMHCR (5' -CCTTGGGGTAGAAGCCGTG -3') (Table 3) were designed within well-conserved regions of the alignment in exons 3 and 4 respectively, from MHC class I transcripts from tuatara, anole, chinese soft-shelled turtle, water snake, ameiva lizard and gecko. These primers are designed within separate exons, so amplification from genomic DNA results in a fragment which includes an intron (~1 kb), while amplification from cDNA results in a 122 bp fragment (excluding primers). Primers were used to amplify from uterine cDNA of both non-reproductive and late pregnant/gravid females from each of six species. PCR conditions were as described above, but using an annealing temperature of 62 ºC.

Isolation of MHC class I α_2 sequences from a viviparous skink

Primers 2MHCF (5'- CAGCAGATGTATGGCTGTGA -3') and 2MHCR (5'- GCAGATCTCCTCCAGGTAG -3') (Table 3) were designed within exon 3 of class I genes to target a 168 bp fragment (excluding primers). Sequences from the α_2 domain of the class I alpha chain were amplified from the genomic DNA and uterine cDNA of a single vitellogenic *Pseudemoia entrecasteauxii* female using the PCR conditions described above, but annealing at 60 ºC. PCR products from both the genomic and cDNA templates were cloned; 70 positive clones containing genomic PCR products and 30 positive clones containing cDNA PCR products were sequenced.

Data analysis

Sequences were edited and assembled using Sequencher 4.2 (GeneCodes Corporation, Ann Arbor, Michigan) and aligned using BioEdit 7.0.1 (Hall 1999) with manual adjustments. Neighbour joining trees were constructed in MEGA version 4 (Tamura et al. 2007) using amino acid sequences.

Results

Characterisation of MHC class I genes from skinks

From each of four skink species, four positive clones containing genomic α_3 fragments were sequenced. Homology of the sequences to MHC class I genes was confirmed using tblastx. Two different amino acid sequences were present in each species (Figure 1). Within each skink species, each sequence differs by only one or two residues (1-2 base pairs), and percent amino acid identities between species range from 76.1% to 100%. One sequence is shared by both *P. entrecasteauxii* and *C. taeniolatus*. The α_3 region of class I molecules contains residues that interact with the β_2-microglobulin domain (positions 238, 240-242, 248 and 250) and CD8 molecules (positions 229-235), both of which facilitate binding between an MHC molecule and T-cells (Otten et al. 1992; Apasov and Sitkovsky 1993). Skink sequences were aligned with classical and non-classical sequences from other vertebrates to analyse residues that interact with β_2-microglobulin and CD8 molecules. At most of these interaction sites, each skink sequence displays a residue also found at the corresponding site of functional class I molecules in other vertebrates, suggesting that these skink sequences represent functional class I genes. Sites at which skinks display a unique residue appear to be particularly variable across vertebrates.

Percent amino acid identities between skink sequences and other reptile and avian sequences range from 30.4% to 69.6% (Table 4). Skink sequences are most similar to sequence predictions from the green anole (58.7-69.6% sequence identity). Skink sequences are more similar to other squamate sequences (52.2-69.6%) than sequences of non-squamate reptiles (30.4-41.9%), although skinks show a greater sequence similarity to the water snake (55.9-64%) than the ameiva lizard (45.7-52.2%). Percent amino acid identities between the eight skink sequences range from 73.3% to 100% (Table 5). Both *E. tympanum* sequences are more similar to each other than to sequences from other species. This is also the case for both *S. equalis* sequences. However, there is high similarity between sequences from *C. taeniolatus* and *P. entrecasteauxii*, and the same sequence is shared by these two species.

Table 4. Pairwise comparisons of MHC class I α_3 amino acid identity between skink and other reptile and avian sequences. Details of sequences used for comparisons are listed in Figure 1. Only one sequence from each skink species was used for comparisons. *Ctta*-1, *Ctenotus taeniolatus*; *Psen*-1, *Pseudemoia entrecasteauxii*; *Saeq*-1, *Saiphos equalis*; *Euty*-1, *Eulamprus tympanum*.

Species	*Ctta*-1	*Psen*-1	*Saeq*-1	*Euty*-1
Green anole (*Anca*-1)	65.2	69.6	58.7	63.0
Ameiva (*Amam*-LC25)	45.7	45.7	52.2	52.2
Water snake (*Nesi*-SC1)	55.9	58.8	64.7	61.8
Turtle (*Trsi*-B01)	41.9	41.9	39.5	39.5
Tuatara (*Sppu*-U*01)	34.8	32.6	30.4	34.8
Great reed warbler (*Acar*-cN3)	41.3	43.8	39.1	39.1
Chicken (*Gaga*-B-FIV)	43.4	41.3	39.1	34.8

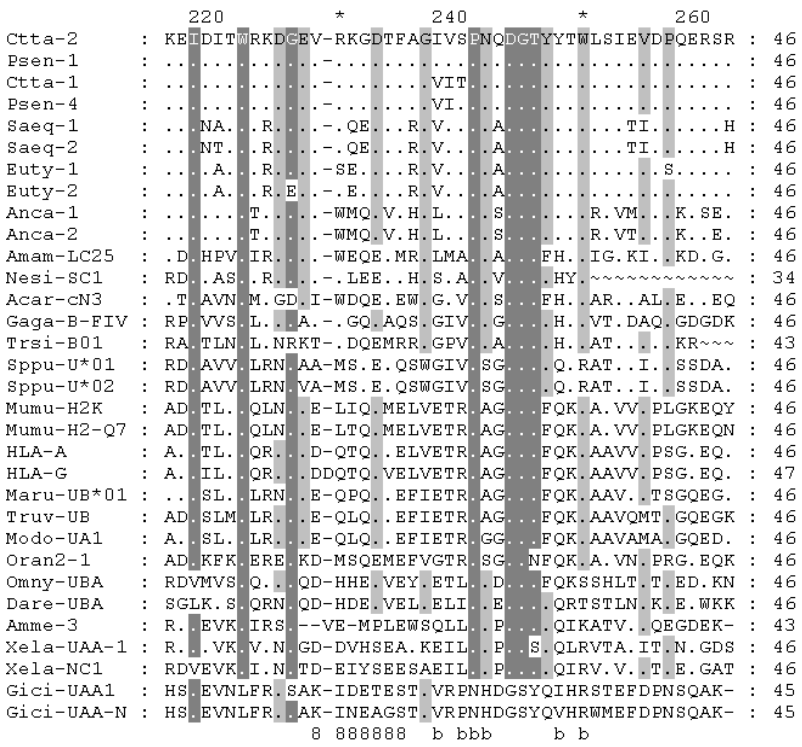

Figure 1. Amino acid alignment of classical and non-classical class I α_3 sequences. α_3 sequences from skinks (*C. taeniolatus*, *P. entrecasteauxii*, *E. tympanum* and *S. equalis*) were amplified from genomic DNA using MHC1F and MHC1R primers, cloned and sequenced. Skink sequences were structurally analysed at CD8 and β_2-microglobulin interaction sites and compared to the α_3 domain of class I genes from other vertebrates. At most of these interaction sites, each skink sequence displays a residue also found at the corresponding site of functional class I molecules in other vertebrates, suggesting that these skink sequences represent functional class I genes. Sites at which skinks display a unique residue appear to be particularly variable across vertebrates. Sequences from other vertebrates were obtained from GenBank. Species names in all figures have been abbreviated to the first two letters of their genus and species names, except HLA-A (human leukocyte antigen A) (U07161) and HLA-G (M32800). Anole (*Anolis carolinensis*) *Anca-1*, *Anca-2* (gene predictions by Ricardo Godinez, Harvard University); ameiva lizard (*Ameiva ameiva*) *Amam-LC25* M81097; water snake (*Nerodia sipedon*) *Nesi-SC1* M81099; great reed warbler (*Acrocephalus arundinaceus*) *Acar-cN3* AJ005503; chicken (*Gallus gallus*) *Gaga-B-FIV* AF013491; chinese soft-shelled turtle (*Pelodiscus sinensis*) *Trsi-B01* AB185243; tuatara (*Sphenodon punctatus*) *Sppu-U*01* DQ145788, *Sppu-U*02* DQ145789; mouse (*Mus musculus*) *Mumu-H2K* U47328, *Mumu-H2-Q7* NM010394; wallaby (*Macropus rufogriseus*) *Maru-UB*01* L04952; brushtail possum (*Trichosurus vulpecula*) *Truv-UB* AF359509; opossum (*Monodelphis domestica*) *Modo-UA1* AF125540; platypus (*Ornithorhynchus anatinus*) *Oran-2-1* AY112715; rainbow trout (*Oncorhynchus mykiss*) *Omny-UBA* AF287487; zebrafish (*Danio rerio*) *Dare-UBA* NM131471; axolotl (*Ambystoma mexicanum*) *Amme-3* U83137; African clawed frog (*Xenopus laevis*) *Xela-UAA-1f* L20733, *Xela-NC1* M58019; nurse shark (*Ginglymostoma cirratum*) *Gici-UAA1* AF220063, *Gici-UAA-NC1* AF357923. Residue position numbers are based on the HLA-A sequence (Koller and Orr 1985). Dots are conserved residues, tildes are missing sequence and dashes are potential gaps. Residues of interest are indicated below the sequence (Siddle *et al*. 2006). 8, CD8 interaction sites; b, β_2-microglobulin interaction sites.

Table 5. Pairwise comparisons of MHC class I α_3 amino acid identity between skink sequences. Two different sequences from each skink species were cloned and sequenced. *Euty*-1 and -2, *Eulamprus tympanum*; *Ctta*-1 and -2, *Ctenotus taeniolatus*; *Saeq*-1 and -2, *Saiphos equalis*; *Psen*-1 and -4, *Pseudemoia entrecasteauxii*.

Sequence	*Euty*-2	*Ctta*-1	*Ctta*-2	*Saeq*-1	*Saeq*-2	*Psen*-1	*Psen*-4
Euty-1	93.3	80.0	82.2	82.2	80.0	82.2	82.2
Euty-2		82.2	84.4	84.4	82.2	84.4	84.4
Ctta-1			93.3	73.3	73.3	93.3	97.8
Ctta-2				75.5	75.5	100.0	95.5
Saeq-1					97.7	75.5	75.5
Saeq-2						75.5	75.5
Psen-1							95.5

MHC class I genes are expressed in the uterus of both oviparous and viviparous skinks

To investigate expression of MHC class I genes in the uterus, IMHCF and IMHCR primers were used to amplify a 164 bp fragment from class I transcripts present in uterine cDNA. This fragment was amplified from the uterus of oviparous and viviparous skinks, and is expressed in the uterus of all six species examined. In these six species, MHC class I genes are expressed during non-reproductive stages, during late gravidity in oviparous species and during late pregnancy in viviparous species.

Multiple MHC class I loci are expressed in the uterus of a viviparous skink

Twelve unique genomic sequences and nine unique expressed sequences were isolated by cloning fragments of the α_2 domain of MHC class I genes from a *Pseudemoia entrecasteauxii* female (Figure 2). A neighbour-joining phylogenetic analysis was performed using unique sequences from both genomic DNA and cDNA (Figure 3). Because some of the unique sequences will be due to *Taq* error during PCR (van Oosterhout et al. 2006), only those cDNA sequences that were represented in both genomic and cDNA sets were included in the analysis. Two cDNA sequences were not included in the analysis because they had no genomic equivalent and appeared to be artefacts of recombination during PCR or cloning. Another cDNA sequence was excluded because of an obvious *Taq* error. Only genomic sequences that were found more than once were included in the analysis, and sequences differing by only 1-2 bp were excluded. Phylogenetic analysis shows seven distinct clusters that contain both genomic and cDNA sequences (Figure 3), suggesting six or seven putative loci expressed in the uterus of *P. entrecasteauxii*, with this individual heterozygous for at least two loci. Three more distinct clusters contained only genomic sequences.

Expressed skink sequences were aligned with their genomic equivalents (Figure 2) and with classical and non-classical sequences from other vertebrates (Figure 4), to analyse residues important for peptide binding and interactions with the β_2-microglobulin domain. In general, skink sequences were very different from mammalian sequences at peptide-binding residues (Figure 4). For example, position 126 is a tyrosine in all mammals included in the alignment, but is substituted for a phenylalanine in skinks and in all other non-mammalian sequences. Furthermore, the highly conserved lysine at position 149 is an arginine at the majority of skink sequences. This lysine and other positively-charged residues in the peptide-binding region (PBR) bind to the negative –COOH end of the antigenic peptide (Bjorkman et al. 1987). It is unlikely that the lysine to arginine substitution affects peptide binding, as arginine is also positively charged, and this same substitution occurs in an otherwise classical-like class I sequence in the tammar wallaby (Siddle et al. 2006).

Three of the nine expressed sequences (*Psen-160Ut*, *-155Ut* and *-164Ut*), which constitute two putative loci, are quite divergent in the phylogenetic tree (Figure 3). The three most divergent skink sequences differ from other skink sequences at peptide-binding and β_2-microglobulin interaction sites (Figure 2). Particularly non-conservative substitutions occur at these sites in the divergent *Psen-160Ut*/*Psen-78G* sequence. The tryptophan at position 150, which is conserved in all vertebrate sequences in the alignment except in the human non-classical class I HLA-G, is substituted for a leucine. The polar glutamine residue at position 118 is substituted for hydrophilic leucine, and acidic residues aspartate and glutamate at position 122 are replaced by an uncharged glutamine. Interestingly, at two β_2-microglobulin interactions sites (positions 119 and 125) and two peptide-binding sites (positions 146 and 150), the three most divergent skink sequences display residues conserved in mammals and other aligned vertebrates, while other skink sequences possess unique but functionally conservative substitutions at these sites.

Clusters containing only genomic sequences were also analysed. The two sequences that comprised one of these clusters (*Psen-82G* and *-101G*) show high amino acid similarity (94.5%) but differ in two of the four peptide-binding sites along the sequence (positions 149 and 150). At these two sites, *Psen -82G* displays the arginine and tryptophan residues characteristic of most of the other skink sequences, while *Psen-101G* displays lysine and leucine residues at these positions, characteristic of the three divergent skink sequences. Another unexpressed sequence *Psen-79G* displays residues at β_2-microglobulin interaction sites and in the PBR that are the same as those in non-divergent skink sequences.

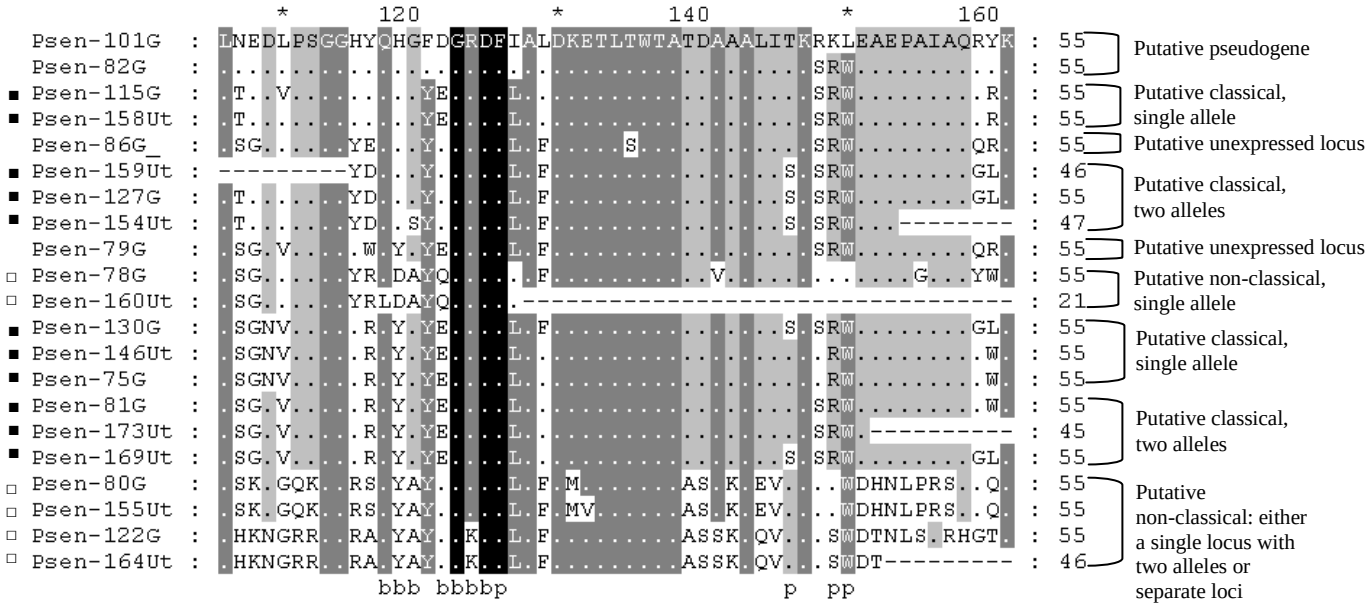

Figure 2. Amino acid alignment of α_2 sequences from a vitellogenic *P. entrecasteauxii* female. α_2 sequences were amplified from genomic DNA and uterine cDNA using 2MHCF and 2MHCR primers, and exhaustively cloned and sequenced. Putative classical and non-classical loci are labelled. G, sequence amplified from genomic DNA; Ut, sequence amplified from uterine cDNA. Residue position numbers are based on the HLA-A sequence (Koller and Orr 1985). Dots are conserved residues and tildes represent regions that could not be sequenced cleanly. Residues of interest are indicated below the sequence (Siddle *et al*. 2006). p, peptide-binding sites; b, β_2-microglobulin interaction sites; ■, putative classical class I sequences; □, putative non-classical class I sequences.

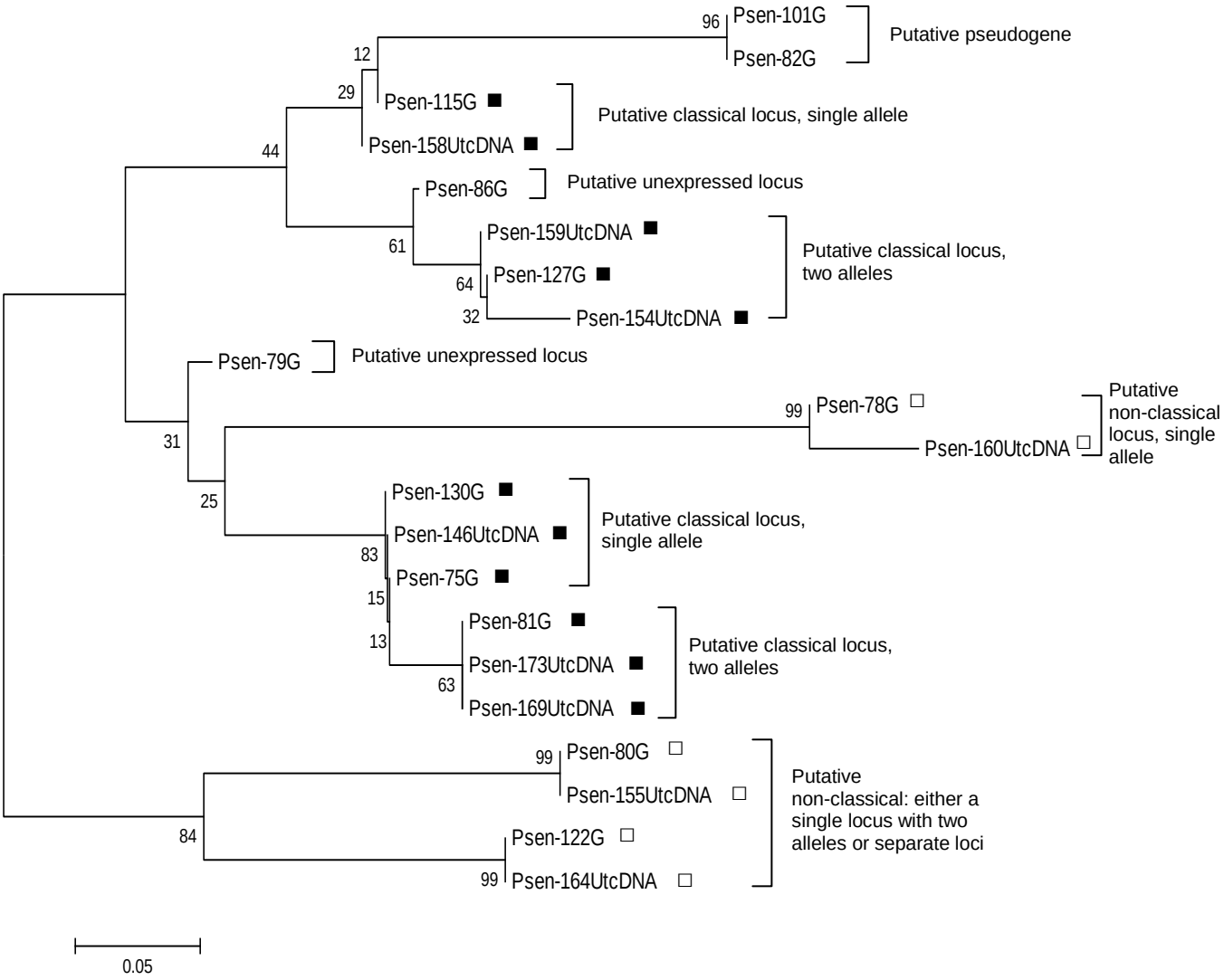

Figure 3. Neighbour-joining analysis of sequences from the α_2 domain of the class I alpha chain amplified from genomic DNA and uterine cDNA from the same vitellogenic *Pseudemoia entrecasteauxii* female. Bootstrap values based on 500 replicates are shown. Putative classical and non-classical class I loci are labelled. G, sequence amplified from genomic DNA; UtcDNA, sequence amplified from uterine cDNA; ■, putative classical class I sequences; □, putative non-classical class I sequences.

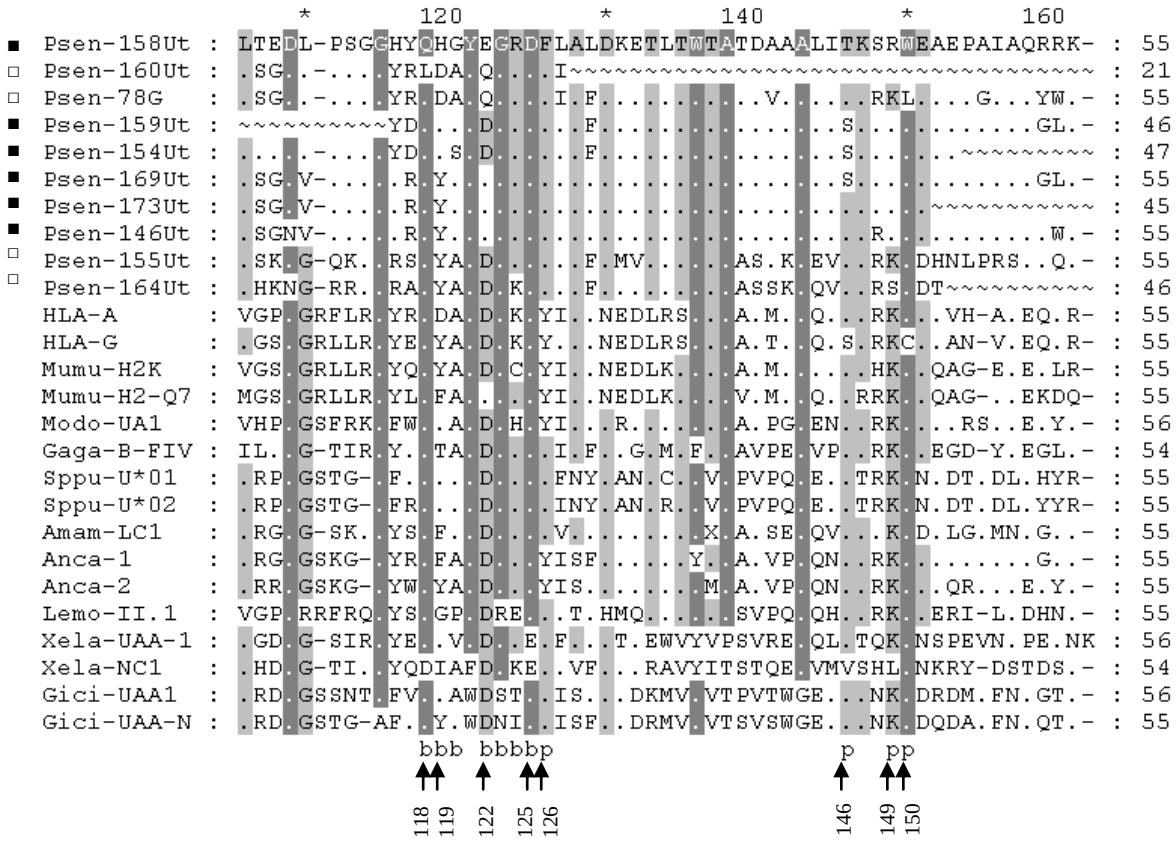

Figure 4. Amino acid alignment of α_2 sequences expressed in *P. entrecasteauxii* uterus and classical and non-classical sequences from other vertebrates. As *Psen-160Ut* is a truncated sequence, its matching genomic sequence (*Psen-78G*) was included in the alignment. G, sequence amplified from genomic DNA; Ut, sequence amplified from uterine cDNA. Putative non-classical class I sequences were more divergent in phylogenetic analyses and exhibited more non-conservative substitutions at peptide-binding and β_2-microglobulin interaction sites than putative classical class I sequences. Species names have been abbreviated to the first two letters of their genus and species names, except HLA-A (human leukocyte antigen A) (U07161) and HLA-G (M32800). Mouse (*Mus musculus*) *Mumu-H2K* U47328, *Mumu-H2-Q7* NM010394; opossum (*Monodelphis domestica*) *Modo-UA1* AF125540; chicken (*Gallus gallus*) *Gaga-B-FIV* AF013491; tuatara (*Sphenodon punctatus*) *Sppu-U*01* DQ145788, *Sppu-U*02* DQ145789; ameiva lizard (*Ameiva ameiva*) *Amam-LC25* M81097; anole (*Anolis carolinensis*) *Anca-1*, *Anca-2* (gene predictions by Ricardo Godinez, Harvard University); gecko (*Lepidodactylus moestus*) *Lemo-II.1* U58160; African clawed frog (*Xenopus laevis*) *Xela-UAA-1f* L20733, *Xela-NC1* M58019; nurse shark (*Ginglymostoma cirratum*) *Gici-UAA1* AF220063, *Gici-UAA-NC1* AF357923;. Residue position numbers are based on the HLA-A sequence (Koller and Orr 1985). Dots are conserved residues, tildes represent regions that could not be sequenced cleanly and dashes are potential gaps. Residues of interest are indicated below the sequence (Siddle *et al*. 2006). p, peptide-binding sites; b, β_2-microglobulin interaction sites; ■, putative classical class I sequence; □, putative non-classical class I sequence. Arrows indicate residues discussed in the main text.

Discussion

This study comprises the first characterization of MHC class I sequences in skinks. We isolated two genomic α_3 sequences from each of four species of skink (*C. taeniolatus*, *E. tympanum*, *P. entrecasteauxii* and *S. equalis*) and found evidence for four putative classical and at least two putative non-classical loci expressed in the uterus of a placentotrophic, viviparous species.

Amplification of class I transcripts from six skink species indicate that MHC class I genes are expressed in the uterus of both oviparous and viviparous skinks at both non-reproductive and late gravid/pregnant stages. Expression of different MHC class I loci is likely to change during gestation due to interactions with the developing embryo. Appropriate housekeeping genes and gene-specific primers suitable for use in real-time PCR are needed to determine whether the uterine expression of all or a select few class I loci are regulated during pregnancy.

At least six class I loci are expressed in the vitellogenic uterus of *P. entrecasteauxii*. There are another three branches on this phylogenetic tree (Figure 3) that are not expressed in the uterus and these may represent either unexpressed loci or pseudogenes. A possible total of at least nine loci (at least six expressed) in *P. entrecasteauxii* is greater than estimates of number of class I loci in avian and in other non-avian reptiles, but within the range observed across vertebrates (Table 1); there are an estimated three to four class I loci in tuatara (Miller et al. 2005, 2007), four class I genes in chicken (two at each of the *B-F/B-L* and *Rfp-Y* regions) (Guillemot et al. 1989; Kaufman et al. 1995; 1999) and four transcribed loci in the great reed warbler (Westerdahl et al. 1999). Seven class I genes are identified in the quail (Shiina et al. 1999; 2004) and four loci are expressed in the ameiva lizard (Grossberger and Parham 1992). Most of these reptile studies have counted the number of unique full length sequences extracted from cDNA libraries and evaluated the frequency of these sequences in many individuals to estimate loci number. Our technique consisted of amplifying and cloning an α_2 fragment from genomic DNA and cDNA of a single individual and using phylogenetic analysis to examine how expressed sequences cluster together with genomic sequences into putative loci. Attempts to isolate full-length class I sequences using 5' RACE and amplify the α_1 domain using consensus primers were unsuccessful (data not shown). High polymorphism in the α_1 domain may have resulted in a lack of conservation that rendered consensus primers insufficiently specific for this region of the class I alpha chain in skinks.

The expressed sequences represent four putative classical and at least two putative non-classical loci. In classical molecules, the residues that interact with the antigenic peptide and the β_2-microglobulin domain in the α_2 region tend to be highly conserved across mammals (Lam et al. 2001; Miska et al. 2002; 2004). Since non-classical genes are less likely to be involved in antigen presentation, the selective pressures on these residues are reduced and substitutions at these sites are more likely to be tolerated than in classical molecules. In mammals, classifying α_2 sequences as classical and non-classical is usually done by comparing the residues found at the PBR and β_2-microglobulin interactions sites with those in human classical and non-classical genes. However, because skink sequences are quite divergent from mammalian sequences (36.4-47.3% amino acid identity between skink sequences and HLA-A), it is difficult to separate sequences into putative classical and non-classical loci by analysing these interaction sites alone. Furthermore, because non-classical loci are not under the same selective pressures as antigen-presenting classical molecules, not only do they differ from classical class I loci at the PBR but they tend to be divergent from conspecific classical sequences in phylogenetic analyses. We suggest that non-classical sequences in lizards are more divergent than classical sequences and therefore are most divergent on the phylogenetic tree. Analysis of conserved residues in the PBR and β_2-microglobulin sites supports this hypothesis, since the three divergent sequences display more non-conservative substitutions at these sites than other skink sequences.

We suggest that three sequences expressed in the uterus may encode non-classical molecules (*Psen-160Ut*/*Psen-78G*; *Psen-155Ut* and *Psen-164Ut/Psen-122G*). Non-classical loci tend to be invariant or less polymorphic than classical class I genes and show tissue-specific expression patterns (Geraghty 1993; Gouin et al. 2006). Since sequences were obtained from one individual only and only one tissue was investigated, we cannot gauge levels of polymorphism or tissue-specificity except to predict homozygosity or heterozygosity at each putative locus in this individual. While one predicted putative non-classical locus is represented by a single sequence (*Psen-160Ut*/*Psen-78G*), the other two sequences (*Psen-155Ut* and *Psen-164Ut/Psen-122G*) may represent either two separate loci or two alleles of a single locus. While the difference between these two sequences is larger than the difference between putative classical sequences that have been considered separate genes, these sequences also cluster tightly with each other in phylogenetic analyses. Since non-classical genes in mammals often lack polymorphism at the PBR, for example, the non-classical *Modo-UG* locus in the opossum (Gouin et al. 2006), it is more likely that these two sequences represent two separate loci instead of a single heterozygous locus. However, without investigating the tissue-specificity and polymorphism of these transcripts in multiple individuals, description of loci as homozygous or heterozygous, and classical or non-classical, can be only putative.

In addition to describing six expressed loci, we have described three clusters of genomic α_2 sequences that lack expressed equivalents as putative unexpressed loci. The first of these loci is represented by two genomic sequences (*Psen-82G* and *-101G*). There is high conservation between the two sequences but low conservation at the PBR, which may indicate that this locus does not encode a functional class I molecule and may be a pseudogene. The second putative unexpressed locus is represented by a single genomic sequence (*Psen-79G*). This sequence displays the same residues at β_2-microglobulin sites and

the PBR as putative classical class I sequences, which indicates this second putative unexpressed locus may be an unexpressed classical class I gene. While none of the genomic or cDNA α_2 sequences contain premature stop codons or frameshift mutations, full-length sequences of skink class I genes will be isolated in future studies to determine which loci are functional and which are pseudogenes.

The sequences obtained in this study will form the basis of future research on skink MHC. In the absence of previous MHC sequences for skinks, they contribute to current research on the effect of MHC on animal behaviour and mate choice. Heterozygosity at the MHC affects mate choice in fish (Reusch et al. 2001; Aeschlimann et al. 2003), mice (Penn and Potts 1999) and humans (Wedekind et al. 1995). These sequences will be valuable for investigations of mate choice in skink species that demonstrate high mate fidelity, such as Cunningham's skink, *Egernia cunninghami* (V. Repaci pers. comm.) and shinglebacks, *Tiliqua rugosa* (Bull 1988).

We have demonstrated that putative non-classical class I genes are encoded and expressed in a viviparous skink, *Pseudemoia entrecasteauxii*. As *P. entrecasteauxii* exhibits a complex epitheliochorial placenta, it is feasible that embryos from this species down-regulate classical class I expression and upregulate expression of non-classical class I genes to evade the maternal immune system, as occurs in mammals with epitheliochorial placentae. The next step will be to isolate full-length sequences for MHC class I genes from an existing *Eulamprus tympanum* cDNA library using α_2 and α_3 probes. Investigating embryonic expression of non-classical class I genes in oviparous and viviparous species will determine whether the mammalian phenomenon of "hiding" the embryo from the maternal immune system is a consequence of viviparity and the evolutionary loss of the eggshell. The sequence variation at the α_2 domain of class I genes in *P. entrecasteauxii* suggests that locus-specific amplification in PCR is achievable, and robust quantitative comparisons of non-classical class I expression in the embryo could be made between oviparous and viviparous skinks. As cells on the outside of the embryo in contact with the uterine epithelium are most likely to express non-classical class I molecules, *in situ* hybridisation using probes specific for non-classical genes will be also be useful to detect very localised regions of gene expression in the embryo.

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Table 1. Number of major histocompatibility complex (MHC) class I loci characterised in representative eutherian and marsupial mammals, birds, reptiles, amphibians and jawed fishes. Numbers of classical and non-classical class I genes are known only in some species. Total number of class I loci includes pseudogenes as well as classical and non-classical class I genes.

Species name	**Common name**	**Number of class I loci**			**References**
		Classicals	Non-classicals	Total	
Homo sapiens	Human	3	3	18	Geraghty et al. 1992
Mus musculus	Mouse	3	~40	~50	Weiss et al. 1984; Stroynowski and Lindahl 1994
Bos taurus	Cow	?	?	>10	Anderson and Davies 1994
Capra aegagrus	Goat	2	?	10-13	Anderson and Davies 1994
Monodelphis domestica	Opossum	3	6	11	Belov et al. 2006
Macropus eugenii	Tammar wallaby	?	?	>11	Siddle et al. 2006
Sarcophilus harrisii	Tasmanian devil	?	2	>7	Siddle et al. 2007
Gallus gallus	Chicken	2	2	4	Guillemot et al. 1989; Kaufman et al. 1995; 1999
Coturnix japonica	Quail	?	?	>12	Shiina et al. 1999; 2004
Acrocephalus arundinaceus	Great reed warbler	?	?	>4	Westerdahl et al. 1999; 2000
Ameiva ameiva	Common ameiva lizard	?	?	>4	Grossberger and Parham 1992
Sphenodon punctatus	Tuatara	2-3	1	3-4	Miller et al. 2005; 2007
Xenopus laevis	African clawed frog	1	>9	?	Flajnik et al. 1993; Shum et al. 1993
Gadus morhua	Atlantic cod	?	?	2-5?	Persson et al. 1999
Xiphophorus spp.	Swordtail fishes	?	?	>7	Figueroa et al. 2001
Salmo salar	Atlantic salmon	1	?	>8	Miller et al. 2006
Ginglymostoma cirratum	Nurse shark	1	1	2	Ohta et al. 2000
Triakis syllia	Houndshark	1	?	2	Okamura et al. 1997