CHAPTER FOUR:

DEVELOPMENT OF A TYPING SYSTEM TO DETERMINE MHC HAPLOTYPES IN THE ZEBRA

Part of this chapter was published in: *Sixth Havemeyer International Equine Genome Workshop Programme. Ireland July 11-14th 2005.*

ABSTRACT

The outbreak of sarcoid in the Cape mountain zebra herds in South Africa, as the herds were becoming more inbred, raised the possibility of genetic influence. Since susceptibility to sarcoid has been described in different equine breeds all over the world in association with MHC haplotypes these herds were investigated for MHC types. Papillomavirus-induced tumours possessing associations with MHC class II haplotypes have also been encountered in other species. To establish an appropriate typing test, sufficiently simple to test a large amount of zebras, single strand conformational polymorphism (SSCP) of MHC class I and class II genes of zebras was investigated. Primers designed to amplify MHC class I and II genes on DNA samples of horses have been used for the amplification of MHC class I and II genes on DNA samples of *Equus zebra hartmannae*. The results demonstrated that class I genes were highly complex, class II DRA were not polymorphic among zebras and the primers for DQA failed to amplify products in the zebra. DRB and DQB could however be used to score SSCP gels with and without sarcoid.

Keywords: MHC, sarcoid, Cape mountain zebra, SSCP, class II genes DRB and DQB.

4.1 INTRODUCTION

Since 1937, after facing near extinction Cape mountain zebra herds are protected in a few game parks. One fundamental strategy for conservation of a species is to maintain the genetic diversity in a population so that the risk of the population going extinct is minimized, as loss of genetic diversity leads to decreased resistance to disease. The South African Cape mountain zebras are descendants of a nucleus of 11 individuals (Bigalke 1952) and the possibility exists that they become inbred with resultant loss of genetic diversity making them more vulnerable. The major histocompatibility complex (MHC) class I and class II genes play a key role in the initiation of the immune reponse (Klein 1986) and exhibit a high level of genetic polymorphism.

In 1995, equine sarcoid-like lesions appeared in 24.7% of Cape mountain zebras from the Gariep Dam Nature Reserve (Free State Province) (Nel *et al*. 2006) and in 1998 in the Bontebok National Park (Western Cape Province) in 53% of animals (Lange 2004). An isolated case of a zebra that was euthanized due to the severity of sarcoid lesions was reported in 2004 in the Mountain Zebra National Park (Eastern Cape Province) (personal communication: Dr Dave Zimmerman).

Sarcoid susceptibility in horses was found associated with MHC class I or class II antigens in family studies (Marti *et al*. 1996). In particular, horses with MHC class II haplotypes possessing the ELA-W13 serological specificity had an increased risk for sarcoid tumours (Lazary *et al*. 1985). Associations between class II MHC genes and papillomavirus-induced tumours have also been described in other species. In humans, papillomavirus-induced squamous cell carcinoma of the cervix is more common in women carrying the HLA-DQW3 class II MHC allele (Wank & Thomssen 1991). Apple *et al*. (1994) found a papillomavirus type-specific association between HLA DR-DQ haplotypes in this carcinoma of the cervix. Shope papillomavirus, which induces the development of tumours in the rabbit, are associated with class II MHC genes (Han *et al*. 1992).

The association of sarcoid tumors with MHC genes in horses was determined using serological typing for class I and class II MHC genes (Lazary *et al*. 1985; Broström *et al*. 1988). Equine MHC class I and class II genes are closely linked and exhibit linkage disequilibrium. Therefore, it was not possible to determine whether the association was for class I genes, class II genes or another of many genes linked to the MHC. Class I genes are a likely candidate for the association since they are implicated in recognition of virally infected cells (Klein, 1986). However, it is just not possible to determine which genes are implicated because of linkage disequilibrium and the close association of class I and class II genes in a limited number of haplotypes.

Serological studies of MHC in zebras were not possible since the existing reagents for horses did not recognize antigenic structures in zebras (Antczak and Bailey, personal communication). Therefore, MHC typing in zebras needed to be based on molecular approaches. The DNA sequences of genes are highly conserved among mammals. Molecular probes for human and mouse MHC class I and class II genes were effective to study horse MHC genes (Alexander *et al*. 1987). Likewise, PCR primers developed for human class II genes were effective to study horse class II genes (Szalai *et al*. 1993; Szalai, 1994; Szalai *et al*. 1994). Therefore, the premise for these studies was that the molecular tools, effective when used between humans and horses, would also be effective when used for zebras.

Several approaches were available at the beginning of this study, the most sensitive being the cloning and sequencing of MHC genes. However, tens of class I and class II genes were known to exist for horses (Alexander *et al*. 1987) and the cost and labor of approaching the work from this direction would be prohibitive. Fraser (1998) and Fraser and Bailey (1996; 1998) described a rapid and inexpensive approach to investigating genetic variation of equine MHC class II genes by using single strand conformational polymorphism (SSCP).

Single strand conformational polymorphism reproducibly detects genetic variation in DNA and has been used effectively to investigate MHC class II among horses (Fraser 1998). Therefore, the use of SSCP in an experimental approach to determine the extent of genetic variation for the MHC class II genes of zebras was also investigated.

These studies were designed to determine whether the approaches used to characterize variation for the equine MHC class II genes would also be efficient for zebras.

4.2 MATERIALS AND METHODS

4.2.1 Zebras and horse DNA samples for comparison

DNA samples (n=14) from Hartmann's mountain zebra (*Equus zebra hartmannae*) were received at Gluck Equine Research Center, Kentucky, from the Center for Reproduction of Endangered Species in San Diego California mainly for the development of a typing system of the zebra MHC. No family data was available on these zebra. For comparison, DNA isolated from peripheral blood cells from five unrelated domestic horses (*Equus caballi*) were used as controls. Comparisons of these two equid species were conducted at the Maxwell H. Gluck Equine Research Center at the University of Kentucky, USA.

4.2.2 Conventional PCR amplification of a second exon class II

Amplification of the class I and II (DRA, DRB, DQA and DQB) genes was performed using the primers as shown in **Table 5**. The amplified regions lie within the second exon of both genes and were used in genomic amplifications for SSCP typing. In class I, two zebras were used, in class II, DRA, two horses and nine zebra, in DRB two zebras and in DQB three zebras were used.

Table 5 Primers used for amplification of class I and II gene products.

Primer	Primer sequence	Bp	Reference
Classs I-2F	(5'-GCT CCC ACT CCA TGA G)	269 bp	
Class I-2R	(5'-GGC CTC GCT CTG GTT)		(Fraser 1998)
Class II DRA Be1	(5'-GGA TCC AGG CTG AGT TCT ATC TG)	229 bp	
Class II DRA-Be2	(5'-GGC TTA AGA GTG TTG TTG GAG CGC TT).		(Bailey 1994)
Class II DRB 2a	(5'-CTC TGC AGC ACA TTT CCT GGA G)	276 bp	
Class II DRB 2b	(5'-CGC CGC TGC ACC AGG AA)		(Fraser & Bailey 1996)
Class II DQA 2e	(5'-CTG AIC ACI TTG CCT CCT ATG)	246 bp	
Class II DQA 2f	(5'-TGG TAG CAG CAG IAG IGT TG)		(Fraser & Bailey 1998)
Class II DQB-GH28	(5'-CTC GGA TCC GCA TGT GCT ACT TCA CCA ACG)	210 bp	
Class II DQB-GH29	(5'-GAG CTG CAG GTA GTT GTG TCT GCA CAC)		(Szalai 1994)

The amplification mixture consisted of 2.0 µl DNA (~75ng), 0.2 µl *Taq* DNA polymerse (New England BioLabs inc) 1 µl 10X buffer with $MgCl_2$, 0.5 µl of each primer (Integrated DNA Technologies, Inc, Coralville, Iowa, USA), 1 µl (100 µM) dNTP's (Invitrogen, Carlsbad, California, USA) and nuclease free water to a total volume of 10 µl. The thermal profile for the amplification was as follows: initial denaturation was performed at 95 °C for 10 minutes. This was followed by 30 cycles, each consisting of 95 °C for 30 seconds, annealing at 56 °C for 30 seconds and extension was at and 72 °C for 30 seconds followed by a final extension at 72 °C for 10 minutes before cooling to 4 °C. The amplification was performed in an automated thermocycler (PerkinElmer, Foster City, CA).

4.2.3 Cloning and sequencing

The PCR amplicons obtained were purified and cloned into plasmid vectors using Invitrogen TA (Carlsbad, CA) cloning kit, and transformed into competent *Esherichia coli* cells (One Shot® INVαF' Invitrogen). Recombinant plasmid DNA was extracted and directly sequenced using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 3100 sequencer. Consensus sequences were compared to sequences obtained from GenBank using BLAST.

4.2.4 MHC Typing: Single strand conformational polymorphism analysis

Analysis was based on the SSCP method originally described by Orita *et al*. (1989). The SSCP gels consisted of a 10% w/v acrylamide:bis-acrylamide (37.5:1) (BioRad, USA) 0.5X TBE buffer (44.5mM boric acid (Merck, USA), 1mM Na_2-EDTA(Merck, USA), pH=8.4), 0.093% w/v ammonium persulphate (Merck, USA) and 0.08% v/v N,N,N',N'-tetramethylethylenediamine (TEMED) (Merck, USA). The samples consisted of PCR products from genomic DNA as well as cloned PCR products.

Samples (8 µl water + 3 µl loading dye { 95% formamide, 20mM Na_2-EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF}, + 2 µl PCR products) were denatured in a heating block at 90 °C for 10 minutes and put immediately on ice until loaded. The gels were electrophoresed in 0.5X TBE buffer on a Protean II xi cell gel apparatus (BioRad Laboratories Inc., Melville, NY) at 100 volts for 20 hours at 20 °C. The SSCP gel was visualized by staining for 10 minutes in 0.5X TBE with 1 ul of a 1 mg/ml stock solution of ethidium bromide, and photographed under UV trans-illumination. Gels were scored by counting the number of fragments visible on the gel (**Table 7**).

4.3 RESULTS

4.3.1 Sequencing

The equine-specific primers successfully amplified the 2nd exon of the MHC class I genes and class II genes DRA, DRB and DQB. These primers were not effective in amplifying the 2nd exon of DQA in the zebras.

4.3.1.1 Class I

The alignment of nucleotide sequences for the 2nd exon of the MHC class I genes for clones from two Hartmann's mountain zebras (*Ezh*) number 509 (four clones) and number 1568 (five clones) is shown in **Figure 13** as well as domestic horses {*Equus caballus (Ec)*} accession numbers DQ083420 and X79892.

Among four clones for zebra 509, there were clearly three different class I sequences. Among five clones for zebra 1586 there were four different sequences. A BLAST search showed that the highest similarity (~96%) occurred with MHC class I antigen *Ec* DQ083420 and (~94%) with *Ec* X79892, confirming that the products are MHC class I.

Figure 13 Alignment of nucleotide sequences for the 2nd exon of the MHC class I clones used for developing a typing system (designated "I2-animal number-clone number") from (*Ezh*) 509 and 1586. *Ec* accession numbers (DQ083420) and (X79892) are used as references.

4.3.1.2 Class II

4.3.1.2.1 DRA

All sequence data were consistent with a single locus for DRA. Clones (15) from three unrelated zebras (*Ezh* 132, 509, 510) were sequenced and no more than two different sequences were found. Subsequent sequencing of genomic DNA of five zebras (*Ezh* 10949, 6819, 3183, 454, 2040) in this study produced data consistent with a single locus. Sequences for clones or genomic sequences for Hartmann's zebras and control horses are demonstrated in **Figure 14** with reference sequences of *Ec* accession number M60100 and *Equus grevyi (Eg)* accession numbers EU930116, EU930124, EU930125. BLAST results revealed the highest similarity (~99%) with MHC class II DRA gene in *Eg* accession number EU930116 and (~98%) with *Ec* accession number M60100 and *Eg* accession number EU930125 and *Eg* accession number EU930124 confirming the products are MHC class II DRA. Alignment is raw data. Past sequence 210, the sequence overlaps with the site for primer Be2. The sequence reflects a discrepancy between the Be2 primer sequence and the genomic DNA sequence. Three alleles from 28 sequences are found.

```
                     10        20        30        40        50        60        70        80        90       100
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DRA-509-3i   ----------------------------AANNCTGGNGA-TCAGGGGN-TTTATGTTTGACTTTGATGGTGATGAGATTTTTCATGTGGATATGGACAAG
DRA-510-3i   ----------------------------....N......-........-...................................................
DRA-510-2i   ----------------------------...........-........-...................................................
H2455        ----------------------------....T......-.N...N..-...................................C...............
g132i        -----------------------------......N...-.....N..-...................................N...............
EU930125     CGTGATCATCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
EU930124     CGTGATCATCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
EU930116     CGTAATCATCCAGGCTGAGTTCTATCTG...........C.....A..G...................................C...............
M60100       CGTAATCATCCAGGCTGAGTTCTATCTG...........C.....A..G...................................C...............
DRA-10494    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-509-7    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-509-9    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-509-2    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-510-ii   CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-132-a    ----------------------------------------.....AA.T..A--TG..TG........................................
DRA-132-b    ----------------------------------------.....AA.T...--..............................................
DRA-6819     TGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-g509     CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-g510     CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-132-4    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-509-6    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-132-7    CGTGA-C-TCCAGGCTGAGTTCTATCTG.......A...C.....A..G...................................C...............
DRA-132-9    CGTGA-C-TCCAGGCTGAGTTCTATCTG.......A...C.....A..G..................................GC...............
DRA-g132     CGTGA-C-TCCAGGCTGAGTTCTATCTG.......A...C.....A..G...................................N...............
DRA-132-ii   CGTGA-C-TCCAGGCTGAGTTCTATCTG.......A...C.....A..G...................................C...............
DRA-2040     CGTGA-C-TCCAGGCTGAGTTCTATCTG.......A...C.....A..G...................................C...............
DRA-g3183    CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-3183     CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-509-ii   CGTGA-C-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-454      CGTGATC-TCCAGGCTGAGTTCTATCTG...........C.....A..G...................................................
DRA-132-4i   ----------------------------------------------------------------------------------------------------

                    110       120       130       140       150       160       170       180       190       200
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
DRA-509-3i   AAGGAGACGGTCTGGCGGCTTGAAGAATTTGGACGTTTTGCCAGCTTTGAGGCTCAGGNTGCTTTGNCCAATATAGCTGTGGACAAAGCCAACCTGGAGA
DRA-510-3i   ...........N...N....................................................................................
DRA-510-2i   ...........N.............................N..........................N.......N.......................
H2455        .......................................A............................................................
g132i        .........................................................................................N..........
EU930125     .........................................................................................T..........
EU930124     .........................................................................................T..........
EU930116     ....................................................................................................
M60100       .......................................A............................................................
DRA-10494    ....................................................................................................
DRA-509-7    ....................................................................................................
DRA-509-9    ....................................................................................................
DRA-509-2    ....................................................................................................
DRA-510-ii   ....................................................................................................
DRA-132-a    ....................................................................................................
DRA-132-b    ....................................................................................................
DRA-6819     ....................................................................................................
DRA-g509     .........................................................................................N..........
```

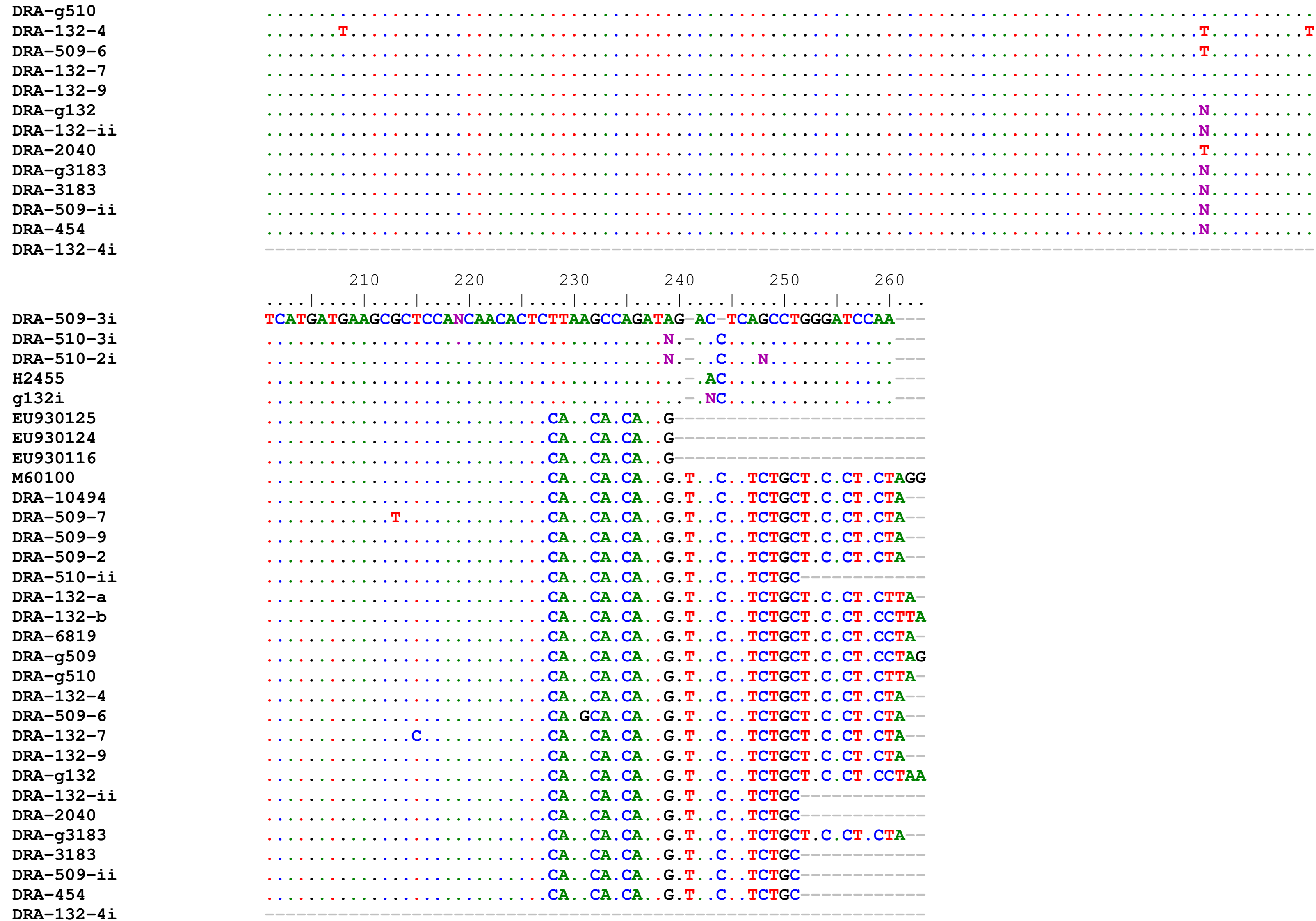

Figure 14 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DRA clones (designated "DRA-animal number–clone number") or genomic sequence (designated "g" followed by animal number) from *Ezh* 132, 509, 510, 3183, 454, 10494, 2040, 6819, and horses H2455 and H2449. Reference sequences of *Ec* accession number (M60100) and *Eg* accession numbers (EU930116, EU930124, EU930125) are used.

In **Table 6** a summary of known DRA 2nd exon allele sequences plus the DRA alleles found among Hartmann's Zebras (prefix *Ezh*) and two horses, when typed by sequencing clones or sequencing genomic DNA are shown. The base present at different positions for each allele is shown with a listing of position number for sequence position obtained in this study followed by the position from Albright-Fraser *et al.* (1996) listed in parentheses. The Hartmann's zebras possessed the alleles previously identified by Fraser (1998) and Brown *et al.* (2004) as 0501, 0601 and JBZ185. The types for *Ezh* numbers 132, 509 and 510 were confirmed by sequencing clones of exon 2. The genotypes from the others appeared to be the result of a simple polymorphism at a single site (DNA base 188). The type for *Ezh* 7040 was deduced from genomic DNA as the simplest explanation for the genotype with the known DRA alleles; the type needs to be confirmed by cloning and sequencing.

Table 6 Summary of known DRA 2nd exon allele sequences plus the DRA alleles found among Hartmann's Zebras (prefix *Ezh*) and two horses when typed by either sequencing clones or sequencing genomic DNA.

ALLELE	**34(11)**	**83(93)**	**133(142)**	**138(144)**	**182(193)**	**188(199)**	**TYPE**
0101	G	C	G	G	C	C	0101
0201	G	C	G	A	C	C	0201
0301	G	C	A	G	G	C	0301
0401	G	C	A	G	C	C	0401
0501	G	T	G	G	C	C	0501
0601	A	C	G	G	C	C	0601
JBZ185	G	T	G	G	C	T	JBZ185
JBH11	G	C	G	G	C	T	JBH11
EQUID							
Ezh 10494	G	T	G	G	C	C	0501
Ezh 132	A/G	C/T	G	G	C	C/T	0601/JBZ185
Ezh 3183	G	T	G	G	C	C/T	0501/JBZ195
Ezh 454	G	T	G	G	C	C/T	0501/JBZ185
Ezh 509	G	T	G	G	C	C/T	0501/JBZ185
Ezh 510	G	T	G	G	C	C	0501
Ezh 6189	G	T	G	G	C	C	0501
Ezh 7040	A/G	C/T	G	G	C	T/C	?JBZ185/?601
Horse 2449	G	C	G/A	G/A	G/C	C	0201/0301
Horse 2455	G	C	G	A	C	C	0201

4.3.1.2.2 DRB

In the sequencing data of nine clones from zebra 510 and one clone from zebra 509 evidence for only two sequences was found. It is likely that zebra 509 has but a single DRB locus and shares an allele with zebra 510. The sequencing data are demonstrated in **Figure 15**. Two different *Ezh* consensus sequences (represented by clones 510-6 and 510-1) were compared to GenBank sequences using BLAST. No exact homology was found. However, the two sequences were most similar to DRB alleles found in Przewalski horses (*Equus ferus przewalski*) *Eqpr*-DRB*2 and *Eqpr*-DRB*5.

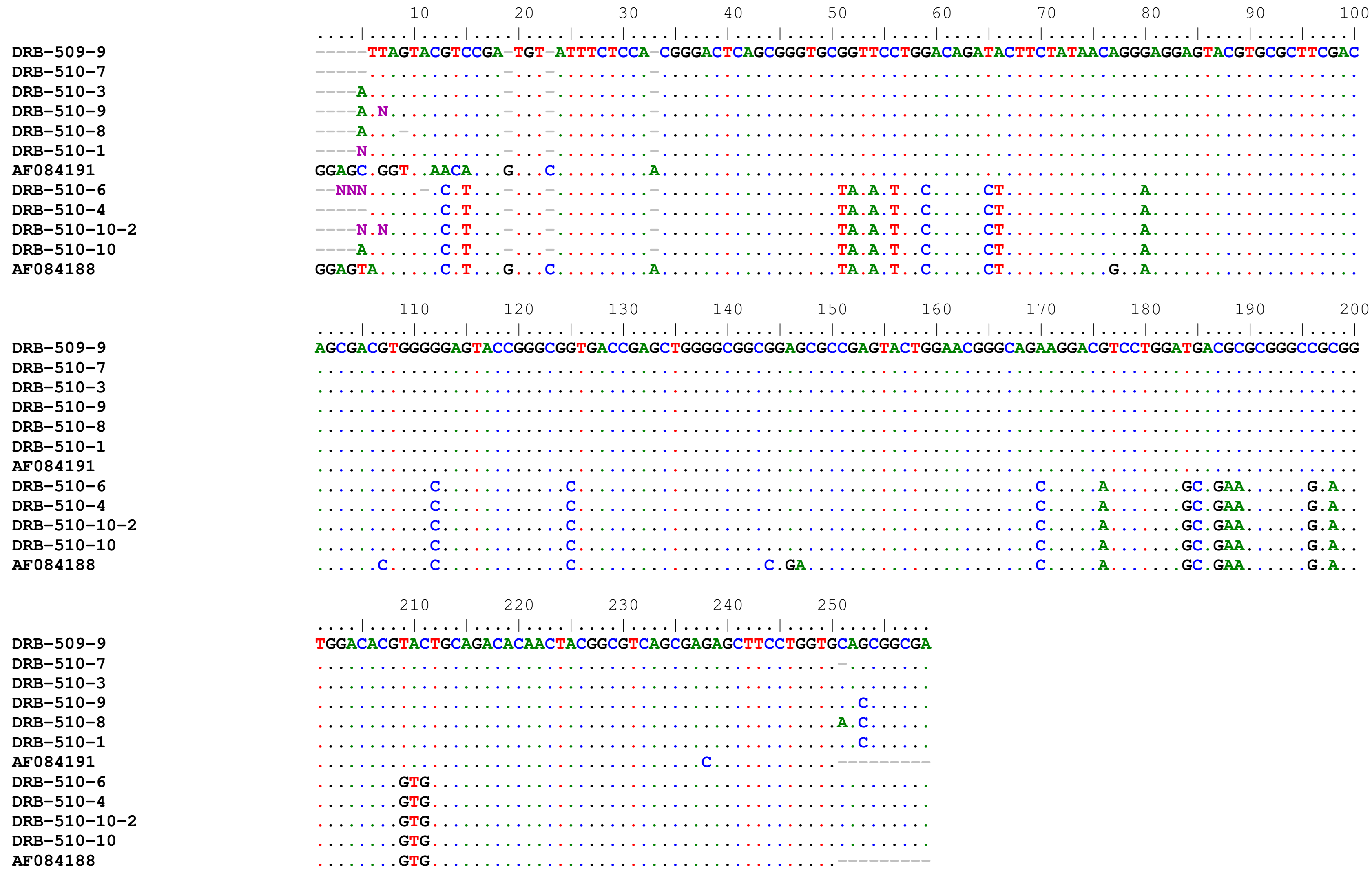

Figure 15 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DRB clones (designated "DRB-animal number-clone number") from *Ezh* 510 and 509 as well as *Eqpr*AF 084188 and AF08491.

4.3.1.2.3 DQB

The data for DQB are consistent with the fact that two loci for DQB in these zebras exist. Zebra 132 had four sequences among seven clones tested, zebra 509 had four sequences among nine clones tested and zebra 510 had three sequences among six clones tested. The sequencing data are demonstrated in **Figure 16**. The data do not exclude the existence of more loci but are minimally compatible with two loci and complete heterozygosity for 132 and 509. As reference sequences *Equus asinus (Ea)* accession number (AF034125) and *Ec* accession numbers (L33910) are used with which BLAST results revealed highest similarity: *Ea* (~94%) and *Ec* (~93%) confirming that the products are MHC class II DQB.

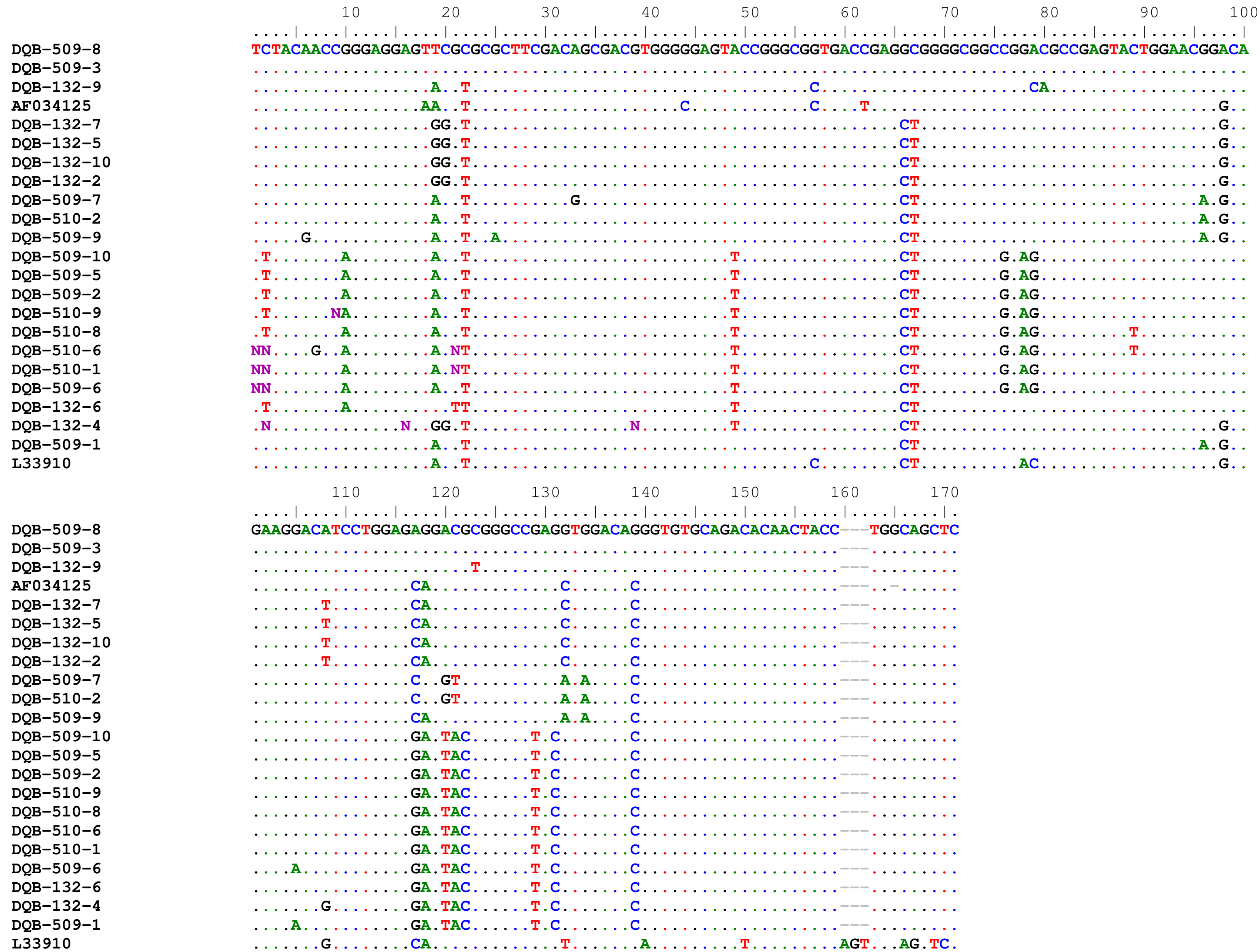

Figure 16 Alignment of nucleotide sequences for the 2nd exon of the MHC class II DQB clones (designated "DQB-animal number-clone number") from *Ezh* 132, 509 and 510. *Ea* accession number AF034125 and *Ec* accession number L33910 are used as references.

4.3.2 Single strand conformational polymorphism

4.3.2.1 DRB

Single strand conformational polymorphism testing was conducted with eight Hartmann's zebras and four horses. Polymorphic patterns were identified as shown in (**Figure 17**).

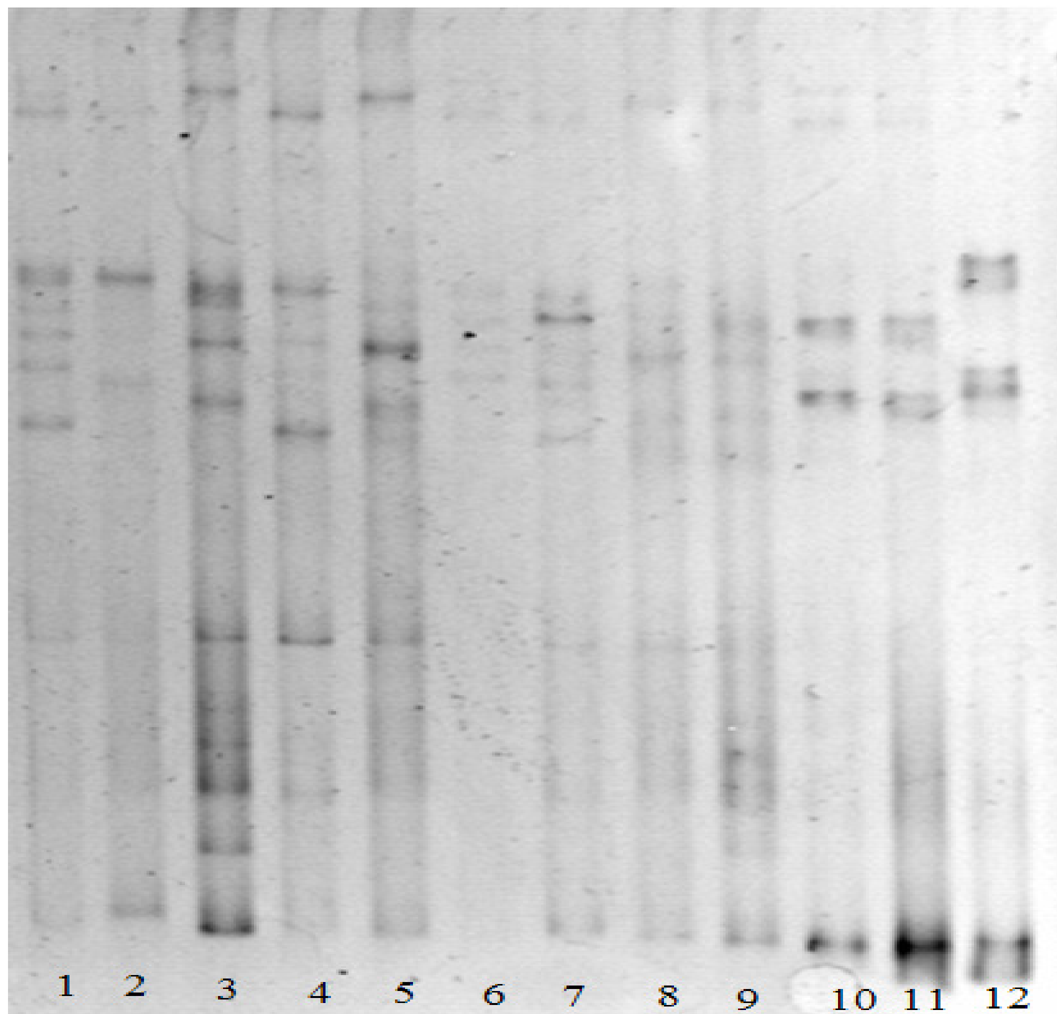

Figure 17 SSCP gel image of DRB patterns for zebras (*Ezh*) and horses (*Ec*) demonstrated in reverse contrast to the ethidium bromide stained gel: Lanes 1-12 represent *Ezh* 3183, *Ezh* 132, *Ec* 90, *Ezh* 510, *Ec* 2449, *Ezh* Solo, *Ezh* 509, *Ec* 190, *Ec* 172, *Ezh* 3861, *Ezh* 7041, *Ezh* 6819.

4.3.2.2 DQB

Single strand conformational polymorphism testing was conducted with 11 Hartmann's zebras and four horses. Polymorphic patterns were identified as shown in (**Figure 18**).

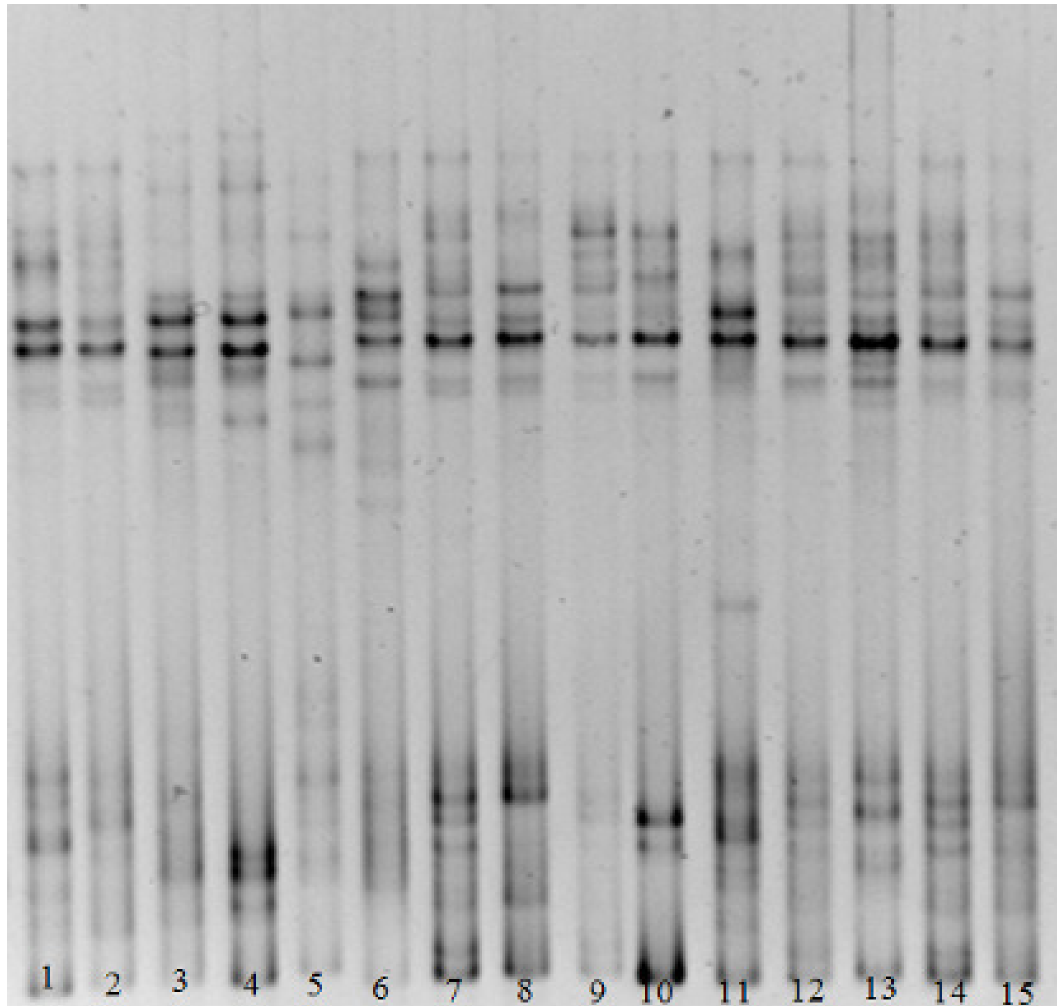

Figure 18 SSCP patterns for DQB in horses (*Ec*) and zebras (*Ezh*) demonstrated in reverse contrast to the ethidium bromide stained gel: Lanes 1-15 represent: *Ezh* 7040, *Ezh* Solo, *Ec* 172, *Ec* 90, *Ec* 190, *Ezh* 132, *Ezh* 509, *Ezh* 510, *Ezh* 3867, *Ezh* 7041, *Ezh* 6819, *Ezh* 454, *Ec* 2449, *Ezh* 3186, *Ezh* 10494.

Comparing gels from samples obtained from the zebra and the horse, the range and averages were similar but the zebras did exhibit slightly fewer bands than the domestic horses (**Table 7**). The identity of the DNA fragments as DRB products was confirmed by DNA sequencing for two individuals *Ezh* 509 and 510 (**Figure 15**) and the identity of DQB products was confirmed by DNA sequencing for three individuals *Ezh* 509, 510 and 132 (**Figure 16**).

Table 7 SSCP results from domestic horses *Equus caballus* (*Ec*) and Hartmann's zebra *Equus zebra hartmannae* (*Ezh*) for DRB and DQB.

Species	Range DRB: Numbers of SSCP bands	Ave. DRB	Range DQB: Numbers of SSCP bands	Ave. DQB
Ec	8-14	12.6	9-13	11.6
Ezh	6-13	10.2	6-12	10.6

4.4 DISCUSSION

Class I sequences demonstrated that among four clones tested for zebra 509, there were clearly three different class I sequences. Among five clones tested for zebra 1586 there were four different sequences. While one or two of the differences could be the result of cloning artifacts, there were multiple differences demonstrating that these were related but clearly different genes.

Based on projections from horses (Ellis *et al*. 1995; Tallmadge *et al*. 2005) and other species; (Aoyagi *et al*. 2002; Moon *et al*. 2005; Xu *et al*. 2007) and based on these preliminary studies, the class I genes are likely to be highly complex and require significantly greater work to characterize haplotypes in zebras than investigations of other MHC genes, such as class II loci.

In class II loci, the DRA gene is considered almost monomorphic in most species tested (Fraser *et al*. 1994; Zhou *et al*. 2007; Xu *et al*. 2007; Ballingall *et al*. 2010), but polymorphic in horses and other equids (Bailey 1994; Albright-Fraser *et al*. 1996; Brown *et al*. 2004; Luis *et al*. 2005; Arbanasic *et al*. 2009). The genetics of the DRA in zebras is unknown except that the methods used to characterize DNA sequences of horse exon two DRA are effective in other equine species.

All sequence data were consistent with a single locus for the DRA gene. Clones from three unrelated zebras were sequenced and none found to have more than two different sequences. If three different sequences were confirmed, this would suggest the existence of more than one locus. Subsequent sequencing of genomic DNA for five more zebras produced data consistent with a single locus. The sequencing data suggested that there were three alleles among the eight Hartmann's zebras (0501, 0601 and JBZ185) (**Table 6**).

Single strand conformational polymorphism typing led to the characterization of the alleles identified by Albright-Fraser *et al*. (1996) but did not allow clear assignment of genotypes for the

three different alleles. Specifically, separation was not obtained to distinguish between alleles 0501, 0601 and JBZ185. Therefore the alleles had to be distinguished by direct DNA sequencing. It can also be distinguished by another method, perhaps a method involving hybridization of target molecules.

As a result of not being polymorphic, the DRA gene cannot be used for typing in the zebra for differentiating between sarcoid and healthy zebra.

In the horse DRB has as many as three loci (Fraser & Bailey 1996) and possibly only two for some horses. In the Przewalski horse there appear to be only two loci (Hedrick *et al*. 1999). In the horse, this was too complex to type using SSCP and Fraser (1998) simply reported the existence of more than 23 alleles without being able to identify which loci were responsible. Diaz *et al*. (2001) confirmed multiple DRB copies in Argentine Creole horses, but were unable to identify specific alleles with specific loci.

In the absence of family data or of discrete molecular data for each individual, it is not possible to make a direct assignment of genotype from the phenotypes observed on the SSCP gels. However, one can compare the limited sequencing data with the SSCP phenotypes to determine whether the limited polymorphism found for sequencing corresponds to limited variation on the stained gel. We determined that zebra 510 is a heterozygote and that zebra 509 shares at least one allele. From the gel in **Figure 17** we can see that zebra 510 and zebra 509 each have four dark staining bands and additional faint bands. There is one pair of dark staining bands in zebra 510 that does not appear in zebra 509 and vice versa. Therefore, this is consistent with sharing one allele but each having a second different allele at that locus. Likewise, the phenotypic patterns observed when testing additional zebras are intermediate in complexity when compared to that observed for the horse / zebra DRA locus and the DRB locus in horses which is known to possess three loci (Fraser & Bailey 1996).

When examining the phenotypes for nine Hartmann's zebra, there are clear differences in phenotypes among them but there are also many bands shared between them. It is possible to score each zebra on each gel for the presence of fragments in common between different pairs of sets of zebra, reflecting the sharing of alleles at the DRB locus. This approach can be used to compare zebras with sarcoid tumours to those without. When a fragment is found more commonly among one group, it will be subjected to further study by cloning, sequencing and application of more specific typing of all zebras.

In horses the DQA locus was found to comprise a single locus in the MHC with at least 17 alleles and probably more (Szalai *et al*. 1994). Typing on SSCP resulted in patterns that reflected the great diversity of the locus but were relatively simple to interpret (Fraser 1998). Unfortunately, the primers that amplified the 2nd exon of DQA in horses were not effective in amplifying the product in zebras. Specifically, multiple products were produced and it was not possible to make the reaction more specific by increasing stringency. It is likely that success in typing for this locus would require the development of *Ezh* specific primers. Nevertheless, as a result of these difficulties this approach was not pursued, especially since the DRB and DQB appeared to be more effective.

In the horse Szalai *et al*. (1993) described a single locus with 13 alleles, for the DQB locus although Fraser (1998) found that the amplification strength of the alleles varied tremendously. Hořin and Matiašovic (2002) described two DQB loci and at least two groups of alleles were shown to exist. This data are consistent with the existence of two loci for DQB in these zebras. Zebra 132 had four different sequences among seven clones tested, zebra 509 had four sequences among nine clones tested and zebra 510 had three sequences among six clones tested. The data do not exclude the existence of more loci but are minimally compatible with two loci and complete heterozygosity for zebra 132 and zebra 509.

The DQB SSCP image (**Figure 18**) has two zones with DNA fragments. The pattern on the top is different from the pattern on the bottom so they represent entirely different gene products and not simply the positive and negative strand of DNA. This phenotypic appearance may reflect locus differences in the migration of allele fragments from the two (minimum) loci for DQB in zebras. Future genetic studies could be directed toward determining the relationship of alleles, loci and the SSCP pattern.

As noted from the sequencing it can be anticipated that DQB genes have at least two loci. This is reflected in the more complex SSCP phenotypic pattern observed when compared to the pattern for DRB (presumed one locus) shown above (**Figure 15**). As described for DRB, the bands can be scored and compared for the zebras on each gel and homologous and non-homologous fragments identified, reflecting similarities and difference in DQB MHC types between the individuals.

In conclusion, to establish the haplotypes in the zebra, class II MHC genes DRB and DQB could be used as a typing system in the sarcoid-affected zebra herds.

4.5 REFERENCES

Albright-Fraser DG, Reid R, Gerber V, Bailey E, 1996. Polymorphisms of DRA among equids. Immunogenetics 43:315-317 erratum. *Immunogenetics* 44:487.

Alexander AJ, Bailey E, Woodward JG, 1987. Analysis of the equine lymphocyte antigen system by Southern blot hybridization. *Immunogenetics* 25:47-54.

Aoyagi K, Dijkstra JM, Xia C, Denda I, Ototake M, Hashimoto K, Nakanishi T, 2002. Classical MHC class I genes composed of highly divergent sequence lineages share a single locus in rainbow trout (*Oncorhynchus mykiss*). *The Journal of Immunology* 168:260-273.

Apple RJ, Ehrlich HA, Klitz W, Manos MM, Becker TM, Wheeler CM, 1994. HLA DR-DQ associations with cervical carcinoma show papillomavirus-type specificity. *Nature Genetics* 6:157-162.

Arbanasic H, Galov A, Salajpal K, Curik I, 2009. Diversity of equine major histocompatibility complex class II locus in Posavina and Croatian Coldblood horse: a new polymorphism detected. *Italian Journal of Animal Science* 8:77-79.

Bailey E, 1994. Variation within the antigen binding site of the major histocompatibility complex DRA gene of domestic horses. In: *Equine Infectious Diseases VII*. Nakajiama H, Plowright W, (eds), R & W Publications, Newmarket, UK, 123-126.

Ballingall KT, Rocchi MS, McKeever DJ, Wright F, 2010. Trans-species polymorphism and selection in the MHC classII DRA genes of domestic sheep. *PLoS ONE* 5(6): e11402.

Bigalke R, 1952. Early history of the Cape mountain zebra. (*Equus zebra zebra* Linn). *African Wildlife* 6:143-153.

Broström H, Fahlbrink E, Dubath ML, Lazary S, 1988. Association between equine leucocyte antigens (ELA) and equine sarcoid tumors in the population of Swedish half breds and some of their families. *Veterinary Immunology and Immunopathology* 19:215-223.

Brown JJ, Clegg P, Eyre S, Kennedy LJ, Matthews J, Carter S, Ollier WER, 2004. Polimorphisms of the equine major histocompatibility complex class II DRA locus. *Tissue Antigens* 64:173-179.

Diaz S, Giovambattista G, Dulout FN, Peral-Garcia P, 2001. Genetic variation of the second exon of ELA-DRB genes in Argentine Creole horses. *Animal Genetics* 32:257-263.

Ellis SA, Martin AJ, Holmes EC, Morrison WI, 1995. At least four MHC class I genes are transcribed in the horse: phylogenetic analysis suggests an unusual evolutionary history for the MHC in this species. *European Journal of Immunogenetics* 22:249-260.

Fraser DG, 1998. Studies on the organization and the polymorphism of equine MHC Class II genes. *PhD Thesis*, University of Kentucky, Lexington, Kentucky. 115p.

Fraser DG, Bailey E, 1996. Demonstration of three DRB loci in a domestic horse family. *Immunogenetics*. 44:441-445.

Fraser DG, Bailey E, 1998 Polymorphism and multiple loci for the horse DQA gene. Immunogenetics 47:487-490.

Fraser DG, Craigmile S, Russell GC, 1994. Molecular cloning and sequencing of a cattle DRA cDNA. *Immunogenetics* 40:311.

Han R, Breitburd F, Marche PN, Orth G, 1992. Linkage of regression and malignant conversion of rabbit viral papillomas to MHC class II genes. *Nature* (London). 356:66-68.

Hedrick PW, Parker KM, Miller EL, Miller PS, 1999. Major histocompatibility complex variation in the endangered Przewalski horse. *Genetics* 152:1701-1710.

Hořin P, Matiašovic J, 2002. A second locus and new alleles in the major histocompatibility complex class II (ELA-DQB) region in the horse. *Animal Genetics* 33:196-200.

Klein J, 1986. *Natural history of the major histocompatibility complex*. John Wiley & Sons, Inc., New York.

Lange AL, 2004. Ongoing investigation into an outbreak of sarcoid tumours in the Cape mountain zebra of the Bontebok National Park, Swellendam, *A report presented to SANParks*.

Lazary S, Gerber H, Glatt PA, Straub R, 1985. Equine leucocyte antigens in sarcoid affected horses. *Equine Veterinary Journal* 17:283-286.

Luis C, Cothran EG, Oom MM, Bailey E, 2005. Major histocompatibility complex locus DRA polymorphism in the endangered Sorraia horse and related breeds. *Journal of Animal Breeding and Genetics* 122:69-72.

Marti E, Szalai G, Antczak AF, Bailey E, Gerber H, Lazary S, 1996. The Equine Major Histocompatibility Complex. In: *The Major Histocompatibility Complex Region of Domestic Animal Species*. Shook LB, Lamont SJ, (eds), CRC Press, Inc., Boca Raton, Florida. 245-267.

Moon DA, Veniamin JA, Parks-Dely, Magor KE, 2005. The MHC of the duck (*Anas platyrhynchos*) contains f*ive* differently expressed class I genes. *The Journal of Immunology* 175:6702-6712.

Nel PJ, Bertschinger H, Williams J, Thompson PN, 2006. Descriptive study of an outbreak of equine sarcoid in a population of Cape mountain zebra (*Equus zebra zebra*) in the Gariep Nature Reserve. *Journal of the South African Veterinary Association* 77:184-190.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekija T, 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proceedings of the National Academy of Sciences of the United States of America* 86:2766-2770.

Szalai G, 1994. Studies of the equine major histocompatibility complex DQ genes. *PhD Thesis*, University of Berne, Berne, Switzerland 82p.

Szalai G, Antczak DF, Gerber H, Lazary S, 1994. Molecular cloning and characterization of horse DQA cDNA. *Immunogenetics* 40:457.

Szalai G, Bailey E, Gerber H, Lazary S, 1993. DNA sequence analysis of serologically detected ELA class II haplotypes at the equine DQB locus. *Animal Genetics* 24:187-190.

Tallmadge RL, Lear TL, Antczak DF, 2005. Genomic characterization of MHC class I genes of the horse. *Immunogenetics* 57:763-774.

Wank R, Thomsson C, 1991. High risk of squamous cell carcinoma of the cervix for women with HLA-DQw3. *Nature* (London). 352:723-725.

Xu S, Sun P, Zhou K, Yang G, 2007. Sequence variability at three MHC loci of finless porpoises (*Neophocaena phocaenoides*). *Immunogenetics* 59:581-592.

Zhou H, Hickford JG, Fang Q, Byun SO, 2007. Identification of allelic variation at the bovine DRA locus by polymerase chain reaction-single strand conformational polymorphism. *Journal of Diary Science* 90:1943-1946.