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A Comparative Analysis of Genetic Diversity at Mhc *DRB* Loci in Some Ruminant Species

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Abstract

In this thesis the highly polymorphic bovine Mhc class II *DRB3* locus (*BoLA-DRB3*) and homologous loci in other ruminant species have been characterized. The aim of the work was to broaden the knowledge about the evolution and significance of Mhc diversity. *DRB* exon 2 polymorphism was investigated by single strand conformation polymorphism (SSCP) analysis, and by DNA sequence analysis. The *DRB* exon 2 sequences showed considerable variation in cattle, bison, reindeer, and red deer whereas, moose, muscox, roe deer, and fallow deer, exhibited low or no polymorphism. Both European and North American populations of moose exhibit very low levels of genetic diversity at the expressed *DRB1* locus. The data imply that the moose has lost Mhc diversity in a population bottleneck prior to the divergence of the two subspecies, more than 100,000 years ago. Thus, a restricted Mhc diversity may be compatible with long-term survival. A comparative analysis of the *DRB3* polymorphism in bison and cattle revealed an extensive sharing of sequence motifs. The result clearly showed a trans-species persistence of sequence motifs in the two species. A deletion of codon 65 was found in three *BoLA-DRB3* alleles, and in one roe deer *DRB1* allele. It could not be resolved whether the presence of the deletion in cattle and roe deer was due to common ancestry or parallel mutations. Analysis of the pattern of sequence polymorphism in ruminant *DRB* alleles suggests that both positive selection for polymorphism and interallelic recombination have contributed to the generation of Mhc diversity at this locus.

Keywords: Mhc, class II, BoLA, DRB, cattle, deer, polymorphism, evolution, deletion

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In this thesis the highly polymorphic bovine Mhc class II *DRB3* locus (*BoLA-DRB3*) and homologous loci in other ruminant species have been characterized. The aim of the work was to broaden the knowledge about the evolution and significance of Mhc diversity. *DRB* exon 2 polymorphism was investigated by single strand conformation polymorphism (SSCP) analysis, and by DNA sequence analysis. The *DRB* exon 2 sequences showed considerable variation in cattle, bison, reindeer, and red deer whereas, moose, muscox, roe deer, and fallow deer, exhibited low or no polymorphism. Both European and North American populations of moose exhibit very low levels of genetic diversity at the expressed *DRB1* locus. The data imply that the moose has lost Mhc diversity in a population bottleneck prior to the divergence of the two subspecies, more than 100,000 years ago. Thus, a restricted Mhc diversity may be compatible with long-term survival. A comparative analysis of the *DRB3* polymorphism in bison and cattle revealed an extensive sharing of sequence motifs. The result clearly showed a trans-species persistence of sequence motifs in the two species. A deletion of codon 65 was found in three *BoLA-DRB3* alleles, and in one roe deer *DRB1* allele. It could not be resolved whether the presence of the deletion in cattle and roe deer was due to common ancestry or parallel mutations. Analysis of the pattern of sequence polymorphism in ruminant *DRB* alleles suggests that both positive selection for polymorphism and interallelic recombination have contributed to the generation of Mhc diversity at this locus.

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III. Mikko, S., Spencer. M., Morris, B., Stabile, S., Basu, T., Stormont, C., and Andersson, L. (1997) A comparative analysis of the Mhc *DRB3* polymorphism in the American Bison (*Bison bison*). *Journal of Heredity, in press.*

IV. Mikko, S., Lewin, H., and Andersson, L. (1997) A phylogenetic analysis of cattle *DRB3* alleles with a deletion of codon 65. *Submitted.*

V. Mikko, S., Røed, K., Schmutz, S., and Andersson, L. (1997) Genetic diversity at major histocompatibility complex *DRB* loci in some domesticated and wild ruminant species. *Manuscript.*

VI. Neighbor-joining phylogenetic tree of the ruminant *DRB* alleles included in this thesis. To be found on the inside of the back cover.

Abbreviations

Am.	American English
APC	Antigen Presenting Cell
BoLA	Bovine Leukocyte Antigens
Br.	British English
IEF	Isoelectrical Focusing
Mhc	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
PBS	Peptide Binding Site(s)
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
SSCP	Single Strand Conformation Polymorphism
SSO	Sequence Specific Oligotyping

Taxonomic classification

Order: Artiodactyla	
Family: Bovidae	
Subfamily: Bovinae	
Bos primigenius taurus	European cattle
Bos primigenius indicus	African cattle
Bison bison	North American bison
Subfamily: Caprinae	
Ovibos moschatus	Muskox
Capra aegrius	Domestic goat
Ovis aries	Domestic sheep
Family: Cervidae	
Subfamily: Odocoileinae	
Alces alces	Moose (Am.), elk (Br.)
Capreolus capreolus	Roe deer
Rangifer tarandus	Reindeer
Subfamily: Cervinae	
Cervus dama	Fallow deer
Cervus elaphus	Red deer (Br.), wapiti (Am.)

Introduction

The major histocompatibility complex (Mhc) was first recognized due to its role in the rejection of tissue grafts (Gorer 1936). It is now known to be involved in the specific immune response (Benacerraf and McDevitt 1972; Benacerraf 1981). Two types of molecules are recognized, class I and II, depending on differences in structure and function. The class I molecules are expressed on the surface of most cell types, and they present endogenously derived peptides to $CD8^+$ cytotoxic T-cells. The class II molecules are mainly present on antigen presenting cells (APC) like macrophages, lymphocytes and dendritic cells. They present processed exogenous antigens to $CD4^+$ T-helper cells (reviewed by Cresswell 1994; Jensen 1995, Harding 1995) (Figure 1). Foreign peptides are only recognized by T-cells if presented by Mhc molecules, a process called Mhc restriction (Zinkernagel and Doherty 1974). Most studies of Mhc structure and function have focused on the human and murine Mhc system (Klein 1986), however Mhc immunogenetics is also an important topic in domestic animals due to the possible association between Mhc polymorphism and quantitative traits like immune response, growth, and reproduction (Schook and Lamont 1996).

The Mhc class II molecule

The structure of the class II molecule was first proposed by Brown et al. 1988, from the known structure of the class I molecule. A few years later the class II molecule was crystallized and the proposed structure confirmed (Brown et al. 1993). The Mhc molecules belong to the immunoglobulin superfamily (Williams and Barclay 1988). An evolutionary model where the class II genes evolved prior to the class I has been suggested by Klein and O'hUigin (1993). The class II molecule is a dimeric transmembrane glycoprotein consisting of an α- and a β-polypeptide chain, which are non-covalently associated to each other (Figure 2). Recent studies have shown that the Mhc class II may exist as dimers of $\alpha\beta$ heterodimers, linked by a CD4 molecule (Schafer et al. 1995).

The outer domains of the α- and the β-chains together form the peptide binding groove of the Mhc molecule. The groove is built as a "floor" consisting of a β-pleated sheet (half from each α- and β- chain), and an α-helix from each chain lying on top of the "floor", with two open ends (Figure 3). These two open ends enable the molecule to bind peptides of 13 to 25 amino acids (Rudensky et al. 1991; Chicz et al. 1992), and the groove provides greater opportunities for building up forces of attraction with the antigen than a flat surface would. The α_2 and β_2 domains, close to the cell membrane, assume characteristic immunoglobulin folds and are highly conserved. Moreover, they interact with the CD4 molecule in the complex

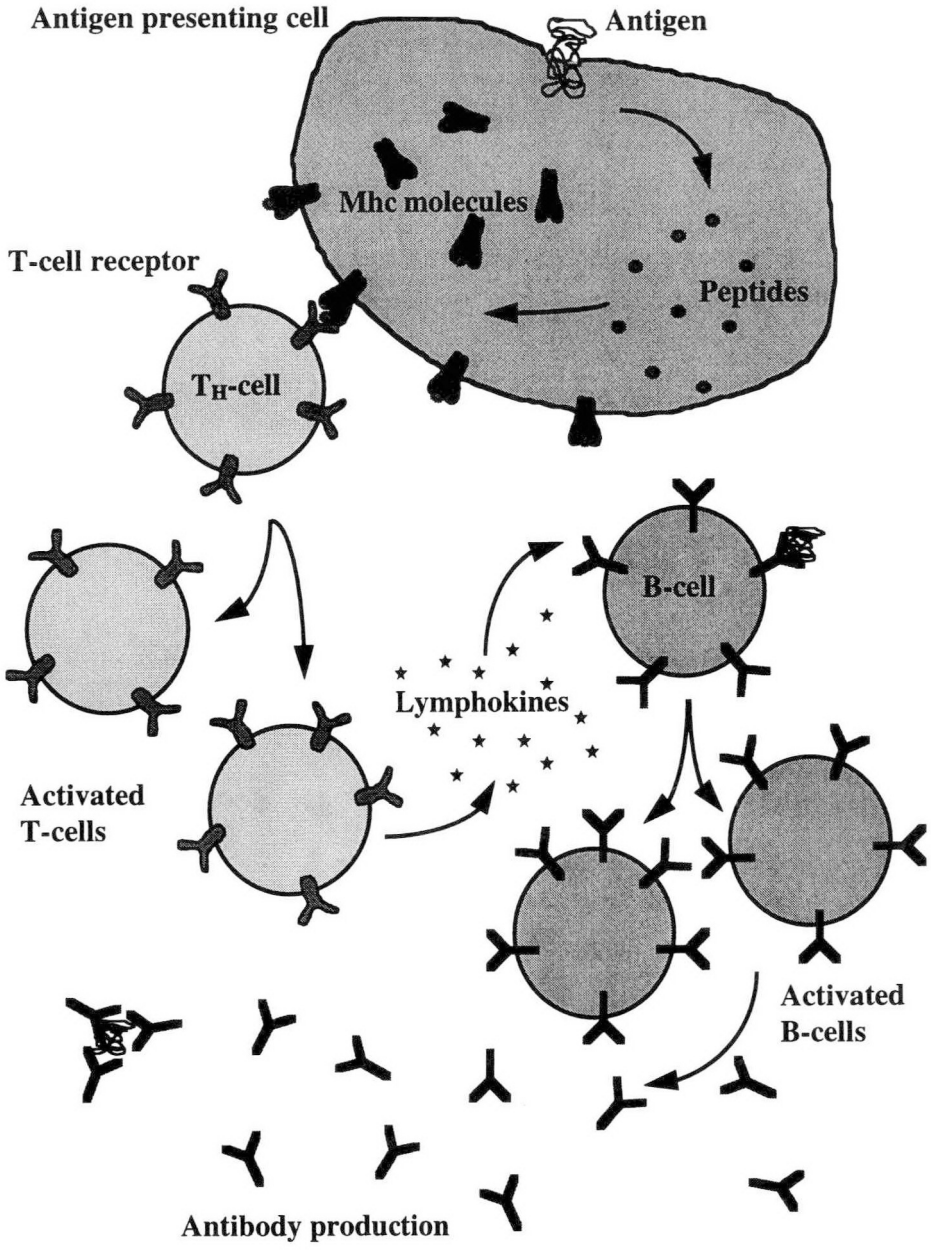
Antigen presenting cell
Antigen
Mhc molecules
T-cell receptor
Peptides
T_H-cell
B-cell
Lymphokines
Activated
T-cells
Activated
B-cells
Antibody production

Figure 1. The main pathways of the specific immune response mediated by the Mhc class II molecules. The antigen is ingested by antigen presenting cells, that is for example macrophages and B-cells. Through an endolythic pathway, the antigen is processed and degraded into peptides. These peptides can bind to the Mhc class II molecule and is subsequently transported to the cell surface where the Mhc/peptide-complex is presented to the T-helper cells. When the T-cell receptors recognize this complex, the T-cells become activated. They start to divide and secrete lymphokines which in turn activates the B-cells, and antibodies specific for the antigen are produced. The figure is an adaptation from Nossal (1993).

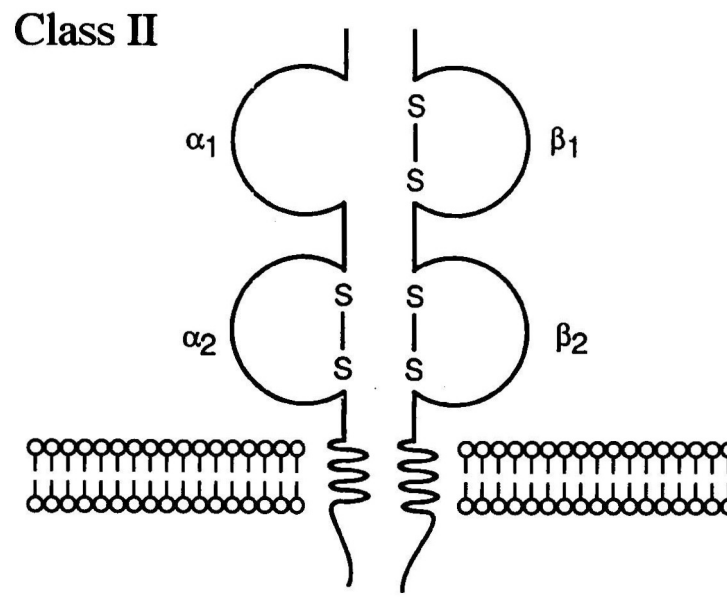

Figure 2. A schematic representation of the Mhc class II α- and β-chain, anchored to the cell surface.

binding to the T-cell receptor. The CD4 molecule is thought to strengthen the binding of low affinity Mhc/peptide-complexes, to the T-cell receptor (Schafer et al. 1995). Further, the class II α- and β-chains comprise a transmembrane and a cytoplasmic region acting to anchor them to the cell surface of the antigen presenting cells.

Chromosomal organization of the bovine Mhc

At the genomic level the Mhc consists of many loci, which are located in clusters over a large area of the chromosome (chr. 6 in humans, chr. 17 in rodents and chr. 23 in cattle). Linkage disequilibrium is frequent within the Mhc. This means that the haplotype frequency of pairs of alleles from linked loci deviates significantly from the product of their individual allele frequencies.. A genetic map of the cattle Mhc, i.e. the *BoLA* complex, is shown in figure 4. The class I and class II regions include the classical Mhc loci, while the class III region comprises a variety of genes such as complement factors (BF, C4), 21 hydroxylase (CYP21), and heat shock protein 70 (HSP70). The order of the genes within the three classes has not been fully resolved. The class II region is divided into IIa and IIb, of which IIa is located close to class I/III, while IIb is distant from all the others (Andersson et al. 1988; Skow et al. 1996). The *DQ* and *DR* loci within the class IIa are tightly linked. The number of *DQA* and *DQB* genes varies between haplotypes (Andersson and Rask 1988). There is one *DRA* and at least three *DRB* genes in the cattle class IIa region (Andersson and Davies 1994). All exons in the *DQB* and *DRB* loci are highly conserved except exon 2 which codes for the antigen binding domain. This exon has a combination of conserved and variable regions. The conserved regions maintain the structure of the molecule, while the variable regions are involved in peptide binding. The peptide binding sites (PBS), or antigen recognition sites (ARS) are all orientated towards the inside of the antigen binding groove. For efficient peptide binding, hydrophobic and charged amino acids are preferred in the PBS (Lundberg and McDevitt 1992).

The MHC polymorphism

Organization of the Mhc polymorphism

The most striking genetic feature of the Mhc is the extensive polymorphism found in many species. However, the degree of polymorphism varies considerably between Mhc loci. For instance *DRA* in cattle appears to be monomorphic while *DRB3* is extremely polymorphic. In addition to sequence polymorphism, there is variation in expression of the different loci. The *BoLA-DRB1* locus is a pseudogene, the *BoLA-DRB2* locus is expressed at low levels, while the highly polymorphic *DRB3* locus is expressed at a high level (Burke et al. 1991). An outstanding feature of Mhc alleles is the large genetic distance between them. For example, the average number of amino acid substitutions is 13.7±0.1, and the average genetic distance is 0.087±0.001, in pairwise comparisons of cattle *DRB3* exon 2 alleles (Paper IV). Another common feature of Mhc polymorphism is that there is no predominant allele, i.e. the allele frequency distribution is even.

However, there are several examples of natural populations with low Mhc diversity are cheetah (O'Brien et al. 1985; Yuhki and O'Brien 1990), beavers (Ellegren et al. 1993), Southern elephant seal (Slade 1992), and whales (fin and sei; Trowsdale et al. 1989). The cheetah and the Scandinavian beaver also show a very low degree of variation at other loci investigated. On the other hand the elephant seals, whales, and Russian beavers have moderate to high allozyme heterozygosities compared to other mammals (see references above).

Within each locus the alleles are grouped into major allelic lineages, which in cattle are defined to have five or more amino acid substitutions compared to any other allele (Davies et al. 1997). An allele with fewer substitutions is considered as a subtype to the closely related major type. The allelic lineages show a trans-species persistence with polymorphism predating speciation events (Klein 1987; Klein et al. 1993). Klein and coworkers (1993) argue that allelic lineages can be more than 40 million years old. In human *DRB1*, which is the homologue to the cattle *DRB3* locus, the average age of alleles within a lineage may only be about 230,000 years old (Erlich et al. 1996).

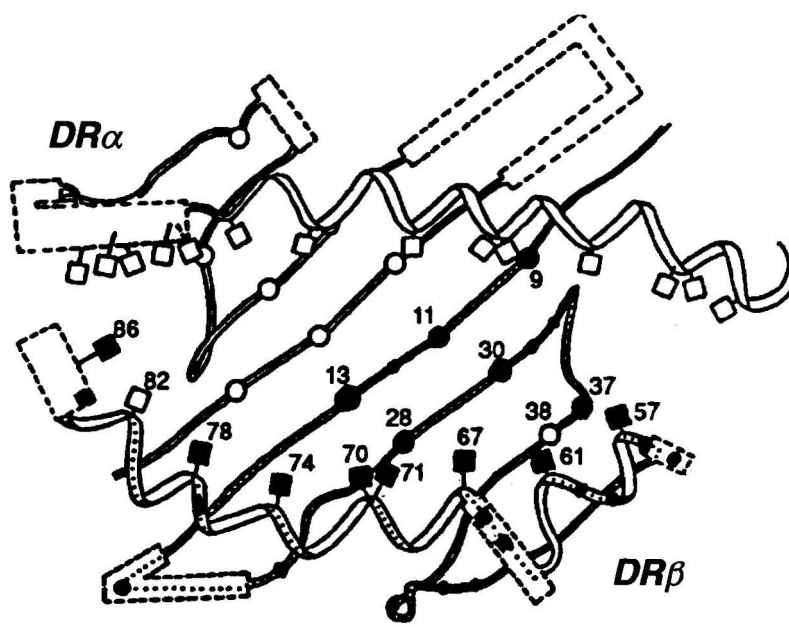

Figure 3. A schematic representation of the Mhc class II antigen binding groove, as seen from the T-cell receptors' point of view. Residues thought to be involved in antigen binding are marked by squares (□ = α-chain, and ■ = β-chain) in the α-helix, and by circles (○ = α-chain, and ● = β-chain) in the β-pleated sheet forming the "floor" of the antigen binding groove. The figure is adapted from Brown et al. (1988).

Selection for and generation of Mhc polymorphism

There is clear evidence that Mhc polymorphism is maintained by some form of balancing selection (Hedrick and Thomson 1983). The mechanisms suggested as the main cause for this selection can be divided into disease-based and mating-based selection models. The proposed selection mechanisms are heterozygote advantage and frequency-dependent selection. Heterozygote advantage (overdominance) implies that heterozygotes have a

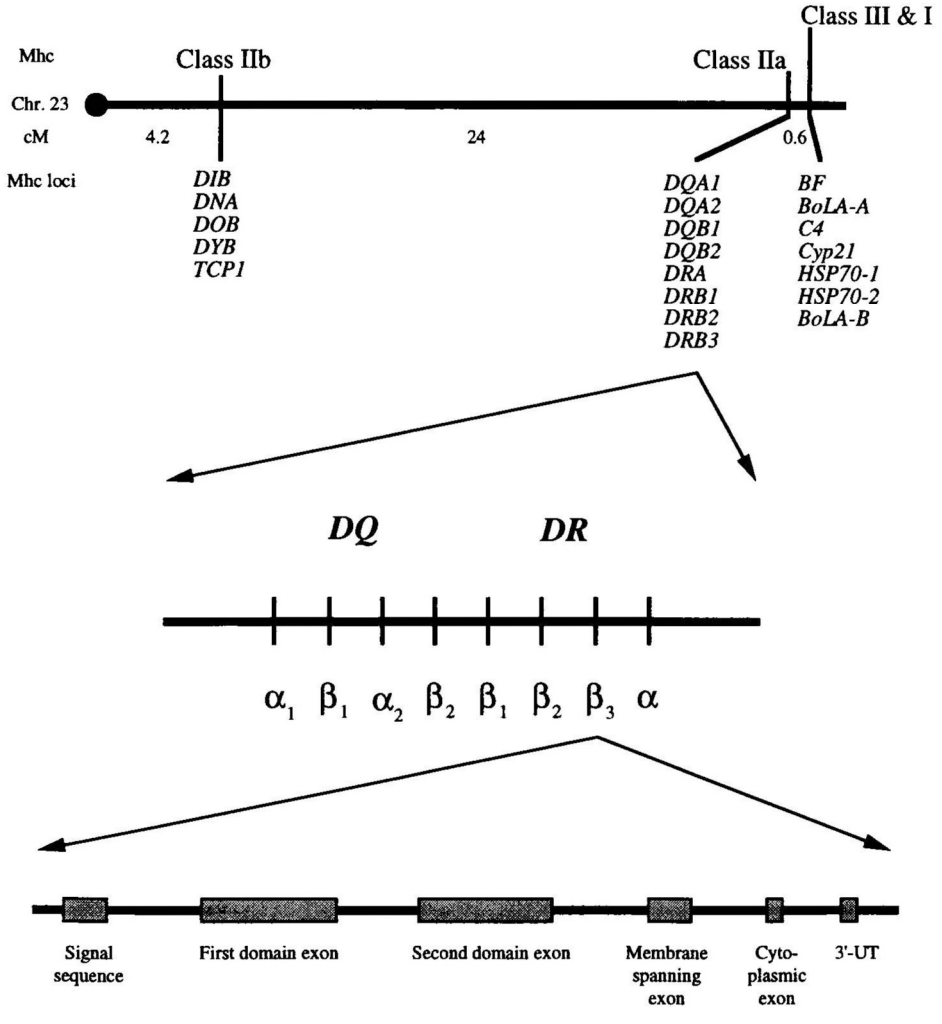

Figure 4. Chromosomal organization of the BoLA system. The relative positions of class I, II, and III is shown. Figures indicate the distance measured in centimorgans (cM). The class IIa region is expanded but the relative order between these genes have not been fully resolved. The exonic organization of the DRB3 locus is expanded. Exon 2 coding for the β_1-domain is the one examined in this thesis.

higher fitness than homozygotes. With regard to disease based selection mechanisms, a heterozygous individual may have a broader immune response repertoire (Zinkernagel and Doherty 1974). Frequency-dependent selection implies that there is an inverse relationship between an alleles' frequency and fitness, i.e. there is selection against common alleles. The coevolution of parasites and Mhc is in agreement with such a model (Bodmer 1972).

Selection mechanisms for mating-based models include maternal-fetal interactions and non-random mating. The hypothesis for maternal-fetal interactions states that fetuses with a different Mhc haplotype to the mother have a higher survival rate. A report that couples sharing Mhc haplotypes tend to more often experience spontaneous abortions (Hedrick 1988), supports this hypothesis. A genetic cause for spontaneous abortions could be due to lethal genes, or genes associated with a lower viability, linked to Mhc genes. On the other hand, an immunological hypothesis suggests that the mother and fetus have to differ in their Mhc for proper implantation and fetal growth to occur (reviewed by Hedrick 1994). Wedekind (1996) suggested several mechanisms why eggs would more often be fertilized by sperm carrying an Mhc haplotype different from the egg haplotype. Non-random mating in terms of Mhc haplotype has been shown to occur in mice (reviewed by Potts and Wakeland 1993). Mice are apparently able to smell Mhc differences (Yamazaki 1983). Odor as a mate preference has also been suggested to occur in humans (Wedekind et al. 1995).

At PBS codons of polymorphic Mhc loci, the relative frequency of nonsynonymous substitutions (d_N) is higher than for synonymous substitutions (d_S). This indicates the existence of positive Darwinian selection for Mhc diversity (Hughes and Nei 1988, 1989). At non-PBS sites the ratio d_N/d_S is less than one, indicating conservative, purifying selection.

Detection of Mhc polymorphism

Polymorphism within the Mhc can be detected in many different ways. At the protein level, the most commonly used methods are serology, mixed lymphocyte reaction (MLR), and isoelectric focusing (IEF) (Davies and Antczak 1991; Davies et al. 1992; Joosten et al. 1989; reviewed by Lewin 1996). Serological typing is performed by incubating lymphocytes from an individual against a panel of sera in the presence of complement. Positive reactions are recognized by cell lysis. Serology is currently the most widely used method for class I typing in cattle, but only five class II specificities have been described (Davies et al. 1994). MLR is a functional test using lymphocytes from two individuals which are mixed and cultured together. A reaction is seen when the Mhc haplotypes of the two cell populations differ from each other. MLR determinants are encoded by class II Mhc molecules (Klein 1986). IEF separates the proteins as a function of their isoelectric point however this method has a restricted resolution as only charge variants

can be detected, thus only 12 IEF variants have been detected at the *BoLA-DRB3* locus.

A higher resolution of genetic polymorphism can be revealed at the DNA level. Restriction fragment length polymorphism (RFLP) is widely used to detect DNA polymorphism. To detect RFLPs, whole genomic DNA is digested using restriction endonucleases; point mutations remove or add restriction sites so that a RFLP can be detected when various probes are hybridized to the DNA. Additionally, insertions, deletions, and duplications can be detected as RFLPs, as was the case with the duplicated *DQ* loci in cattle (Andersson and Rask 1988). RFLPs are abundant in the genome and RFLP testing has proven to be a good method for large scale screenings of genetic polymorphisms. The Mhc of cattle has been extensively studied by RFLP analysis, and a total of 46 *DRB* haplotypes have been identified (Davies et al. 1994; Sigurdardóttir et al. 1988, 1991).

Several PCR based typing methods have been used for detection of Mhc variation in cattle including PCR-RFLP (van Eijk et al. 1992), microsatellite typing (Ellegren et al. 1993), asymmetric PCR followed by single strand conformation polymorphism (SSCP; Russell 1994), and sequence specific oligotyping (SSO) (Sitte et al. 1996). The SSO typing system is based on the hybridization of short oligonucleotides to dot blots. A combination of many oligonucleotides makes it possible to achieve a high resolution.

Mhc and disease associations

It is well known that allelic Mhc molecules differ with regards peptide binding (Nepom et al. 1996 and references therein). Such differences may cause Mhc disease associations due to a direct effect of the Mhc polymorphism. Mhc disease associations may also be due to genes linked to the Mhc. Linkage disequilibrium in the Mhc region may complicate association studies, as it is possible to find association to one locus while the "true" gene is actually in linkage disequilibrium with the one detected. Many of the human diseases sharing an Mhc association are autoimmune diseases such as insulin-dependent diabetes mellitus (IDDM; Noble et al. 1996), multiple sclerosis (MS; Allen et al. 1994; Spurkland et al. 1991) and rheumatoid arthritis (Begovich et al. 1989). Some infectious diseases, like human papilloma virus (HPV) -induced cervical carcinoma, malaria, tuberculosis, and leprosy, have also been shown to be associated with HLA polymorphism (Allen et al. 1996; reviewed by Hill 1996). In chickens, there is a significant association between Mhc genotype and resistance to Marek's disease in White Leghorn (Briles et al. 1983; Hepkema et al. 1993). Marek's disease is a herpes virus induced lymphoma.

Numerous diseases and production traits have been suggested to be associate with Mhc polymorphism in cattle (see Andersson and Davies 1994).

The associations are often complex and conflicting results are frequently obtained in different breeds or populations (Mejdell et al. 1994), and few studies have obtained strong correlations. Although the etiology of mastitis is complex, association of class I and II polymorphisms have been detected in Norwegian Red cattle (Våge et al. 1992), Swedish Red and White cattle (Lundén et al. 1990), and Holstein cattle (Weigel et al. 1990). Bovine Leukemia Virus (BLV) is a retrovirus causing lymphosarcoma and persistent lymphocytosis (PL). Dominant resistance to PL is linked to the presence of glutamic acid and arginine at positions 70 and 71 in the *DRB3* molecule (Xu et al. 1993). BoLA class II haplotypes are associated with high and low response to synthetic foot and mouth disease virus (FMDV) peptides (Glass et al. 1991).

Present investigations I-V

Aims of the thesis

- To broaden the knowledge about the evolution of Mhc diversity in cattle and its close relatives.
- To develop simple and rapid typing methods for detecting genetic variation
- To study how Mhc polymorphism is generated and maintained both in domesticated and natural populations of ruminants.

Objectives of Paper I-V

I. The objective was to extend the knowledge about the *BoLA-DRB3* polymorphism in taurine cattle, and in the closely related indicus subspecies. We searched for differences in *BoLA-DRB3* variability and allele frequencies between the taurine and indicus subspecies. Such variation could possibly explain differences in resistance to diseases and parasites.

II. Because of the occurrence of a fatal disease in southwestern Sweden we wanted to characterize Mhc polymorphism in the moose. The *DRB* locus, which is the most polymorphic class II locus in cattle and humans, were chosen for this study. Genetic analysis of genomic DNA was complemented with RT-PCR studies to ensure that we are investigating an expressed locus. Samples from different populations in Scandinavia and North America were collected to enable comparisons of polymorphism both within and between populations.

III. To further characterize the Mhc polymorphism in ruminants the North American bison was chosen because; i) it is a close relative to cattle, allowing us to study the dynamics of the evolution of *DRB* alleles; ii) it has experienced a population bottleneck.

IV. The objective of this study was to investigate the evolution of a group of *BoLA-DRB3* alleles with a novel sequence motif, comprising a deletion of codon 65.

V. The aim of this study was to characterize the degree of Mhc *DRB* polymorphism in selected wild populations of ruminants. Differences in the heterozygosity, and number of Mhc *DRB* alleles in populations with different life style and population history, were examined.

Materials and methods

Species included in the present studies

Cattle (Paper I, IV, V)

Domestic cattle are divided into two subspecies *Bos primigenius indicus* and *B. p. taurus*, commonly referred to as *B. taurus* and *B. indicus*. The *B. p. indicus* is recognized by the presence of a hump, while the taurine cattle are humpless. Linneaus (1758) as well as Darwin (1859) thought they were separate species even though they are fully interfertile. Extensive crossbreeding has occurred during domestication of cattle. Molecular studies of the bovine mitochondrial D-loop (Loftus et al. 1994), and Y-specific markers (Bradley et al. 1994) have provided further insight into the relatedness of the two subspecies. The taurine cattle generally do poorly in the African environment, with a heavy load of parasites and high susceptibility to tropical diseases. The N'Dama, which is a taurine breed, is an exception and are well suited to the African environment, and it shows tolerance to trypanosomiasis.

In paper I, sequence analysis of *BoLA-DRB3* was conducted on nine West African Zebu of the Fulani type (*B. p. indicus*), and nine N'Dama cattle (*B. p. taurus*). Their *DRB3* exon 2 polymorphism were then compared to 18 European cattle (*B. p. taurus*) from four different breeds. The cattle sequences were subsequently compared to *DRB* sequences found in other ruminants (Paper II, III, IV, V).

Bison (Paper III)

The North American bison (*Bison bison*) is a close relative to cattle withan estimated divergence time of about 1-1.5 million years ago (Loftus et al 1994). The bison population experienced a severe bottleneck in the 1800's when the number of animals dropped from several millions to fewer than 1,000 (Hall et al. 1959). Since then about 20 generations have reconstituted the population to its present status of about 100,000 animals. This study provided the opportunity to evaluate and characterize Mhc polymorphism in a species with a well documented and recent bottleneck. The close relationship between cattle and bison provided the chance to study the evolution of Mhc. Twenty North American bison were selected based on previous PCR-RFLP typing. They originated from three different herds in Western USA.

Muskox (Paper V)

The muskox (*Ovibos moschatus*) is a species well adapted for the arctic climate. It is a relatively stationary animal living in quite small herds. The few genetic studies that have been performed on muskox indicate that the overall level of variation is low. The proportion of microsatellite loci which are polymorphic, is about one third of that found in other bovidae and cervidae (Engel et al. 1996). The degree of heterozygosity was found to be ten times lower in the muskox compared to the other species in the same study. To further evaluate the Mhc polymorphism of natural populations of ruminants, muskox samples were collected from Canada and West Greenland.

Moose (Paper II, V)

The moose (*Alces alces*) is the largest deer in the world. It evolved in Eurasia and entered into North America through the Bering land bridge during a glaciation period. Interglacial periods allowed the moose population to disperse and four American subspecies were formed during this time, they are *A. a. gigas*, *A. a. americana*, *A. a. andersoni*, and *A. a. shirasi* (Coady 1982). The North American and Scandinavian (*A. a. alces*) subspecies differ in chromosome numbers (70 and 68, respectively; Gustavsson and Sundt 1967; Hsu and Bernirschke 1969). Differences are also present within mitochondrial DNA where the D-loop of the Scandinavian moose has a duplicated fragment of 75 bp, compared with the American subspecies (Paper I). The overall genetic variability in moose is fairly high as verified by allozyme data (Ryman et al. 1980), and DNA fingerprinting (Ellegren et al. 1996).

Due to a high mortality within the moose population in southwestern Sweden (Steen et al. 1993), and the suspicion of a retroviral infection suppressing the immune system (Merza et al. 1994) we wanted to investigate Mhc variation in the moose. The susceptibility to an immunosuppressive retroviral infection has previously been found to be associated with *DRB3* polymorphism in cattle, as shown for Bovine Leukemia Virus (BLV) (Lewin and Bernoco 1986; Lewin et al. 1988). In the present study, only the work done on healthy moose is included. An investigation of disease association to Mhc polymorphism will be published elsewhere (Mikko et al. in preparation). Moose samples originating from different localities in Sweden were collected during the annual hunt. Samples from Norwegian, Canadian, and Alaskan moose populations were collected by others (Paper V).

Other deer species (Paper IV, V)

The deer population in Scandinavia is heavily influenced by climatic changes (Lepiksaar 1986). As the ice retreated after the last ice age, the more cold adapted species, like reindeer, and European bison colonized ice free land areas first. The reindeer may have entered the Scandinavian peninsula both from the south (*Rangifer tarandus tarandus*) and from the north via Finland

(*R. t. fennicus*). A few thousand years later, when the climate was warmer, the red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), and moose (*Alces alces*) entered from the south. The animals entered Norway mainly along the coasts. During the so called "Little Ice Age" about 500-150 years ago, the stock of species adaptive to a more southern habitat (red deer and roe deer), were heavily reduced. The fallow deer was introduced to Sweden from England, during the 16th century, thus it is not a natural species in Scandinavia.

The genetic variation of transferrin and other loci in reindeer populations has been extensively studied, and has shown considerable variation in most of the populations (Røed). The Svalbard reindeer (Røed et al. 1985) exhibit intermediate levels of genomic variability. The fallow deer populations in Europe do not possess a significant amount of overall genetic variation (Emerson and Tate 1995; Pemberton and Smith 1985). The roe deer show a low Mhc and transferrin polymorphism in Scandinavia (Gyllensten et al 1980), but a significant amount of allozyme diversity at the European continent (Hartl et al. 1991).

DNA from about twenty animals of each deer species was collected. Three reindeer populations were investigated, one originating from Svalbard which is a separate subspecies (*R. t. plathyrynchus*) from the wild and domestic reindeer in Norway (*R. t. tarandus*), from where the other two populations samples were collected. Two Norwegian, and one Swedish roe deer populations as well as fallow deer herds from both countries, were included in the study. Samples of Norwegian red deer were also included and compared to red deer from New Zealand (Swarbrick et al. 1995).

Detection of MHC polymorphism

PCR based typing methods

In this thesis, exon 2 of *BoLA-DRB3* as well as of *DRB* gene(s) in several other ruminants were PCR amplified using primers specific for *BoLA-DRB3* exon 2. The primers were designed to anneal to the flanking exon-intron borders of exon 2, with their 3'-ends at conserved sites to increase the chance of amplification of all alleles. In most species a single locus was amplified as is the case for cattle. However, in red deer and fallow deer, two loci were amplified.

One method to detect variation within the PCR product is to digest it with a panel of restriction enzymes, and the allelic status can be determined from the specific fragment length patterns formed, i.e. PCR-RFLP. Since the sequence of many *BoLA-DRB3* alleles is already known, suitable endonucleases can be chosen. In cattle the enzymes *Rsa*I, *Hae*III, and *Bst*YI have shown to be appropriate for typing of the *BoLA-DRB3* locus. So far, most major allelic types of *BoLA-DRB3* exon 2, can be resolved by using these three enzymes in PCR-RFLP (van Eijk et al. 1992). Due to the close

relationship between cattle and bison *DRB3* sequences, the same enzymes also work well in bison (Morris et al. 1994). As for ordinary RFLP the PCR-RFLP technique can primarily detect variation within the restriction sites. The individuals included in paper II and IV had previously been typed by PCR-RFLP

The single strand conformation polymorphism (SSCP) method, was first described by Orita et al. (1989a,b). PCR products are denatured by heating in a formamide containing loading buffer and then quickly cooled on ice. The denatured fragments are separated on a non-denaturing polyacrylamide gel with low cross-linking. As soon as the fragments enter the gel matrix they fold into different conformations depending on the sequence. Theoretically, each DNA strand results in one band on the gel, i.e. a homozygote will produce two bands, while a heterozygote will produce four bands. A single base pair difference can be detected in a several hundred base pair long fragment. Optimal fragment lengths are less than 400 bp, which make *DRB* exon 2 (about 300 bp) well suited for this method.

SSCP is a very sensitive and robust method but the typing conditions need to be constant to obtain reproducible results. Some important issues are: i) A constant temperature during the electrophoresis is very important since altered conformations can result from temperature changes. ii) Excess of primers can interfere with the DNA strands and give different SSCP profiles (Cai and Touitou, 1993). iii) Complex band patterns may occur because of incomplete denaturation. iv) Multiple alternative single-strand conformers can be formed from the same DNA fragment, giving two or more bands or even a smear of bands. The SSCP technique is well suited for routine typing of Mhc loci showing low to moderate polymorphism. SSCP of the *HLA-DPB* locus have been suggested as a method for typing before transplantations (Hoshino et al. 1992). However, the presence of many alleles makes reliable scoring of all alleles very difficult.

Heteroduplex analysis can be combined with SSCP analysis as homo- and heteroduplexes are formed after denaturation and are separated in the non-denaturing gel. The coding strand of the first allele has annealed with the complementary strand of the other allele and vice versa. These heteroduplexes will migrate slower than the homoduplexes in a non-denaturing gel. In this way, homozygotes and heterozygotes can often be easily distinguished.

The polymorphism of a microsatellite positioned in intron 2 of the *BoLA-DRB3* gene is tightly associated to the polymorphism in *DRB3* exon 2 (Ellegren et al. 1993). This microsatellite has successfully been amplified using cattle primers, in bison, muskox, and red deer. More than twenty *DRB3* microsatellite alleles have been identified in cattle (Ellegren et al. 1993, Mikko et al. unpublished) This typing method is very useful but is restricted by the fact that a microsatellite allele of a specific length can be associated with more than one exon 2 sequence. This problem may be solved by adding typing data from the *DRBP1* microsatellite which is located within the *BoLA-*

DRB1 pseudogene. The *DRBP1* and *DRB3* microsatellites are closely linked and thus provide haplotype information (Gwakisa et al. 1994). The microsatellite analysis was used as an additional screening method when no variation was found in exon 2, as in the muskox (Paper V), or to screen for certain alleles (e.g. *DRB3*0201* in Paper IV). Another useful application of the DRB3 microsatellite is to screen microsatellite-containing subclones to be able to detect and select the two alleles of an individual prior to sequencing.

Sequence analysis

The ultimate method for detecting genetic polymorphism is by DNA sequence analysis. PCR products from homozygotes can be sequenced directly after the excess of PCR primers and dNTPs has been removed. The best results are obtained if a nested primer is used as sequencing primer, but in this thesis in this thesis we used the same primers as in the original PCR. Cycle sequencing or solid phase sequencing (SPS) are the preferred methods when sequencing double stranded PCR products. Direct sequencing of PCR products from a heterozygous individual will result in double peaks and the allelic status need to be determined by subcloning. Sequence reactions can be performed in several ways. Manual sequencing generally demands large amounts of material, but on the other hand the output is generally a high quality sequence. Cycle sequencing is the generally preferred sequencing method as it requires much less template. To visualize the sequence reactions on a polyacrylamide gel, it has to be labeled in some way. Either the primer, ddNTPs or one of the dNTPs can be labeled. Some of the sequences obtained in Paper I were manually sequenced using ^{35}S-dATP. All other sequence reactions were performed as cycle sequencing with fluorescently labeled primer or ddNTPs. Separation and detection was then done using a sequencing instrument (ABI373 or ABI377).

Statistical methods

All *DRB* sequences obtained were subjected to general sequence statistical analysis. At first a GenBank search was conducted to evaluate if there were any *DRB* alleles already sequenced from the species studied. Sequences found in GenBank were compiled into species specific databases. Most within population calculations were performed using the MEGA program (Kumar et al. 1993), except the ones in table 2, Paper IV, where the program SEND (kindly provided by Dr. M. Nei) was used. All between population calculations as well as the average Jukes Cantor distances for all populations were calculated using the SEND program. First, the number of nucleotide and amino acid differences were calculated to deduce if the specific sequences from my studies were new major alleles, new subtypes or had been sequenced before. Second, the distances (d) between *DRB* alleles within species were calculated to document the degree of sequence variation. Third,

the relative frequencies of nonsynonymous (d_N) and synonymous (d_S) substitutions were calculated to further evaluate the relationship betweenalleles. The relative frequencies of d_N and d_S were estimated according to the method of Nei and Gojobori (1986) applying Jukes and Cantor's (1969) correction for multiple hits. The standard errors of the mean frequencies of nucleotide substitutions among sequences were estimated according to Nei and Jin (1989).

Results and discussion

Degree of Mhc-DRB polymorphism

Species with extensive *Mhc-DRB* diversity

A compilation of the total number of *DRB* alleles found in the species included in this thesis is shown in figure 5. The number of *BoLA-DRB3* alleles are steadily increasing as more animals are typed around the world. At present, 68 alleles have been sequenced (Davies et al. 1997; Russell et al. 1997). Of these, as many as 41 are considered as major types with five or more amino acid substitutions to its closest relative. This is extraordinary compared to the situation in the human homologue, *HLA-DRB1*, where 184 alleles are divided into only 13 major allelic lineages (Bodmer et al. 1997). Although the European cattle breeds comprise a significant amount of Mhc variation, the African breeds seem to exhibit an even higher degree of variation (Paper I). The most common alleles among the Swedish animals, were not found in the small sample of African cattle (Paper I). A previous study has shown that *DRB3* allele frequencies are evenly distributed in Swedish Red and White Breed (SRB) (Sigurdardóttir et al. 1988), which results in a high level of heterozygosity. Sigurdardóttir and coworkers (1988) estimated the expected heterozygosity to 0.82 from *DQ* haplotype information.

Nine alleles were found at the bison *DRB3* locus (Paper III). As many as seven of these were major types, which is approximately the same proportion as in cattle. The bison may have lost a considerable amount of alleles during the dramatic bottleneck in the late 19th century. The Norwegian red deer also exhibits a considerable amount of variation, however analysis was complicated by coamplification of a second DRB locus which made SSCP typing impossible. The variation detected was however comparable with the situation in a sample of 50 red deer in New Zealand, where a total of 49 alleles were found (Swarbrick et al. 1995). The reindeer (except the population at Svalbard) was found to comprise an amount of Mhc *DRB* variation comparable to bison. In two Norwegian populations, seven alleles were sequenced, and six of them turned out to be major allelic types (Figure 5). The SSCP analysis revealed two more alleles, that were not

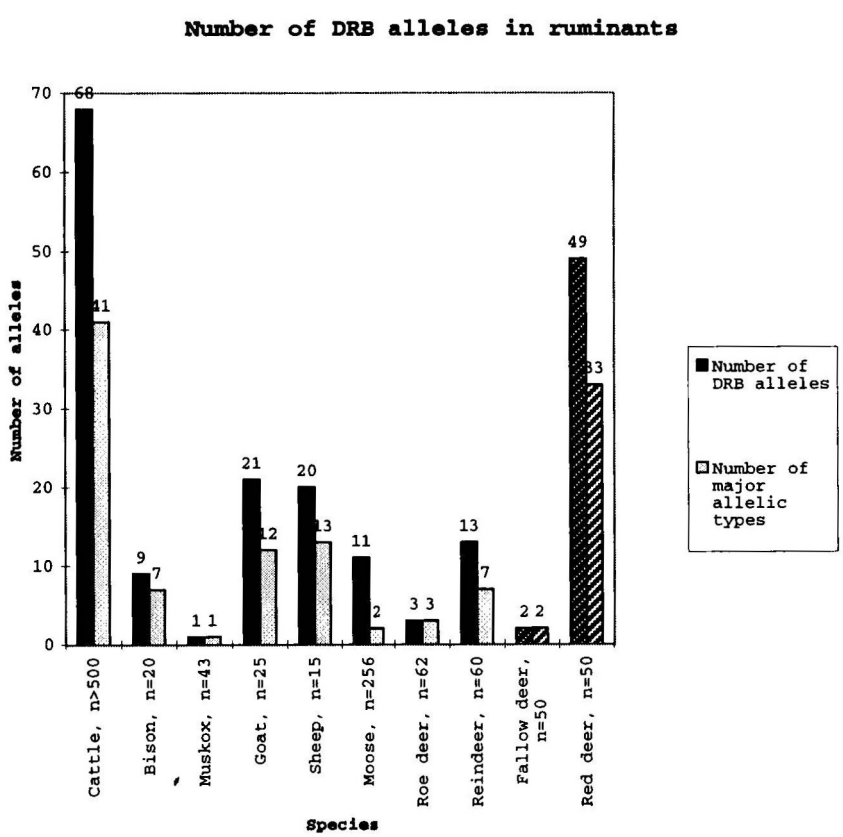

Figure 5. Number of Mhc *DRB* alleles in ruminant species. Black bars indicate the total number of alleles found. White bars show the number of "major" allelic types. That is alleles which do not differ by more than four amino acid substitutions. Hatched bars indicate that the results are derived from two *DRB* loci.

sequenced, resulting in a total of nine reindeer alleles on the Norwegian mainland. The two sequenced allelic subtypes could not be distinguished from each other by SSCP analysis. The heterozygosity levels were very high in both of these two populations (Figure 6), and comparable to the situation in cattle and bison. The allele frequencies are evenly distributed within the Norwegian reindeer populations except for one allele that is very common in the domestic population (Figure 7). In the Svalbard reindeer population one predominant allele was found. A single animal was heterozygous for an allele which was a subtype of one allele found in the subspecies in Norway.

Species with low *Mhc-DRB* diversity

In a total of 256 moose typed, 11 alleles were discovered. Six and seven alleles were found in the Swedish and Norwegian populations, respectively. In Canadian and Alaskan populations, as few as four and three alleles were

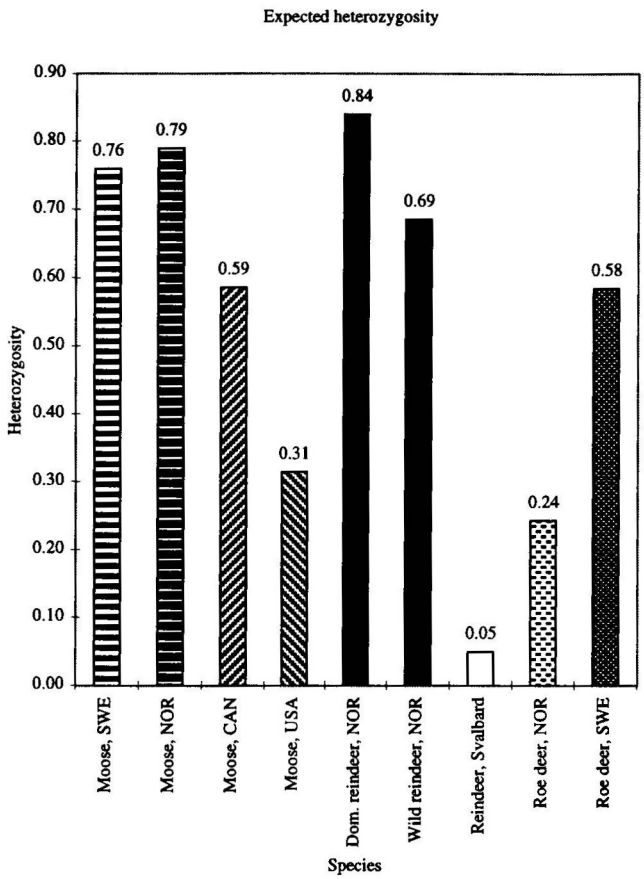

Figure 6. The expected heterozygosity in different moose, reindeer, and roe deer populations.

found, respectively. In most moose populations there is an even allele frequency distribution (Figure 8), causing fairly high levels of heterozygosity (Figure 6). A consequence of one of the three alleles within the Alaskan population being very common is a lower heterozygosity (H_{exp}=0.314) compared with the Canadian population.

Four *DRB1* alleles were recognized in the sample of 62 roe deer from Sweden and Norway. Three of these alleles were more or less common while the fourth was found in a single heterozygous individual (Figure 9). The sample of Swedish roe deer also exhibited a relatively high degree of heterozygosity compared to the sample of Norwegian roe deer (Figure 6).

Fallow deer was monomorphic at the two *DRB* loci examined, even though as many as 50 deer from more than seven Norwegian and Swedish populations were typed. The muskox *DRB* exon 2 was also completely

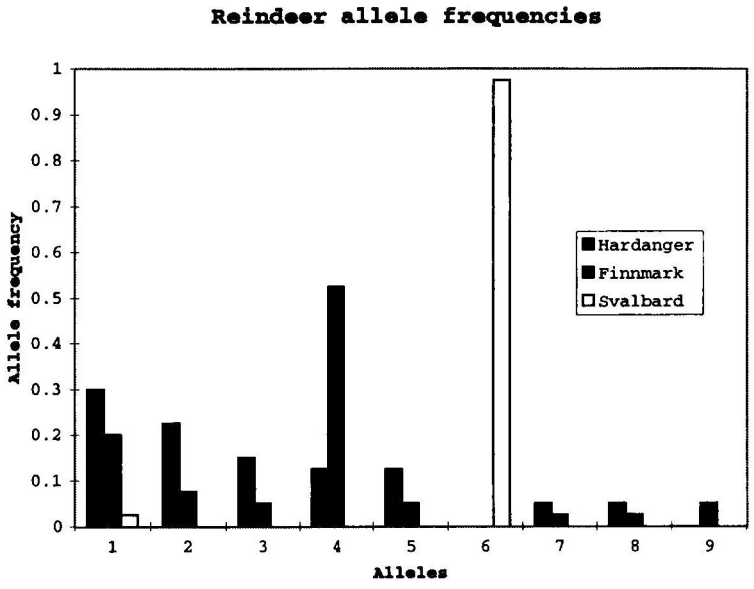

Figure 7. Allelefrequencies in the three reindeer populations studied. The Hardangervidda population is a wild herd in Central Norway, while the population from Finnmark in Northern Norway is a domesticated herd. The Svalbard population belongs to another subspecies (*R. t. plathyrynhus*).

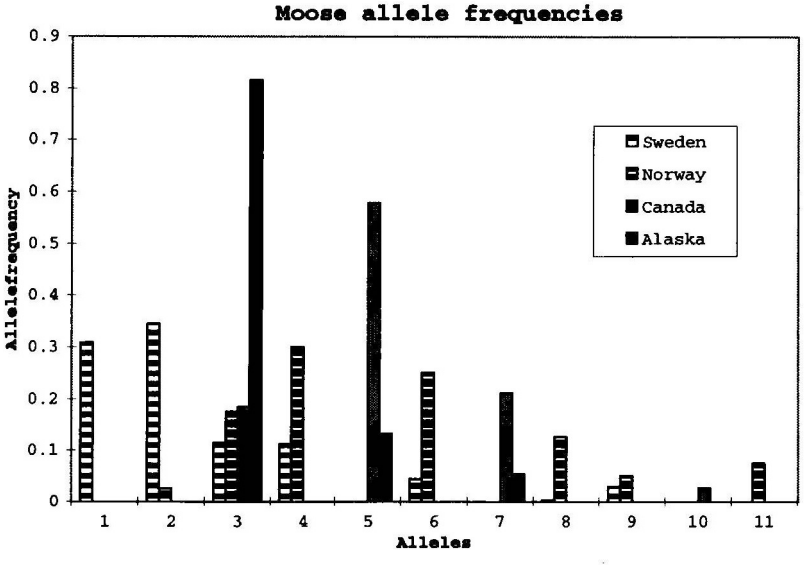

Figure 8. Allele frequencies in four moose populations. The Swedish and Norwegian moose belong to the *A. a. alces*, and the Alaskan moose to the subspecies *A. a. gigas*. The Canadian moose population beong to the subspecies *A. a. americana* or *A. a. andersoni.*

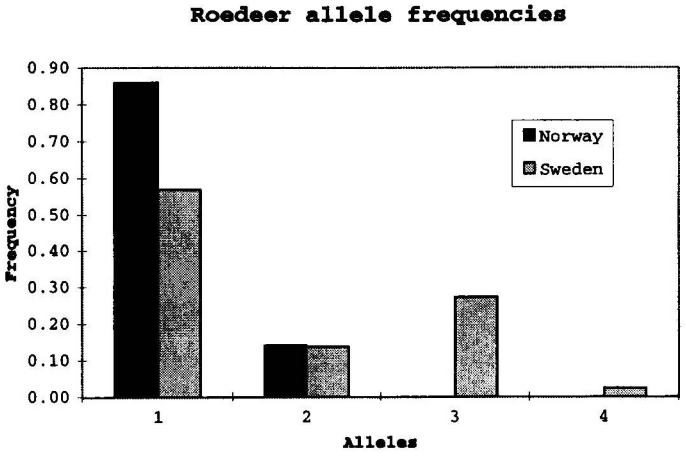

Figure 9. Allele frequencies in Norwegian and Swedish roe deer. The Norwegian animals were collected from two populations in souther Norway, and the Swedish animals were from the Mälardalen area.

monomorphic, but some polymorphism was revealed by RFLP and microsatellite analysis (Paper V, data not shown). The low variation in muskox is not a phenomena of the order Caprinae since both goat and sheep exhibit a considerable amount of variation in their Mhc (Obexer-Ruff et al. 1996; Schwaiger et al. 1994, 1996).

Genetic distances between alleles

The number of major allelic types shows a strong positive correlation to the genetic distances between alleles. The distances between *DRB* alleles are high in cattle, bison, goat, and red deer (Paper V and Figure 10). The distances between alleles in sheep, roe deer and reindeer are on average lower than among alleles in these four species, and similar to the distance found between the two monomorphic loci in fallow deer. Within locus distances will thus be zero for the two fallow deer loci, as for the muskox. The moose alleles are remarkably similar to each other, with an average distance between alleles of 0.018±0.001. In fact, nine of the ten alleles sequenced belong to the same major allelic type (Figure 5). The average distance between the red deer alleles is higher than all the others, but this figure reflects an average between two expressed loci (Swarbrick et al. 1995). The genetic distances between alleles are dependent on the long-term effective population size as it may take time to generate Mhc polymorphism which has been lost in a severe population bottleneck.

In all ruminant species investigated, the relative frequency of synonymous substitutions (d_S) are about half of the frequency of non-

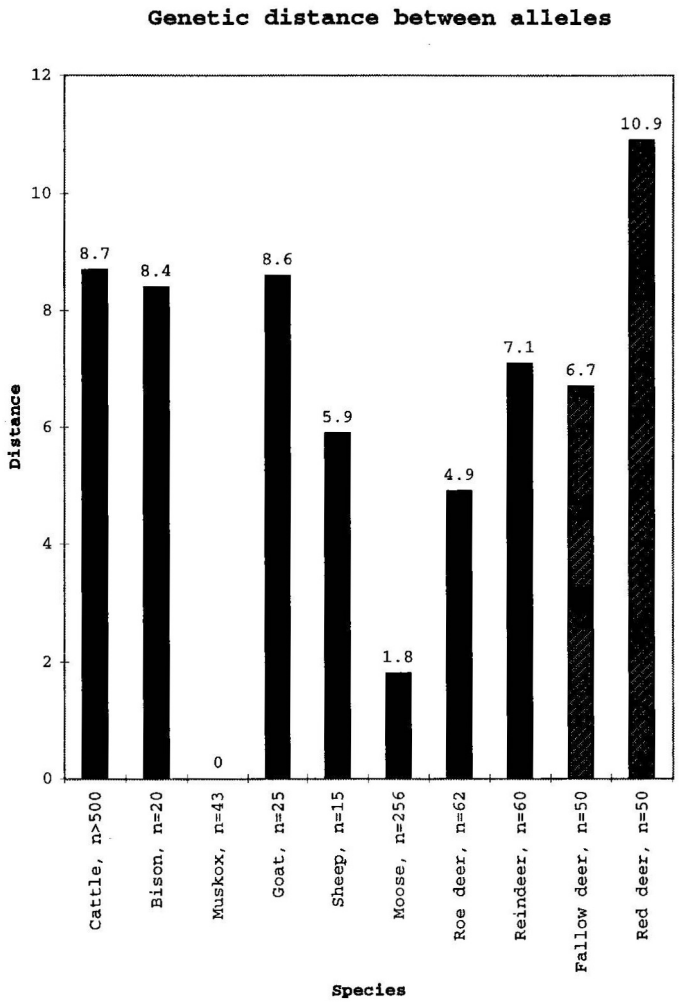

Figure 10. Genetic distances between *DRB* alleles within each ruminant species. The distances are calculated using Jukes and Cantors' correction for multiple hits. The hatched bars for fallow deer and red deer indicate that the results are averages from two *DRB* loci.

synonymous substitutions (d_N). The differences are even more striking if only the peptide binding sites are considered (Paper V, Table 2). This finding strongly suggests that the loci amplified in the present studies are functional, i.e. they are not pseudogenes. In humans the frequencies of d_N and d_S in *DRB1* exon 2, are both as high as the d_N in ruminants. The frequency of synonymous substitutions is expected to reflect the evolutionary distance between alleles thus, the lower number of d_S in ruminants indicates that the alleles are either younger than in humans or that some homogenization has occurred due to interallelic recombination (Paper IV).

In all species studied, the average d_N is higher in the region coding for the α-helix compared to the region coding for the β-pleated sheet (cattle and roe deer, $p<0.001$; moose, $p<0.01$), indicating that there is a higher selection pressure acting on the α-helix, or that more polymorphism is tolerated in this part of the *DRB* exon 2.

Significance of Mhc-DRB polymorphism and selection

Extensive polymorphism in domestic ruminants

Most studies of Mhc polymorphism in ruminants have so far been conducted in domestic species like cattle (Lewin 1996), goat (Obexer-Ruff et al. 1996), and sheep (Schwaiger et al. 1996). All show high diversity at their Mhc as well as in many other loci. Domestic animals are often exposed to a high level of pathogens since they are kept in large herds and dense populations. The significance of mating preference should be minute since the reproduction have been controlled by humans for many generations. Still, some kind of selective fertilization mechanism may be possible as suggested by Wedekind (1996). Such a mechanism would not be possible to avoid in breeding programs. For example, maternal-fetal interactions have been suggested in cattle when the cow and calf have shown class I compatibility (Joosten et al. 1991).

Levels of Mhc diversity differ among wild ruminants

The present study has demonstrated several natural populations of ruminants with low or no Mhc class II DRB diversity (Paper II and V), but with no apparent negative consequences such as poor population growth or marked disease susceptibility. What are the possible explanations for the low levels of Mhc variation in muskox, moose, roe deer, Svalbard reindeer and fallow deer? First, we may not have revealed all the polymorphism present in the Mhc-*DRB* loci amplified. Mutations in the primer sites can result in null alleles, which is detected as an excess of homozygotes in the sample. In these species there may also be other Mhc loci showing more polymorphism, and the locus amplified may be non-functional. The former explanation is unlikely in muskox, and moose since RFLP analysis using both class I and II probes confirmed low levels of polymorphism (Paper V, data not shown; Ellegren et al. 1996). Ten of the eleven moose *DRB1* alleles had already been found when 50 animals from Sweden and Canada had been typed. The additional typing of more than 200 animals only resulted in identification of one new allele. This presents a compelling argument that nearly all the variants present at *DRB1* in moose, have been identified. Moreover, cDNA synthesis from moose mRNA confirmed that the amplified locus was transcribed (Paper II). In cattle the *DRB3* locus is known to be expressed at a high level (Burke et al. 1991). Also, both loci amplified in red deer are

known to be transcribed (Swarbrick et al. 1995). It is not known if the *DRB* loci amplified in reindeer, roe deer, muskox or fallow deer are expressed or not, although the ratio of d_N/d_S (Paper V) indicates that there is positive selection acting on these loci, hence they should be functional.

A second explanation, to account for the low observed levels of Mhc variation may be reduced balancing selection pressure acting on the *DRB* loci in muskox, moose roe deer, Svalbard reindeer, and fallow deer. A reduced selection pressure has been suggested to be one of the reasons for the low Mhc diversity found in marine mammals (Slade 1992). A solitary living species, such as the moose may not need as high Mhc diversity as herd living species. The explanation could be a lower density or reduced transmission of pathogens. This situation may be satisfactory until there is a pathogen that has sufficient transmission potential to invade and persist in the population, which would result in a very high mortality rate. Contradictory to this, the moose is often loaded with parasites but still the population is fully viable, and has increased dramatically during the last 100 years (Cederlund, personal communication). Also the Scandinavian roe deer population has increased considerably since the bottleneck some hundred years ago (Lepiksaar 1986), but nevertheless, it is often manifested by a heavy parasite load (Steen, personal communication). The fallow deer and muskox are herd living animals with low Mhc polymorphism. The muskox is known to suffer from bovine pathogens (Gunn 1982). Fallow deer and to some extent also the red deer are kept under semidomesticated forms for meat production and, thus quite dense populations may occur. Still, they are viable and do not suffer from inbreeding depression, or any marked disease problems. However, their levels of Mhc variation differ considerably. Although the Mhc is often referred to as being under a strong selection pressure, the selection values are actually quite low (< 0.05; Satta et al. 1994). However, the selection pressure my still be increased by the introduction of a new pathogen in the population. A reduced selection pressure is unlikely to be the major cause for the low Mhc polymorphism seen in the species included.

Thirdly, Mhc diversity may be lost by random genetic drift in small populations. In the arctic species, especially muskox and Svalbard reindeer, the harsh climate makes the population sizes fluctuate considerably. Such repeated bottlenecks are possible reasons for the low polymorphism seen. Most probable few animals founded the Svalbard population, hence genetic drift may have had a strong effect. This suggestion is supported by the fact that lower variation is found among allozyme loci (Røed 1985). Additionally, the fallow deer is know from other genetic studies to be a monomorphic species (Emerson and Tate 1995; Pemberton and Smith 1985). This species has experienced several bottlenecks during the introduction of herds from Mesopotamia to Europe. These repeated bottlenecks may be the major cause for the low polymorphism seen, especially if there was already low levels of polymorphism within the ancestral populations. As discussed above the moose and roe deer populations in Scandinavia have experienced bottlenecks

in fairly recent time. Thus, this cannot be the major reason for the low polymorphism seen in moose since the low polymorphism is shared with the North American subspecies which diverged from the European subspecies more than 100,000 years ago.

The results from this thesis indicate that domesticated animals exhibit extensive Mhc variation while the level of diversity differs considerably between wild animal species. A similar situation can be recognized among humans class I genes, where urban populations comprise a significant amount of alleles, while on the contrary, natural populations of Amerindian tribes only possess a portion of that (William and McAuley 1992). The urban populations may have acquired their diversity due to mixing, over many thousands of years, of former subpopulations. Studies in mice, which are considered to be highly polymorphic at its Mhc, have shown that within subpopulations there may be quite low diversity (reviewed by Klein 1987). Our results suggest that the effect of natural selection may be overshadowed by population history, such as population bottlenecks.

Evolution of Mhc-DRB polymorphism

Sharing of alleles and motifs across populations

In a neighbor-joining tree, *DRB3* alleles found in European and African cattle, and in bison are intermingled (see Figure 2 in Paper I and III, respectively). No species specific clustering can be distinguished. The European and the African populations share several alleles but the allele frequencies are strikingly different. Apparently the 1-1.5 million years separating the cattle and bison subspecies are not enough to generate fixed species specific mutations in the *DRB3* exon 2 sequence (Paper III). Despite the high similarity between cattle and bison alleles, no alleles are shared between the two species, instead the similarities are restricted to shared amino acid motifs. Also, the single muskox *DRB* allele found is very similar to cattle and bison, despite its phylogenetic classification among the caprinae (Groves and Shields 1996). There is only one amino acid residue in the muskox sequence that have not been found in cattle or bison *DRB3* alleles. This amino acid, coded by the same nucleotides, is found at the same position in pig *DRB* alleles (Brunsberg et al. 1996).

There is only one shared allele between North American and Scandinavian moose, i.e. allele 3 which is the most common allele in the Alaskan population. Within each continent there is extensive sharing of alleles with few population specific alleles, but the allele frequencies differ considerably (Figure 8). In the two Scandinavian populations the allele frequencies are more evenly distributed within each population while the North American populations each have one predominantly existing allele. Although there is only one shared allele between continents, all but one of the polymorphic amino acid residues are found in both continents. Thus, the

polymorphism seen in moose most probably originated before the split of the Scandinavian and North American subspecies.

Among the three reindeer populations studied (Paper V), no alleles are shared among all three populations, but three are shared between the two Norwegian populations, which belong to the same subspecies. There are also three alleles specific for each of these two latter populations, and two alleles are specific for Svalbard reindeer. As have already been seen in comparisons between cattle and bison, and moose subspecies, the picture is totally different when the amino acid motifs are considered as separate units instead of as whole alleles. Of the 50 amino acids present in the polymorphic positions of reindeer *DRB1*, as many as 30 are shared between all three populations, and only two amino acids are specific for the Svalbard population and the wild reindeer in Central Norway, respectively. No amino acid residues are specific for the domestic reindeer in Northern Norway. Much of the polymorphism seen among the cervids is shared between species although no alleles are shared, as seen in the comparison between cattle and bison *DRB3* alleles. This suggests that, as with bovids, some of the motifs were present before the divergence of the cervidae species.

One of most common *DRB3* alleles in cattle, the **0201*, has the characteristic of a deleted codon, i.e. number 65 according to the numbering proposed by Brown and coworkers (1988, 1993). In Paper IV, two other alleles containing the same deletion motif, were sequenced. Deletion of codon 65 (del65) has also been found in a cattle *DQB* allele (*BoLA-DQB3*1*, Sigurdardóttir et al. 1992) and a roe deer *DRB* allele (*Caca-DRB1*0301*; Paper V). In the α-helical region the cattle del65 alleles were more similar to each other than alleles in general, whereas in the β-strand region they did not show to be more similar. The same result is seen if the roe deer del65 allele is compared to the cattle del65 alleles. This may suggest that cattle and roe deer share an ancestral del65 allele. However, the presence of several species specific substitutions disprove this possibility. Still, the deletion motif itself may originate before the split of the bovids and cervids. The *BoLA-DQB3*1* allele is not strikingly similar to any of the other alleles comprising del65. An interesting aspect is that the deletion of one amino acid from the α-helix of the class II β chain will make it resemble the structure of a class I molecule.

The results of the comparisons above (i.e. cattle vs. bison, Scandinavian vs. North American moose, Norwegian vs. Svalbard reindeer, cervidae *DRB* alleles, and the deletion in codon 65 of the *DRB* exon 2) is not in full agreement with the transspecies hypothesis which postulates that the allelic lineages are older than the species (Klein et al. 1993; Figueroa et al. 1988). Further, when comparing the two ruminant families in this thesis, it is clearly seen that within each of the bovidae and cervidae family there is extensive sharing of motifs, but less between the two families (Paper V, Figure 1). Instead we suggest a transspecies inheritance of short sequence motifs. Non-shared polymorphism is most likely generated after the divergence of cervidae and bovidae. In a neighbor-joining tree this is viewed as weak

species specific clustering (see Appendix VI). Only the species with the lowest Mhc diversity tend to show higher levels of allele clustering, i.e. moose, roe deer, reindeer, and fallow deer. The two loci amplified from fallow deer are more similar to each other than to any other DRB allele, which is not the case for the two loci amplified in red deer. The red deer sequences are scattered on several branches within the cervidae part of the tree. The bovinae and caprinae alleles are similarly mixed within the bovidae, but separate from the cervidae. Thus, the extensive sharing of motifs disrupts the phylogenetic tree within the level of families, but the divergence is strong enough to separate species between families, i.e. bovidae and cervidae.

Generation of Mhc polymorphism

There has been much discussion about the most plausible mechanism for generation of the characteristic "patchwork pattern" of shared sequence motifs, among series of Mhc alleles. The two main hypotheses are that the predominant mechanism for generation of new alleles is either point mutations, or some kind of recombination event. Recombination alone cannot generate new sequence motifs, but is restricted to shuffling already existing sequence motifs into new allelic types. Point mutations must therefore be the ultimate source for generation of new motifs. Point mutations are not restricted to any special location within the gene. However, not all substitutions will be accepted as some will result in loss of function of the molecule, either as lower affinity to peptides, misparing of the α- and β-chains or as a disrupted interaction with the T-cell receptor. Such unfavorable mutations will be removed by purifying selection. In addition, hydrophobic and charged amino acids are required as functional groups in peptide binding (Lundberg and McDevitt 1992), and some amino acids may not fit into the allowed structure of the peptide. Because of these functional requirements assigned to the Mhc molecule, the mutation rate is relatively slow, i.e. 2.4×10^{-8} substitutions per site per generation (Klein et al. 1993).

Interallelic recombination has the possibility to merge motifs from different allelic lineages, forming completely new ones. In this model, already accepted motifs are exchanged between the alleles. Thus, a product from a recombination event is more likely to be selectively advantageous than a new point mutation. Numerous studies have shown that interallelic recombination may contribute to the generation of polymorphism of Mhc genes (Belich et al. 1992; Gyllensten et al. 1991; She et al. 1991; Watkins et al. 1992), but few direct evidences exists. Zangenberg and coworkers (1995) showed, by sperm typing, that interallelic recombination occurs in *HLA-DPB1*.

Generation of new polymorphism by interallelic recombination may be particularly important after a population bottleneck, when the number of alleles have decreased. New alleles are then quickly generated from the few alleles "surviving" the population bottleneck. Occasional point mutations

will increase the number of motifs that can be shuffled around. An example of this is the moose *DRB1* which are restricted to 10 alleles, differing by nine nonsynonymous and a single synonymous substitution (Paper II, Figure 1A and B). A maximum parsimony analysis of these alleles showed that they could be divided into two major groups depending on the sequence motif present in codon 85 and 86 (Andersson and Mikko 1995). The shortest trees could be generated by 18 point mutations, but only 14 mutational events were needed if interallelic recombination was also considered as a possible mechanism for generation of polymorphism.

In cattle, few new sequence motifs were recognized when the *DRB3* polymorphism was analyzed also in African cattle (Paper I). As seen in all other species studied so far, the allelic diversity consisted of various combinations of shared sequence motifs. Closely related alleles often differ by multiple closely located substitutions (see Paper I, Figure 1 and Russell et al. 1997). Only a few of those substitutions are not found in any other allele sequenced so far, and are thus most probably recent point mutations, while the majority (i.e. about four times as many) are substitutions shared with other *BoLA-DRB3* alleles which may have been shuffled around by interallelic recombination. In cattle, the three deletion alleles, show considerable similarities around the deletion, but are not strikingly similar in other regions of the first domain. Therefore the most plausible explanation is that interallelic recombination has caused the motif containing the deletion to be inserted into other alleles, or vice versa. In a study by Gaur and Nepom (1996), the same region of exon 2 (codon 60-80) is involved in a segmental exchange of a deletion in the primate *DRB6* locus.

Another example of a possible recombination is illustrated by the bison specific 59^{Arg}, encoded by AGG, which was found in three out of nine bison *DRB3* alleles but in none of all cattle alleles sequenced so far. The 20 codons around this position, is shared between cattle and bison *DRB3* sequences. This supports the transfer of this sequence motif by a recombination event. A synonymous substitution in position 53 is found in 24 of the 68 cattle alleles but in none of the bison alleles suggesting that the mutation has occurred after the divergence of the cattle and bison species. It is highly unlikely for this mutation to occur seven times as suggested from the topology of the phylogenetic tree (Paper III, Figure 2). It is more likely that recombination have shuffled this motif among cattle alleles. This suggestion is also supported by the fact that 18 of these 24 substitutions are residents within closely related alleles, which diverged quite recently compared to other alleles.

Examples like the ones above are abundant among polymorphic Mhc genes and the common occurrence is a strong argument for the importance of interallelic recombination for the generation of Mhc polymorphism.

Conclusions

Most domestic animals, like cattle and goat, comprises a significant amount of Mhc polymorphism, while sheep exhibit intermediate levels. In wild animals, the level of polymorphism differ considerably between species. Muskox, moose, Svalbard reindeer, and fallow deer exhibit no or very low levels of Mhc polymorphism. The bison and red deer are highly polymorphic, while the reindeer and roe deer, like sheep, possess intermediate levels of Mhc diversity. None of the populations with low Mhc polymorphism show any apparent reduction in viability.

Domestic cattle comprises a significant amount of Mhc variation, particularly in Africa. The *DRB3* allele frequency distribution in the West African N'Dama breed is more similar to the West African Zebu than to the European breeds. This is consistent with recent studies indicating a substantial gene flow between *B. taurus* and *B. indicus* breeds in Africa.

A significant amount of Mhc polymorphism has been maintained through the population bottleneck which bison experienced in the 19th century. There is extensive sharing of polymorphism between cattle and bison *DRB3* loci, although no complete alleles are shared.

Both European and North American moose populations possess low genetic diversity at their Mhc. The data imply that the moose has gone through a population bottleneck where Mhc variation may have been lost. The extensive sharing of polymorphic *DRB1* sequence motifs states that this loss of diversity must predate the divergence between the European and the North American subspecies more than 100,000 years ago, thus a low Mhc diversity is compatible with long-term survival.

Mating type preferences cannot be the major selection mechanism in domestic animals since their reproduction have been controlled by humans for thousands of years. There are no data from domestic animals, arguing against pathogen-driven selection. The data in this thesis suggests that the effect of population history in wild animals may overshadow that of natural selection. However, there is a significant excess of nonsynonymous compared to synonymous substitutions in the PBS of the *DRB* loci amplified, indicating that the polymorphism studied is most likely functional.

The sharing of polymorphisms across species, as well as subspecies, seen in the present study is consistent with a trans-species persistence of sequence motifs rather than allelic lineages. Several examples where obtained supporting the view that interallelic recombination contributes to the generation of Mhc alleles.

Future perspectives

Since the level of polymorphism as well as expression differs considerably between Mhc loci, further investigations are certainly important to fully understand the significance of Mhc polymorphism. The main approach would be to characterize which of the amplified loci are expressed and to find out if other loci comprises the principal polymorphism, when a low variation is found in one locus. The fallow deer is a candidate species for such an investigation. Performance of RT-PCR of class I and II genes would evaluate which loci are transcribed. Screening of a cDNA library could pick up previously unknown loci which may carry major polymorphism.

Further and extended comparisons of the Mhc polymorphism in populations with different ecology would be interesting, as differences in level of Mhc polymorphism is seen among the wild animals in this study. Two main perspectives of this study would be i) To study Mhc diversity in one species but between different environments. ii) To study the Mhc diversity between species within the same environment. A parallel experiment to the moose survey presented in this thesis would be to compare the Mhc polymorphism of European bison, in contrast to the North American bison.

An evaluation of how important natural selection is compared to population history could be performed using experimental populations in controlled environments such as fishes in an aquarium. The development of Mhc polymorphism could then be followed through experimentally controlled population bottlenecks, and at different selection pressures. Additionally, disease resistance could be selected for by adding different quantities of pathogens.

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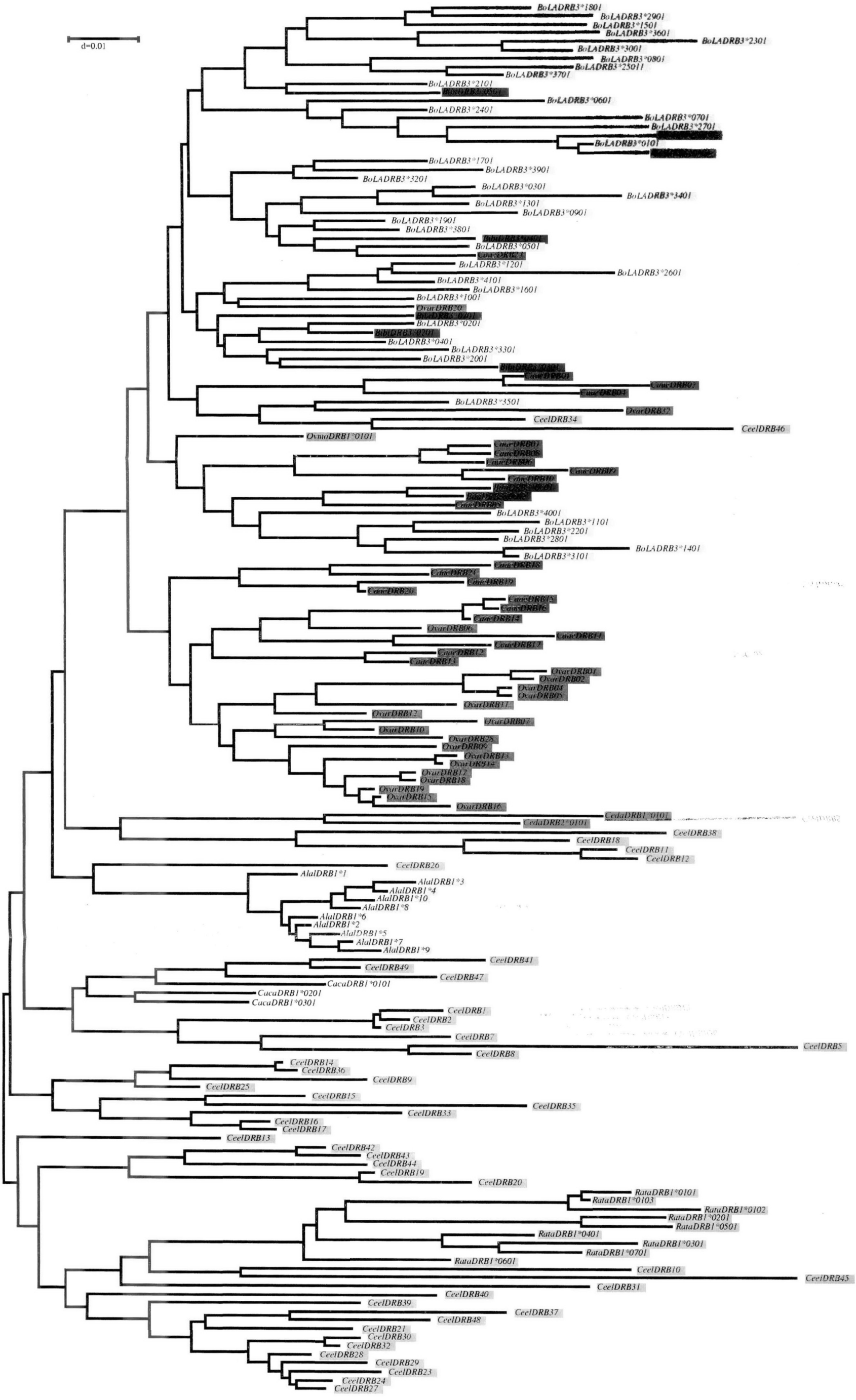
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