Molecular Ecology 1996, 5, 3–9

Limited polymorphism at major histocompatibility complex (MHC) loci in the Swedish moose *A. alces*

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Abstract

The Swedish moose was analysed for genetic variability at major histocompatibility complex (MHC) class I and class II *DQA*, *DQB* and *DRB* loci using restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) techniques. Both methods revealed limited amounts of polymorphism. Since the SSCP analysis concerned an expressed *DRB* gene it can be concluded that the level of functional MHC class II polymorphism, at least at the *DRB* locus, is low in Swedish moose. DNA fingerprinting was used to determine if the unusual pattern of low MHC variability could be explained by a low degree of genome-wide genetic diversity. Hybridizations with two minisatellite probes gave similarity indices somewhat higher than the average for other natural population, but the data suggest that the low MHC variability cannot be explained by a recent population bottleneck. However, since minisatellite sequences evolve more rapidly than MHC sequences, the low levels of MHC diversity may be attributed to a bottleneck of more ancient origin. The selection pressure for MHC variability in moose may also be reduced and we discuss the possibility that its solitary life style may reduce lateral transmission of pathogens in the population.

Keywords: bottleneck, DNA fingerprinting, genetic variation, MHC, moose, SSCP

Received 11 March 1994; final revision received 11 May 1995; accepted 17 May 1995

Introduction

The gene products of the major histocompatibility complex (MHC) system are recognized as key molecules in the immunological recognition of self and nonself (Klein 1986). There are two distinct classes of MHC molecules, class I and class II, which are encoded by separate but tightly linked loci. Both are cell-surface glycoproteins which function as presenters of intracellulary processed peptides. In a majority of the species so far studied, MHC represents the most polymorphic gene system found in the genome. Unlike most other polymorphic genes, MHC variability is characterized by the presence of a large number of alleles that occur at intermediate frequencies (Klein 1986). This is a pattern expected for loci under balancing selection (Hedrick & Thomson 1983) and it is generally believed that the high degree of genetic variability at MHC is maintained by overdominant or frequency-dependent selection (Klein *et al.* 1993). Many investigators argue that this relates to the immunological defence against pathogen-induced diseases (Bodmer 1972; Doherty & Zinkernagel 1975), although other selective forces such as mating preference and maternal–fetal interactions have been suggested as well (Hedrick & Thomson 1988; Potts & Wakeland 1993).

It has been suggested that populations which have experienced extensive bottlenecks, leading to reduced MHC variability, would be particularly vulnerable to infectious disease (O'Brien & Evermann 1988). Preserving MHC polymorphism has in fact been put forward as being a prime objective in all conservation programs (Hughes 1991). In line with this, it has been proposed that the extremely low level of MHC variability in the African Cheetah (O'Brien *et al.* 1985; Yuhki & O'Brien 1990) may be related to a marked susceptibility to a feline coronavirus infection in a group of captive individuals (O'Brien *et al.* 1985). Severe outbreaks of infectious disease in some other

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species speculated to possess reduced MHC variability may represent similar situations as well (O'Brien & Evermann 1988). We are involved in a study of the possible association between genetic variation at MHC loci and susceptibility to a lethal disease syndrome, still of unknown origin, among Swedish moose *A. alces* (Stéen *et al.* 1993). We have now used various molecular techniques to assess the amount and characteristics of MHC class I and class II variability in Swedish moose and in parallel analysed their genome-wide variability by means of DNA fingerprinting. We show here that these animals exhibit low levels of MHC polymorphism concurrent with a fairly high degree of genomic diversity.

Materials and methods

Animals

Two sets of animals were analysed. First, liver samples were obtained from 10 free-living moose collected in four different areas spread from southern to northern Sweden. Second, liver samples were also derived from 18 free-living individuals shot during the autumn hunt in a 35 km^2 area in central Sweden. Apparent relatives were avoided in the latter case by only collecting a single specimen from each shooting location. Pieces of liver were transferred to –20 °C not later than six hours after shooting.

We have previously made extensive genetic studies of the MHC system of cattle (e.g. Sigurdardóttir *et al.* 1988). We will make reference to cattle several times in this study since this species represents the closest relative to the moose for which MHC variation previously has been well characterized. All cattle data cited have been obtained from the Swedish Red and White breed.

RFLP analysis

Genomic DNA was prepared from blood and liver as previously described (Ellegren *et al.* 1991). Southern blots of *Taq*I, *Pvu*II, *Eco*RI or *Hin*dIII digests (10 μg) were prepared following standard procedures, i.e. electrophoretic separation of fragments in 1.0% agarose gels and subsequent transfer to nylon filters (Biodyne B) at alkaline conditions. Hybridization was carried out according to Mariani *et al.* (1992) with a final washing stringency of 0.7 x SSC; 0.1% SDS at + 58 °C. We used four human MHC cDNA clones as hybridization probes: pHLA27 representing *HLA* class I (see Lindberg & Andersson 1988), pII-α-5 representing *HLA-DQA* (Schenning *et al.* 1984), pII-β-1 representing *HLA-DQB* (Larhammar *et al.* 1982) and pII-β-3 representing *HLA-DRB* (Gustafsson *et al.* 1984). The three latter probes correspond to MHC class II genes.

DNA fingerprinting

Filters were prepared as above except that the DNA was digested with *Hin*fI and that 0.7% agarose gels were used. We employed two commonly used minisatellite probes, MS6 (Jeffreys *et al.* 1985) and a 952 bp *Bst*NI fragment from phage M13 (Vassart *et al.* 1987). Hybridization was performed in the absence of competitor DNA and filters were washed at a final stringency of 2 × SSC; 0.1% SDS at + 55 °C.

SSCP analysis

Exon two of the MHC *DRB* gene was amplified by PCR using the cattle specific primers LA31 and LA32 (Sigurdardóttir *et al.* 1991). The 20-μL reactions contained 0.2 mM dNTP, 0.5 μM of each primer, 25 ng of genomic DNA and 1 U *Taq* polymerase. A total of 30 cycles was performed, the three first had + 95 °C for 30 s, + 50 °C for 10 s and + 72 °C for 45 s, the following 27 had + 94 °C for 10 s, + 50 °C for 10 s and + 72 °C for 30 s. The PCR was performed on a Perkin–Elmer–Cetus 9600 instrument.

The SSCP analysis essentially followed Maekawa *et al.* (1992). Briefly, PCR products were mixed with a 1.5 volume denaturing solution containing 95% formamide, 20-mM EDTA, 0.05% BFB and 0.05% xylene cyanol, and were incubated at + 80 °C for 10 min. Samples were subsequently applied to PhastSystem gradient gels 8–25 (previously prerunned at 250 V, 10 mA and 2.5 W for 60 Vh) and electrophoresed with native buffer strips at + 15 °C in a PhastSystem device (Pharmacia, Uppsala) for 330 Vh (using the same settings as above). After separation, the gels were silver-stained with the PhastSystem silver staining kit.

Results

RFLP analysis of moose MHC class I and class II DQA, DQB and DRB homologues

Human cDNA clones encoding *HLA* class I (locus not determined), *HLA-DQA*, *HLA-DQB* and *HLA-DRB* genes were hybridized to moose *Taq*I, *Pvu*II, *Eco*RI and *Hin*dIII digests. The screening was performed on 10 unrelated individuals sampled at various sites in Sweden. The outcome of the experiments is summarized in Table 1. Clearly, the levels of polymorphism were very limited. Completely monomorphic hybridization patterns were revealed in seven out of 16 probe/enzyme combinations. Furthermore, the seven polymorphic class II probe/enzyme combinations did most likely only detect two different *DQ* haplotypes. This conclusion was reached on the basis of that the polymorphic fragments, only one or two in each case, consistently were present in the same

Table 1 Summary of the moose MHC RFLP analysis. The number of polymorphic fragments are indicated for each probe/enzyme combination with the total number of fragments in parentheses. Obviously cross-hybridizing fragments were excluded from the total numbers

	Enzyme			
Locus	*Taq*I	*Pvu*II	*Eco*RI	*Hin*dIII
Class I	1 (11)	0 (12)	1 (12)	0 (13)
Class II *DQA*	2 (6)	1 (4)	1 (5)	1 (4)
Class II *DQB*	1 (3)	2 (5)	0 (5)	0 (3)
Class II *DRB*	0 (3)	0 (4)	0 (3)	0 (2)

four individuals in all seven combinations. The variability seen with the class I probe was similarly low, i.e. monomorphic pattern with two enzymes and one polymorphic fragment with two other enzymes. The latter polymorphisms represented different haplotypes as compared to the class II polymorphisms (the polymorphic *Taq*I fragment was present in five individuals whereas the polymorphic *Eco*RI fragment only occurred in two individuals).

In an extended screening we analysed 18 additional moose sampled in central Sweden. The screening was restricted to *DQB* and *DRB* probes hybridized to *Pvu*II digests. As for the first 10 individuals, a completely monomorphic pattern was revealed with the *DRB* probe. Four out of five hybridization fragments specifically detected by the *DQB* probe were polymorphic in this material but could be interpreted as a simple two-allele polymorphism (Fig. 1a). One allele was composed of 2.4- and 6.9-kb fragments (allele A) whereas the other harboured 4.6 and 5.0-kb fragments (allele B). The fact that only the 4.6 and 5.0-kb fragments were polymorphic in the first 10 individuals indicates that all these animals carried at least one copy of allele A. The frequencies of allele A and B in the total sample of 28 animals were 64% and 36%, respectively. Genotype frequencies were consistent with Hardy–Weinberg equilibrium (χ^2 = 0.29, NS). The observed heterozygosity was 50%.

The low levels of genetic variability found in RFLP analysis of Swedish moose using different class I and class II probes indicate that the overall degree of MHC polymorphism in this species is limited. A comparison with bovine data gives strong support for this conclusion. Sigurdardóttir *et al.* (1988) used the same probes/enzymes as in this study in an extensive survey of MHC class II polymorphism in cattle. They found 21 *DQ* and 25 *DR* haplotypes, while we only found two *DQ* haplotypes in moose. Moreover, among 197 domestic bulls, 185 were *DQ* heterozygotes (94%; Sigurdardóttir *et al.* (1988)). The proportion of *DQ* heterozygotes in cattle and moose shows a highly significant difference (χ^2_1 = 46.2, $P < 0.001$). Furthermore, in a study of bovine MHC class I polymorphism, Lindberg & Andersson (1988) distinguished no less than 21 *Pvu*II haplotypes in a sample of 50 unrelated individuals. There were no more than two class I haplotypes seen with any enzyme in moose. As an illustration of the varying levels of MHC polymorphism in cattle and moose, a blot with bovine *Pvu*II digests hybridized with the same human *DQB* probe as employed in the present study is shown in Fig. 1(b). Clearly, the degree of polymorphism differs considerably between the two species.

SSCP analysis of a moose DRB homologue

The primers LA31 and LA32 specifically amplify exon two of the bovine *DRB3* gene, the most predominantly expressed and the most polymorphic *DRB* gene in cattle. The primer pair was used in amplifications with moose DNA and a single distinct PCR product was revealed for each individual. The size of the amplified product exactly corresponded to that obtained for the bovine *DRB3* gene (297 bp) and subsequent sequence analysis made evident that the product represented a moose MHC *DRB* gene, denoted *DRB1* (Mikko & Andersson 1995). It can be concluded that this locus constitutes an MHC gene of func-

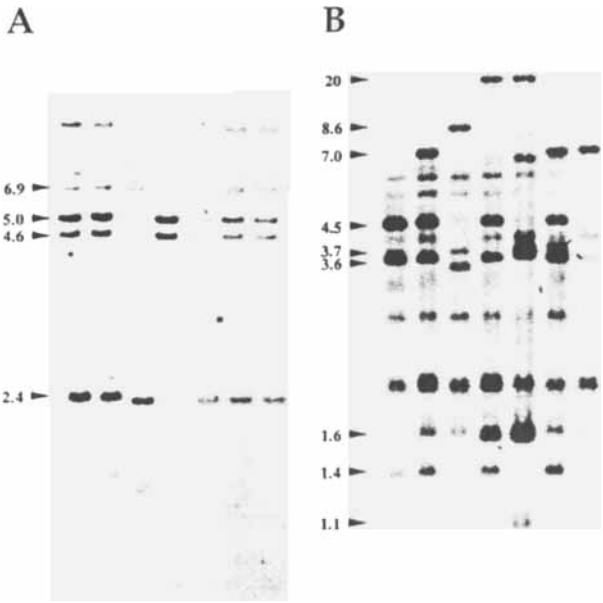

Fig. 1 RFLP patterns of unrelated (a) moose and (b) cattle using a human *HLA-DQB* probe and *Taq*I-digested genomic DNA. Fragment sizes (kb) of polymorphic fragments are indicated to the left of each blot.

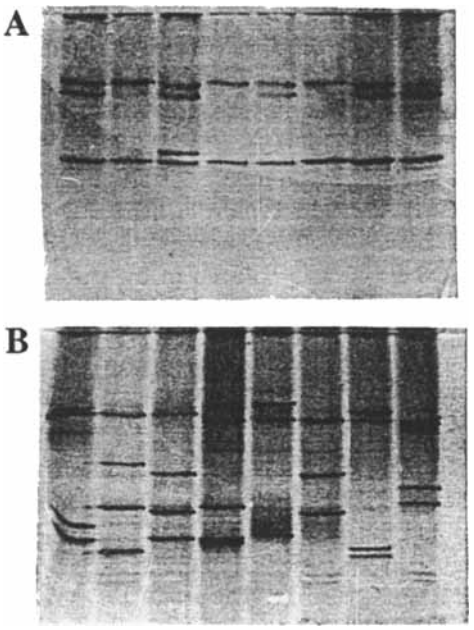

Fig. 2 SSCP analysis of *DRB* alleles in (a) moose and (b) cattle. Presumed *DRB* allelic fragments are indicated by dots. The genotypes are as follows: (a) (1) 1/2, (2) 2/2, (3) 2/3, (4) 2/2, (5) 1/2, (6) 2/2, (7) 1/2 and (8) 1/1. (b) (1) 9A/11, (2) 3A7/A, (3) 8A/9A, (4) 1B/5A, (5) 2A/12, (6) 2C/8A, (7) 1A/6 and (8) 4A/10 (bovine nomenclature following Sigurdardóttir *et al.* 1988).

tional importance since it is the predominantly expressed *DRB* locus in moose according to RT-PCR analysis (Mikko & Andersson 1995).

SSCP analysis of the *DRB1* gene amplified from 16 moose identified three different alleles (Fig. 2a). One allele was obviously rare since it was only found in one heterozygous animal. The frequencies of the other two alleles were 0.53 and 0.41, respectively (H_{exp} = 0.55). Again this indicates that the moose has limited MHC class II polymorphism. A sample of cattle analysed with the same SSCP methodology (i.e. using the same primers) is shown in Fig. 2b.

Linkage disequilibrium between DQB and DRB

A characteristic feature of the MHC is the presence of strong linkage disequilibrium between loci. This was also evident from the present data. Using the method of Hill (1974), a significant linkage disequilibrium between the moose *DQB* RFLP and the *DRB* SSCP alleles was observed ($D = 0.17$; $P < 0.05$). The relative amount of disequilibrium (Hedrick *et al.* 1978) was estimated at 0.68.

Genomic variability of moose

We carried out DNA fingerprinting using two minisatellite probes and *Hinf*I digested DNA to analyse the genome-wide variability of Swedish moose. Statistics derived from these experiments are given in Table 2 and a representative autoradiograph is shown in Fig. 3. Similarity indices (D) calculated from hybridizations with MS6 and M13 were 0.46 and 0.49, respectively. These values are somewhat higher than that previously reported for a number of outbred populations of mammals and birds (Reeve *et al.* 1990), and is more similar to that obtained for several domestic animals (e.g. Georges *et al.* 1988).

Discussion

We have investigated the genetic variability of moose MHC class I and class II genes by means of (i) RFLP analysis using four different human cDNA probes and (ii) by SSCP analysis of exon two of the moose *DRB1* gene. The experiments showed that the Swedish moose exhibits limited amounts of MHC polymorphism at the class I and the class II *DQA*, *DQB* and *DRB* loci. Hybridizations with

Table 2 DNA fingerprinting data from a sample of 18 Swedish moose

Parameter	Probe	
	MS6	M13
Total number of resolvable bands (N)	36	33
Mean number (± SD) of bands per individual (n)	13.0 ± 2.0	12.0 ± 1.5
Mean (± SD) band frequency (x)	0.36 ± 0.23	0.36 ± 0.26
Probability of identity of DNA fingerprints (P)*	1.7×10^{-6}	4.7×10^{-6}
Similarity index (D)†	0.46	0.49
Average percentage difference (APD)‡	0.69	0.66

*$P = x^n$

†$D = 2N_{A+B}/N_A + N_B$.

‡Calculated according to Yuhki & O'Brien (1990).

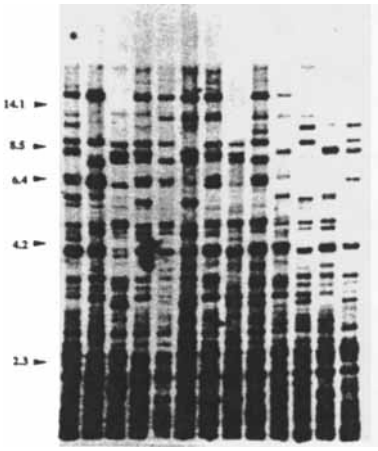

Fig. 3 DNA fingerprinting of unrelated moose using the human MS6 minisatellite probe and *Hin*fI-digested DNA.

class II probes revealed only two different haplotypes whereas the SSCP analysis uncovered three *DRB* alleles, one of which was rare. Although this does not formally prove that the entire moose MHC is less variable than in other species, we find this very likely (see discussion below) and we will for simplicity refer to our findings as just low MHC variability rather than each time specifying the individual loci investigated.

Human MHC cDNA clones have been applied to a number of animal species and are capable of detecting extensive polymorphism in RFLP analysis. Previous analyses have shown that the variability seen by heterologous probes is closely correlated to expressed polymorphism in such divergent species like cattle and chicken (Andersson *et al.* 1987; Lindberg & Andersson 1988; see also Yuhki & O'Brien 1990). This situation and, as we previously have discussed (Ellegren *et al.* 1993), the facts that the polymorphic parts of MHC genes are well conserved even between distantly related mammals and that clones corresponding to some of the most polymorphic human class II genes were employed (*DQB* and *DRB*), argue for that the RFLP method is a reliable indicator of the amount of functional MHC variability in moose. Moreover, in this study we supplemented the RFLP analyses with SSCP, a technique that is one of the most sensitive methods for detecting point mutations. SSCP analysis relies on the fact that the mobility rate of a single-stranded DNA molecule in a nondenaturing gel is not only determined by its size but also by its nucleotide sequence, which, in turn, governs its three-dimensional structure (Orita *et al.* 1989). Even a single nucleotide substitution may slightly alter this structure leading to a differential migration pattern. It has been estimated that SSCP can distinguish between 70% and 95% of all possible mutations in PCR products < 200 bp and about 50% for those < 400 bp (Michaud *et al.* 1992; Sheffield *et al.* 1992).

The finding of low levels of MHC variability in Swedish moose is thus supported by the inherent sensitivity of the SSCP technique. The fact that an expressed moose MHC gene was analysed rules out the possibility that the limited variability could be explained by a reduced selection pressure at a locus of no functional significance. The sequence analysed by SSCP, exon two of *DRB*, encodes the antigen recognition site in the first extracellular domain of class II β chain genes. In other species, the genetic polymorphism of class II gene products is concentrated to particular amino acids involved in antigen binding encoded by this exon (Brown *et al.* 1988). A characteristic feature of this polymorphism, as known from various species, is that there is a considerable amino acid divergence between alleles (Klein 1986; Andersson & Davies 1994). In an accompanying study we have shown that the Swedish as well as the Canadian moose does not only possess limited amounts of *DRB* polymorphism but also has minor sequence variations between alleles (Mikko & Andersson 1995).

What is the causal basis for the low levels of MHC polymorphism among the moose analysed in this study? A possible explanation would be that a previous bottleneck could have reduced the genetic variability. Similar to the situation for the Scandinavian beaver population (Ellegren *et al.* 1993), the size of the Swedish moose population was reduced due to over-hunting in the last century. The population has subsequently recovered and has increased dramatically during the 20th century, currently harbouring some 400 000 individuals. However, the extent of the bottleneck in the 19th century is uncertain (cf. Ryman *et al.* 1980) and our data strongly argue against a severe reduction since DNA fingerprinting patterns were far from monomorphic. The observed band-sharing probabilities (0.46–0.49) are somewhat higher than that previously found among outbred natural populations (e.g. Reeve *et al.* 1990), but do not indicate a dramatic loss of genetic variability. Similarly, Ryman *et al.* (1980) analysed allozyme variability in a large number of moose sampled at various localities in Scandinavia and found a mean heterozygosity, within each population, of 0.020. Again, this does not indicate a dramatically lower variability than among other mammals. Nevo (1978), Baccus *et al.* (1983) and Wooten & Smith (1985) have reported mammalian mean heterozygosities of 0.035–0.040.

The low levels of moose MHC polymorphism may however, be due to a more remote population bottleneck. Minisatellite loci, uncovered by DNA fingerprinting, are characterized by extremely high mutation rates (Jeffreys *et al.* 1988) and fingerprinting variability may thus rather rapidly be regenerated following a bottleneck. In an attempt to date the genetic bottleneck of the African

Cheetah, Menotti-Raymond & O'Brien (1993) calculated that the moderate DNA fingerprinting variability found among modern Cheetahs could have been reconstituted (from complete homozygosity) in the order of 10 000 years. Although the DNA fingerprinting variability in our moose sample was higher than among the Cheetahs, calculations similar to those made by Menotti-Raymond and O'Brien are consistent with that the Swedish moose may have experienced a severe bottleneck in the last 10 000–50 000 years. If so, extensive MHC variability is not to be expected among contemporary moose since the mutation rate at MHC loci is significantly lower than that at minisatellite loci. As evident from interspecific comparisons, extensive MHC polymorphism reflects the accumulation of mutations during millions of years of evolution (Klein *et al.* 1993).

Besides the possible effects caused by an ancient bottleneck, the low levels of moose MHC variability could also be due to a relatively weak selection pressure for polymorphism at these loci. There is a clear difference between the moose and cattle as regards their degrees of MHC polymorphism in relation to their genome-wide genetic variability measured by DNA fingerprinting. The DNA fingerprinting variability of moose is similar to that among several domestic animals, including cattle (Georges *et al.* 1988), and is significantly lower than that in humans. In contrast, the degree of MHC polymorphism in moose is much lower than that in both cattle and humans. Differences in the intensity of pathogen-driven, balancing selection affecting moose and cattle may contribute to the marked differences in their degree of MHC diversity. Domestic cattle are kept in dense herds which obviously facilitates the transmission of pathogens between animals. The moose, on the other hand, has a rather solitary life style. Males' close contacts with other animals are probably restricted to the reproduction period, a situation that would reduce the lateral transmission of pathogens in the population.

The Swedish moose consequently provides an additional example of a viable population with restricted MHC polymorphism. Slade (1992) noticed that three marine mammals from two different orders (the elephant seal, the sei whale and the fin whale) exhibit low degrees of MHC variability despite no apparent loss of allozyme heterozygosity. He proposed that this could be due to a reduced balancing selection pressure in the marine environment, a possible consequence of decreased exposure to pathogens. Trowsdale *et al.* (1989), who analysed the two whale species, argued that the aquatic environment would impose less contact between the animals compared to a terrestrial environment and that this would hamper the spread of pathogens in the population. A similar but controversial idea has been invoked to explain the apparent lack of MHC class I polymorphism in Syrian hamsters (Darden & Streilein 1984); the solitary life style of this species could act as a barrier for pathogen transmission as well (McGuire *et al.* 1985). In this context it could be noted that beavers, of which at least three European populations appear to be devoid of MHC polymorphism (Ellegren *et al.* 1993), also have a rather isolated life style. Although they live in family groups and dispersal may occur in natal drainage systems, large land areas separating different river systems can be seen as barriers for long-range dispersal and hence also for lateral transfer of pathogens.

Most authors, including us, have interpreted the presence of reduced levels of MHC diversity in certain species to be due to previous bottlenecks or relaxed pathogen-driven selection. However, it should be noted that the hypothesis of pathogen-driven selection for MHC polymorphism has not yet been confirmed by experimental data, nor has it been shown that the level of MHC diversity influence the long term survival of natural populations. It is clear that data from a broad range of species will be required to shed light on this important question.

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This work is part of long-term project aimed at understanding the evolution and polymorphism of MHC in different mammals. Hans Ellegren is associate professor, Sofia Mikko is PhD student and Leif Andersson is professor in molecular and disease genetics at the Swedish University of Agricultural Sciences in Uppsala. This particular study arose from a collaboration with Dr Kjell Wallin at the same university who has a keen interest in the biology of moose.
