Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4259–4263, May 1995
Genetics

Low major histocompatibility complex class II diversity in European and North American moose

(evolution/intraexonic recombination/natural selection/mtDNA/population bottleneck)

SOFIA MIKKO AND LEIF ANDERSSON†

Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Biomedical Center, Box 597, S-751 24 Uppsala, Sweden

Communicated by George Klein, Mikrobiologiskt och Tumörbiologiskt Centrum, Stockholm, Sweden, January 23, 1995 (received for review November 14, 1994)

ABSTRACT **Major histocompatibility complex (MHC) genes encode cell surface proteins whose function is to bind and present intracellularly processed peptides to T lymphocytes of the immune system. Extensive MHC diversity has been documented in many species and is maintained by some form of balancing selection. We report here that both European and North American populations of moose (*Alces alces*) exhibit very low levels of genetic diversity at an expressed MHC class II *DRB* locus. The observed polymorphism was restricted to six amino acid substitutions, all in the peptide binding site, and four of these were shared between continents. The data imply that the moose have lost MHC diversity in a population bottleneck, prior to the divergence of the Old and New World subspecies. Sequence analysis of mtDNA showed that the two subspecies diverged at least 100,000 years ago. Thus, viable moose populations with very restricted MHC diversity have been maintained for a long period of time. Both positive selection for polymorphism and intraexonic recombination have contributed to the generation of MHC diversity after the putative bottleneck.**

The major histocompatibility complex (MHC) class I and class II molecules exhibit an extraordinary high degree of genetic polymorphism in many vertebrate species (1). The MHC polymorphism occurs predominantly at residues involved in peptide binding (2–4), and there is compelling evidence that the polymorphism is maintained by some form of balancing selection (5–8). The essential role of the MHC molecules for immunological recognition of foreign peptide antigens implies that the cause for this selection is related to the influence of MHC polymorphism on host defense against pathogens (9, 10). Consequently, it has been suggested that species with low MHC polymorphism may be particularly vulnerable to infectious diseases (11, 12).

The moose is the largest deer in the world and has a circumpolar distribution in the Northern Hemisphere. It evolved in Eurasia and entered America by way of the Bering land bridge during the Pleistocene (13). The moose from the Old and New Worlds are considered to be subspecies (14). Our interest in characterizing MHC polymorphism in the moose is due to the occurrence of high mortality caused by a nematode (*Elaphostrongylus* spp.) parasitizing the central nervous system (15), as well as a wasting disease associated with a recently identified retrovirus (16). We have observed low levels of MHC polymorphism in Swedish moose for both class I genes and for class II *DQ* and *DR* genes by using restriction fragment length polymorphism and single strand conformation polymorphism (SSCP) analyses (H. Ellegren, S.M., K. Wallin, and L.A., unpublished data). The objective of this study was to provide further insights into the level of moose MHC polymorphism by DNA sequence analysis.‡ The *DRB* gene was chosen for this purpose because it is the most polymorphic class II gene in cattle and humans (17, 18). Moreover, to test whether low levels of MHC polymorphism are characteristic for the moose, samples from both the European and North American subspecies were analyzed.

MATERIALS AND METHODS

Samples. Blood or tissue samples were available from 30 Swedish moose from five localities (Lycksele and Laisdalen, county of Lappland; Robertsfors, county of Västerbotten; Sala, county of Uppland; Tranås, county of Småland). Tissue samples from 19 Canadian moose collected in seven different national parks (Yoho, Terra Nova, Jasper, Banff, Glacier, Kootenay, and Prince Albert) were kindly provided by C. Strobeck (Department of Zoology, University of Alberta, Edmonton, Canada). The localities were geographically well distributed within each country.

SSCP and Sequence Analysis of *DRB1* Exon 2. Genomic DNA was prepared, and PCR amplification of *DRB* exon 2 was carried out with the primers LA31 and LA32 as described (19). SSCP analysis was carried out on fragments fractionated by 8% polyacrylamide gel electrophoresis (SE600, Hoefer) at a constant temperature of +20°C. SSCP fragments were visualized by silver staining. PCR fragments corresponding to individual alleles were used for direct sequencing after a second round of PCR with LA31 and a nested primer, F1 (5′-GCTCAC-CTCGCCGCTGCAC-3′). The sequence analysis involved a total of 21 moose representing the 10 alleles revealed by SSCP analysis. For homozygotes, an aliquot of the reaction mixture from the first round of PCR was used as template, while for heterozygotes, allelic fragments were isolated from polyacrylamide gels containing SSCP fragments. Gel fragments were put in 100 μl of sterile water, and the supernatant obtained after repeated freezing and thawing was desalted with the Qiaex kit (Qiagen, Chatsworth, CA). Ten microliters of the desalted eluate was used as template in a second round of PCR. The resulting PCR product was used for DNA sequencing after purification with QIAEX. DNA sequencing on both strands was carried out on an ABI 373A instrument by using cycle sequencing and dye terminators (Applied Biosystem).

cDNA Analysis. Blood samples were collected from two individuals, both of whom turned out to be homozygous for the *DRB1*4* allele. mRNA was prepared from buffy-coat cells by using the QuickPrep *Micro* mRNA purification kit (Pharmacia). First strand cDNA synthesis was performed with random primers by using the Reverse Transcription System (Promega). The product of first strand synthesis was diluted to 20% of its initial concentration and 5 μl of the diluted product was used

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; SSCP, single strand conformation polymorphism; D loop, displacement loop.

†To whom reprint requests should be addressed.

‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X82398 and X83278–83286).

as template for PCR amplification with the highly conserved *DRB* primers LA59 (5′-GATGGATCCGTGCGCTTCGA-CAGCGAC-3′) and LA23 (5′-ATCGAATTCGCTG-CAGGGGCTGGGTCTTG-3′). PCR reactions were carried out with 10 pmol of each primer and 200 μM each dNTP in a reaction buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris·HCl (pH 8.3), and 0.5 unit of *Taq* DNA polymerase in 20 μl. A total of 30 cycles were performed in a thermocycler (Perkin–Elmer model 9600) as follows: 10 s at 94°C, 10 s at 50°C, and 30 s at 72°C, except that the three first cycles had denaturation times of 30 s at 95°C. The PCR product was diluted to 1% of its initial concentration and 2 μl of the diluted product was used for a second round of PCR with the same primers. The amplified product was purified and sequenced from both strands as described above.

mtDNA Analysis. A segment of the hypervariable control region of mtDNA was amplified by PCR by using the primers LmPro (L15997) 5′-GCCATCAACTCCCAAAGCT and TDKT (H16498) 5′-CCTGAAGTAGGAACCAGATG (20); the primer designations are composed of a coded name followed by the human nucleotide reference number. L or H indicates light or heavy strand. PCR and SSCP analyses were carried out essentially as described above. The DNA sequences of the mtDNA types of six Swedish and 10 Canadian moose were determined by cycle sequencing of the PCR products without subcloning.

Phylogenetic Analyses. Neighbor-joining phylogenetic trees (21) were constructed by using the MEGA program (22) and by applying genetic distances estimated with Kimura's two-parameter method (23). The relative frequencies of nonsynonymous (d_N) and synonymous substitutions (d_S) were estimated according to Nei and Gojobori (24) applying Jukes and Cantor's (25) correction for multiple hits. Genetic distances between mtDNA haplotypes were calculated by means of the Kimura two-parameter method using MEGA. The average pairwise distance between mtDNA haplotypes from Swedish and Canadian moose was calculated according to Nei (26).

RESULTS

SSCP and Sequence Analysis of *DRB1* Exon 2. Allelic fragments representing a single locus, denoted *DRB1*, were amplified by PCR from genomic DNA by using cattle-specific primers for *DRB* exon 2; this exon encodes half of the *DR* peptide binding sites and harbors the great majority of polymorphic codons in mammalian *DRB* genes (3). SSCP analysis revealed seven and four *DRB1* alleles among Swedish and Canadian moose, respectively, and only one allele was shared between continents (Table 1). The alleles were further investigated by sequencing exon 2. The polymorphism was restricted to 10 nucleotide substitutions causing altogether six amino acid substitutions (Fig. 1 *A* and *B*). The moose *DRB* diversity is minute compared with that of cattle (Fig. 1*A*) and a number of other species, with respect to the number of alleles and, in particular, the number of substitutions between alleles. However, the polymorphism is most certainly functionally significant, as all substitutions occur at positions involved in peptide binding.

Table 1. MHC *DRB1* allele frequencies in Swedish and Canadian moose

Allele	Sweden (n = 30)	Canada (n = 19)
*DRB1*1*	0.28	0
*DRB1*2*	0.38	0
*DRB1*3*	0.05	0.18
*DRB1*4*	0.12	0
*DRB1*5*	0	0.58
*DRB1*6*	0.13	0
*DRB1*7*	0	0.21
*DRB1*8*	0.02	0
*DRB1*9*	0.02	0
*DRB1*10*	0	0.03

The allele frequencies have been obtained by pooling data from different localities, and there may be allele frequency differences between localities.

The relative frequencies of nonsynonymous and synonymous substitutions among *DRB1* alleles differed significantly at codons for the peptide binding site ($d_N = 0.113 \pm 0.032$; $d_S = 0$; $P < 0.001$). This provides evidence that the accumulation of *DRB* diversity has been promoted by positive Darwinian selection. For instance, alleles 1 and 3 differ by a single synonymous substitution but by eight nonsynonymous substitutions at five codons for residues involved in peptide binding (Fig. 1*B*).

***DRB* cDNA Analysis.** cDNA was isolated from peripheral blood lymphocytes of Swedish moose and subjected to PCR and subsequent sequence analysis. This was done to ensure that our estimate of *DRB* diversity was based on an expressed locus and that PCR of genomic DNA with cattle-specific primers did not fail to amplify a second more polymorphic *DRB* locus. The experiment was expected to amplify all expressed *DRB* genes since the primers used corresponded to highly conserved parts of *DRB* exons 2 and 3. Direct sequencing of the amplified product from two presumed homozygotes showed that the cDNA products were derived from a single predominantly expressed locus identical to that defined by PCR analysis of genomic DNA (Fig. 2).

Phylogenetic Analyses. A phylogenetic-tree analysis of moose, cattle, and human *DRB* alleles illustrates the very restricted moose *DRB* diversity (Fig. 3). Moreover, the frequency of synonymous substitutions between alleles was estimated at 0.087 ± 0.021, 0.046 ± 0.015, and 0.009 ± 0.009 for humans, cattle, and moose, respectively. The striking differences in interallelic genetic distances show that *DRB* alleles in the moose must be a lot younger than human and cattle alleles (Fig. 3); some of the allelic lineages in humans are assumed to be tens of millions of years old (4).

mtDNA Analysis. A part of the hypervariable displacement loop (D loop) of mtDNA was amplified by PCR and sequenced to get an estimate of the time since divergence of European and North American moose populations. SSCP and sequence analyses of the amplified fragments resolved two and seven mtDNA types among the samples of Swedish and Canadian moose, respectively. A striking difference between D-loop sequences from Swedish and Canadian moose was that the former contained an insertion of 75 nucleotides. A total of 28 polymorphic positions, all due to transition mutations, were found among the nine alleles (Table 2). The subspecies status of European and Canadian moose is supported by the clear dichotomy in D-loop sequences from the two populations (Fig. 4); the insertion was not included in the phylogenetic analyses.

We observed an average pairwise nucleotide distance of 3.9% between mtDNA sequences from different continents. Applying the estimated molecular clock rate of 0.118×10^{-6} substitutions per site per year for the D loop in humans (29) to these data, we obtained a divergence time of 165,000 years B.P. for the two subspecies. A higher estimate of about 350,000 years was obtained by relating the moose data with the corresponding D-loop distance between domestic cattle and American bison (10.6%) who are assumed, on the basis of allozyme and paleontological evidence, to have diverged from a common ancestor 1 million years ago (30). We conclude that the divergence time for the European and North American populations included in this study is in the range of 100,000–400,000 years B.P. Thus, the split between the two subspecies must predate the last Pleistocene glaciation, which lasted from about 120,000 to 10,000 years B.P. (31), and the two subspecies have recolonized previously glaciated areas from different

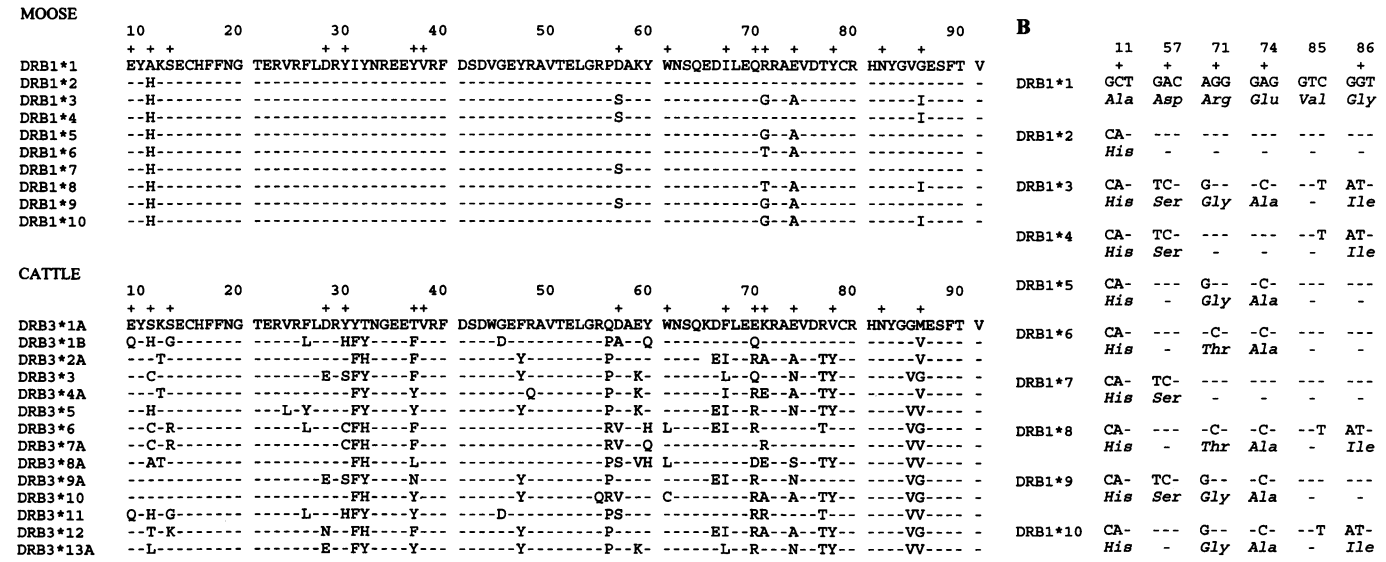

FIG. 1. (*A*) Alignment of moose and cattle (19) DRB amino acid sequences encoded by exon 2. Identity to the master sequence in each species is indicated with a dash. + marks positions in the peptide binding site (3). (*B*) Polymorphic codons with corresponding amino acid residues for the 10 moose *DRB1* alleles.

refugia. The mtDNA diversity revealed here provides a tool to study the phylogeny of moose populations.

DISCUSSION

This study has revealed that both European and North American moose populations possess low genetic diversity at a MHC class II *DRB* locus. The functional importance of this locus is validated by our cDNA analysis, as well as the significant excess of nonsynonymous substitutions, which provides evidence that the locus is under selection for expressed polymorphism. The data imply that the moose, sometime during its population history, has lost most of its *DRB* diversity. This could be due to selection for one or a few favored MHC class II haplotypes or to a population bottleneck. The latter explanation is the most plausible one and is supported by the fact that the heterozygosity at allozyme loci in the moose is low compared with other mammals and in relation to its current effective population size (32); the average heterozygosity has been estimated to be 0.020 compared with 0.143 in humans. The almost complete sharing of *DRB* sequence motifs between Swedish and Canadian moose shows that the inferred loss of MHC diversity must predate the divergence between the European and North American subspecies, which occurred more than 100,000 years B.P. according to our mtDNA analysis.

An important question is whether our estimate of MHC diversity in the moose is representative since it is not trivial to estimate the degree of genetic diversity in a complex system

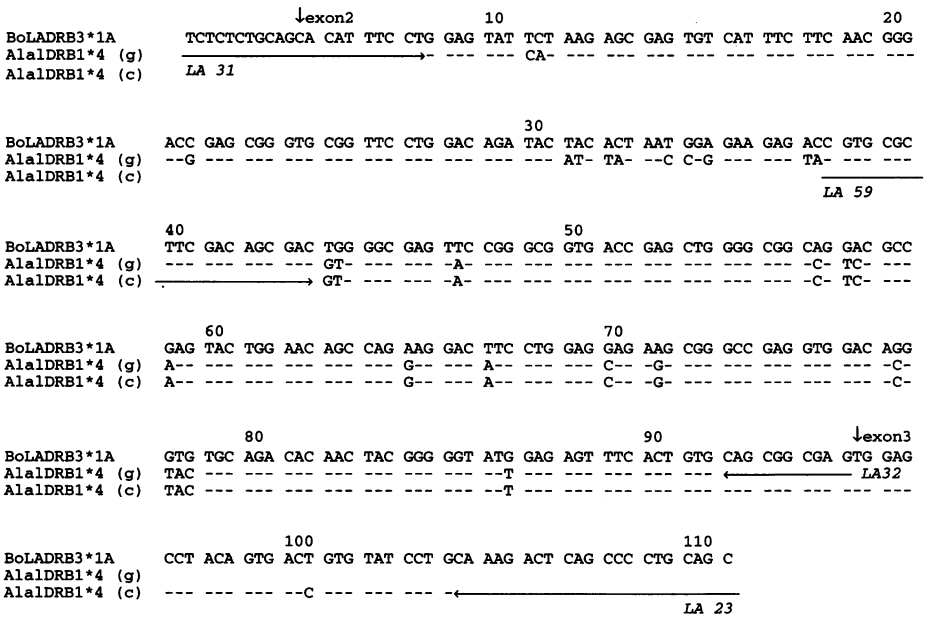

FIG. 2. Comparison of genomic (g) and cDNA (c) sequences obtained for the moose allele *AlalDRB1*4*; the designation of the moose allele follows the proposed nomenclature for MHC genes (27). A genomic cattle sequence, corresponding to clone A1 (28), is used as a reference. The nucleotide sequences corresponding to the primers LA31, LA59, LA32, and LA23 are indicated with arrows. The primer pairs LA31/32 and LA59/23 were used for the PCR amplification of genomic DNA and cDNA, respectively. Intron/exon borders are indicated by vertical arrows.

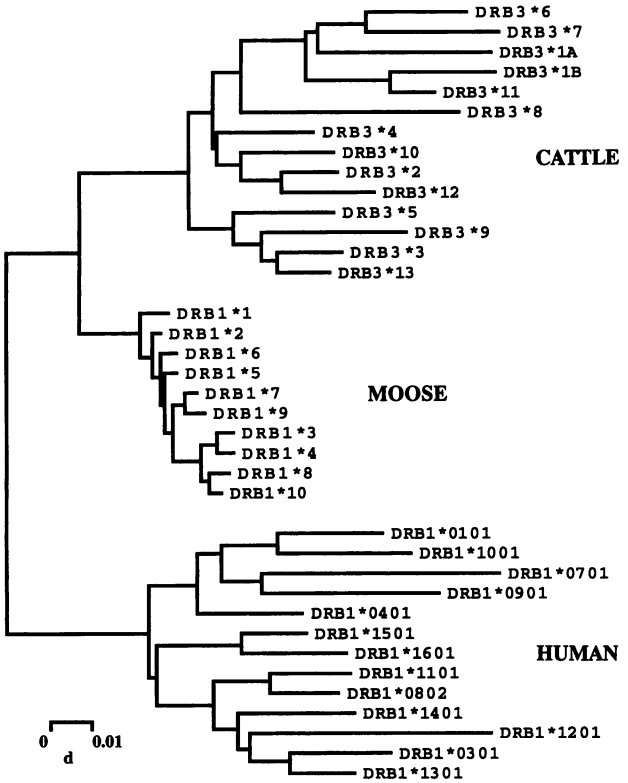

FIG. 3. Neighbor-joining phylogenetic tree of moose, cattle (19), and human (18) *DRB* exon 2 sequences. *d*, Genetic distance.

like the MHC. First, the sample size used was rather small. However, it was adequate for the purpose of this study since we sampled almost 100 chromosomes representing the two major phylogenetic groups of moose. The statistical significance for a markedly lower *DRB* diversity in the moose compared with that in cattle or humans is overwhelming (compare Figs. 1*A* and 3). Second, our cattle-specific primers may not amplify all moose alleles. This could not be a major bias since we got good amplifications from all individuals. Thus, if there are alleles which do not amplify well, they should be rare. Third, there may be other highly polymorphic class II genes. Although this cannot be formally excluded, it appears unlikely. Our cDNA experiment showed that the locus investigated is the predominantly expressed *DRB* locus in moose. In humans and cattle, it is the predominantly expressed *DRB*

Table 2. Mitochondrial D-loop sequences from Swedish and Canadian moose

		Position		
		11122333	3334444444	44445555
		206889124	5570111134	45592223
Sequence	Origin	3050666829	1453123551	88942356
1	S	GATAACTTTA	CTGCTCGCTC	GCTAATTG
2	S	-----T----	-C-T-----T	-T------
3	C	AG-GGT-C-G	--ATC-ATCT	ATC-GCC-
4	C	AG-GGT-C-G	--ATC-ATCT	ATCGGCCA
5	C	AGCGGT-C-G	T-ATCTATCT	ATC-G-C-
6	C	AG-GGT-C-G	--ATC-ATC-	ATC-GCC-
7	C	AG-GGT-C-G	--ATC-ATCT	AT--GCC-
8	C	AG-GGTCC-G	--ATC-A-CT	ATC-GCC-
9	C	AG-GGT-CCG	--ATC-ATCT	ATC-GCC-

The length of the sequenced fragment was 549 nucleotides including an insertion of 75 nucleotides (position 201–275) in sequences 1 and 2 from Swedish moose. Only the 28 polymorphic positions are shown. A dash indicates identity to sequence 1. S, Swedish; C, Canadian.

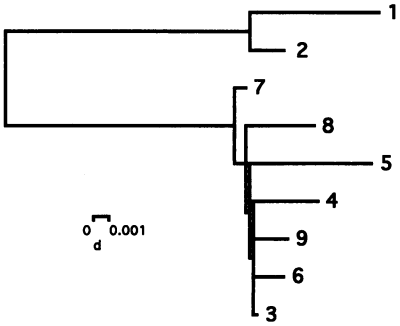

FIG. 4. Neighbor-joining phylogenetic tree of moose mtDNA D-loop sequences. Sequences 1 and 2 were from Swedish moose, and sequences 3–9 were from Canadian moose. *d*, Genetic distance.

locus which is the most polymorphic one (17, 18). Thus, although there may well be other expressed *DRB* genes in the moose, they are unlikely to be highly polymorphic. Finally, the *DRB* sequence data are entirely consistent with our previous restriction fragment length polymorphism studies of Swedish moose showing very restricted class I and class II *DQ* and *DRB* diversity (H. Ellegren, S.M., K. Wallin, and L.A., unpublished data).

The well-established trans-species mode of evolution of MHC polymorphism implies that the accumulation of MHC diversity is a slow process (4). Thus, it will take a long time to restore MHC diversity after a population bottleneck. The moose data are consistent with this hypothesis regarding the tempo in the accumulation of new point mutations. Four of the six amino acid substitutions were shared between the two subspecies, and we can thus infer a maximum of two substitutions which have been established in one of the populations during the last 100,000 years. In contrast, our data imply that the occurrence of new MHC alleles by recombination events can be a much more rapid process.

It is likely that some of the observed alleles have been generated by recombination events subsequent to the divergence from a common ancestor since only one of the ten alleles was shared between continents (Table 1). The interpretation that shuffling of polymorphic motifs by intraexonic recombination has occurred at the moose *DRB1* locus is supported by the striking sharing of polymorphic sequence motifs among the 10 alleles (Fig. 1*A* and *B*). Alleles 3 and 4 provide a strong case for this contention, as they share identical exon 2 sequences, including the synonymous substitution at codon 85, except at codons 71 and 74, where allele 3 is identical to alleles 5, 9, and 10 (Fig. 1*B*). We can exclude the possibility that some of the alleles reported here are due to PCR artifacts, as the data were generated by direct sequencing of amplified products, and the existence of each allele was corroborated by SSCP analysis. This study, together with the observations of recombinant class I alleles in tribes of South American Indians (33, 34), shows that intraexonic recombination plays a significant role in the generation of MHC diversity.

It has often been suggested that the presence of extensive MHC diversity ensures the survival of the population since there will always be some individuals who are able to mount a protective immune response to a new lethal pathogen—e.g., see ref. 35. This view is not based on any empirical evidence and is now challenged by our moose data, which show that low MHC diversity, at least in some species, is compatible with long-term survival of the population. The *DRB* polymorphism found in North American moose was restricted to four amino acid substitutions, and the two most common alleles in Swedish moose, comprising about 65% of the gene pool, differed by a single amino acid substitution (Table 1; Fig. 1). This is expected to cause a restricted repertoire of peptides presented by DR molecules in the moose. Our previous restriction fragment

length polymorphism study of Swedish moose also showed that class I and class II *DQ* genes are only weakly polymorphic in this species (H. Ellegren, S.M., K. Wallin, and L.A., unpublished data). Despite this, viable populations of moose have apparently been maintained for more than 100,000 years, and the species has been able to achieve a circumpolar distribution in the Northern Hemisphere; the moose population in Sweden only has been estimated at about 300,000 animals. Species with low MHC diversity have previously been documented (1, 11, 36, 37), but the moose data are unique in the documented time span involved and that the low degree of polymorphism was verified by DNA sequencing.

It should be stressed that the results of this study in no way contradict that genetic diversity at certain MHC loci is maintained by balancing selection. On the contrary, it has provided evidence for selection since all observed amino acid substitutions occur at residues involved in peptide binding and since there was a significant excess of nonsynonymous substitutions at codons for the peptide binding site. However, the data are in agreement with the interpretation that the selection intensity at MHC loci is quite low (4, 38). The MHC system is only one of many lines of defense against pathogen infections, and the level of MHC polymorphism does not necessarily have a large effect on the long-term survival of a population.

We thank Margareta Stéen and Hans Ellegren for providing samples from Swedish moose and Curtis Strobeck for providing DNA samples from Canadian moose. We also thank Peter Outteridge and Svante Pääbo for discussion. This work was supported by the Swedish Research Council for Forestry and Agriculture.

1. Klein, J. (1986) *Natural History of the Major Histocompatibility Complex* (Wiley, New York).
2. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. & Wiley, D. C. (1987) *Nature (London)* **329,** 506–512.
3. Brown, J. H., Jardetzky, T., Saper, M. A., Samroui, B., Bjorkman, P. J. & Wiley, D. C. (1988) *Nature (London)* **332,** 845–850.
4. Klein, J., Satta, Y. & O'hUigin, C. (1993) *Annu. Rev. Immunol.* **11,** 269–295.
5. Hedrick, P. W. & Thomson, G. (1983) *Genetics* **104,** 449–456.
6. Hughes, A. L. & Nei, M. (1988) *Nature (London)* **335,** 167–170.
7. Hughes, A. L. & Nei, M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 958–962.
8. Takahata, N. & Nei, M. (1990) *Genetics* **124,** 967–978.
9. Bodmer, W. F. (1972) *Nature (London)* **237,** 139–145.
10. Doherty, P. C. & Zinkernagel, R. M. (1975) *Nature (London)* **256,** 50–52.
11. O'Brien, S. J., Roelke, M. E., Marker, L., Newman, A., Winkler, C. A., Meltzer, D., Colly, L., Evermann, J. F., Bush, M. & Wildt, D. E. (1987) *Science* **227,** 1428–1434.
12. Watkins, D., Garber, T. L., Chen, Z. W., Toukatly, G., Hughes, A. L. & Letvin, N. L. (1991) *Immunogenetics* **33,** 79–89.
13. Kurtén, B. (1971) *The Age of Mammals* (Weidenfeld and Nicholson, London).
14. Peterson, R. L. (1955) *North American Moose* (Univ. Toronto Press, Toronto).
15. Stéen, M. & Rehbinder, C. (1986) *Acta Vet. Scand.* **27,** 326–342.
16. Merza, M., Larsson, E., Stéen, M. & Morein, B. (1994) *Virology* **202,** 956–961.
17. Andersson, L., Sigurdardottir, S., Borsch, C. & Gustavsson, K. (1991) *Immunogenetics* **33,** 188–193.
18. Marsh, S. G. E. & Bodmer, J. G. (1990) *Immunogenetics* **31,** 141–144.
19. Sigurdardottir, S., Borsch, C., Gustavsson, K. & Andersson, L. (1991) *Anim. Genet.* **22,** 199–209.
20. Ward, P., Frazier, B. L., Dew-Jager, K. & Pääbo, S. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8720–8724.
21. Saitou, N. & Nei, M. (1987) *Mol. Biol. Evol.* **4,** 406–425.
22. Kumar, S., Tamura, K. & Nei, M. (1993) MEGA, Molecular Evolutionary Genetics Analysis (Penn. State Univ., University Park), Version 1.01.
23. Kimura, M. (1980) *J. Mol. Evol.* **16,** 111–120.
24. Nei, M. & Gojobori, T. (1986) *Mol. Biol. Evol.* **3,** 418–426.
25. Jukes, T. H. & Cantor, C. R. (1969) in *Mammalian Protein Metabolism*, ed. Munro, H. N. (Academic, New York), pp. 21–132.
26. Nei, M. (1987) *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York), Equation 10.21., p. 276.
27. Klein, J., Bontrop, R. E., Dawkins, R. L., Erlich, H. A., Gyllensten, U. B., Heise, E. R., Jones, P. P., Parham, P., Wakeland, E. K. & Watkins, D. I. (1990) *Immunogenetics* **31,** 217–219.
28. Groenen, M. A. M., van der Poel, J. J., Dijkhof, R. J. M. & Giphart, M. J. (1990) *Immunogenetics* **31,** 37–44.
29. Stoneking, M., Sherry, S. T., Redd, A. J. & Vigilant, L. (1992) *Philos. Trans. R. Soc. London B* **337,** 167–175.
30. Loftus, R. T., MacHugh, D. E., Bradley, D. G., Sharp, P. M. & Cunningham, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2757–2761.
31. Sibrava, V., Bowen, D. Q. & Richmond, G. M., eds. (1986) *Quaternary Glaciations in the Northern Hemisphere. Report of the International Geological Correlation Programme, Project 24* (Quaternary Science Reviews, Pergamon, Oxford), Vol. 5.
32. Nei, M. & Grauer, D. (1984) *Evol. Biol.* **17,** 73–118.
33. Bellich, M. P., Madrigal, J. A., Hildebrand, W. H., Zemmour, J., Williams, R. C., Luz, R., Petz-Erler, M. L. & Parham, P. (1992) *Nature (London)* **357,** 326–329.
34. Watkins, D. I., McAdam, S. N., Liu, X., Strang, C. R., Milford, E. L., Levine, C. G., Garber, T. L., Dogon, A. L., Lord, C. I., Ghim, S. H., Troup, G. M., Hughes, A. L. & Letvin, N. L. (1992) *Nature (London)* **357,** 329–333.
35. Lawlor, D. A., Zemmour, J., Ennis, P. D. & Parham, P. (1990) *Annu. Rev. Immunol.* **8,** 23–63.
36. Slade, R. W. (1992) *Proc. R. Soc. London B* **249,** 163–171.
37. Ellegren, H., Hartman, G., Johansson, M. & Andersson, L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8150–8153.
38. Nei, M. & Hughes, A. L. (1991) in *Evolution at the Molecular Level*, eds. Selander, R., Clark, A. & Whittam, T. (Sinauer, Sunderland, MD), pp. 222–247.