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Genetic diversity of the major histocompatibility complex class II in Alaskan caribou herds

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Summary

We have sampled five different herds of caribou in Alaska to ascertain their major histocompatibility complex (MHC) class II diversity, and to assess whether the herds were significantly different in their MHC class II allele profiles. We complemented the MHC results with data from nine neutral microsatellite markers. The results indicate that while the microsatellites are diverse, there are no significant differences between the herds. However, for the MHC, we have shown that there is diversity at three of the four loci studied, the different herds have significantly different MHC class II allele profiles. It is also clear that although some of the herds have overlapping ranges, they are still different for their MHC class II alleles.

Introduction

In all mammalian species the major histocompatibility complex (MHC) plays a central role in the immune response. This includes disease susceptibility/resistance (Todd, 1990; Lie & Thorsby, 2005), response to vaccination (Glass, 2004) and infections. MHC molecules present foreign peptides to the immune system, and thus greater variability for MHC genes allows for a response to a larger range of pathogens. The MHC has also been implicated in mate selection (Piertney & Oliver, 2006; Yamazaki & Beauchamp, 2007), although much of the data are based on studies in mice. However, there are data to suggest that antler development may be associated with pathogen resistance in deer and thus could be a signal of genetic quality (Ditchkoff *et al.*, 2001). Clearly the fact that one male caribou services many females within a herd could have a major influence on the MHC allele profiles within caribou herds.

The MHC has been very well characterized in many mammals, including man, many primate species, mouse and rat. Significant progress has also been made in characterizing the MHC of several domesticated animals including cattle (Russell *et al.*, 1997), pig (Smith *et al.*, 2005), sheep (Arrieta-Aguirre *et al.*, 2006; Dukkipati *et al.*, 2006) goat (Mainguy *et al.*, 2007), horse (Fraser & Bailey, 1996; Gustafson *et al.*, 2003), dog (Wagner, 2003) and cat (Kennedy *et al.*, 2003). As more genomes are sequenced, it becomes possible to approach characterizing the MHC of other mammals. There have been some reports on MHC class II genes in various species of deer, including red deer (elk) (Swarbrick *et al.*, 1995; Swarbrick & Crawford, 1997), white tailed deer (Van Den Bussche *et al.*, 1999), fallow deer and roe deer (Mikko *et al.*, 1999). These investigators have used PCR primers designed for use in cattle, sheep and goats, with varying degrees of success.

To better understand the evolution of MHC loci and to identify selective forces driving the loss of alleles or the fixation of new alleles, it is useful to collect comparative information from diverse species, both those long in domestication as well as wild ones in a variety of natural settings. It is thought that reduced variability, or increased homozygosity, of the MHC causes reduced fitness (O'Brien *et al.*, 1985; Yuhki & O'Brien, 1990). Thus, reduced diversity at MHC loci may impact the likelihood of the survival of threatened and endangered species. In nature, animals are often discontinuously distributed and species are composed of several local populations which are more-or-less isolated from one another. It is possible that the most interesting evolutionary experiments may be the consequence of pathogen-driven selection in such local populations. In this study, we present data on allelic diversity in an interesting wild species, the caribou, which is found across high latitudes of the circumpolar north.

Rangifer tarandus is a deer species with a circumpolar distribution. This species, known as reindeer in

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Eurasia and caribou in North America, and has a worldwide population exceeding five million individuals. Caribou have been present in North America since the Illinoian period prior to the last major glaciation some 500 000 years ago (Kurtén & Anderson, 1980). They survived the Wisconsin glaciation of North America both by retreating south of the glaciers and by inhabiting ice-free refugia in modern Alaska and Canada (Flagstad & Roed, 2003). The descendant population is scattered in 32 different herds across Alaska, ranging in size from <100 to >500 000 individuals. Caribou are very mobile animals and most of the large herds are migratory, moving hundreds of kilometres between winter and summer feeding grounds.

When members of the deer family were transplanted from high to lower latitudes and were found to be highly susceptible to infection, wildlife biologist Valerius Geist (1985) hypothesized that the northern populations showed 'loss of immunity'. Geist's hypothesis is supported by a more recent suggestion that animals living at high latitudes may be exposed to fewer pathogens than those at middle latitudes and that therefore there is less selective pressure for diversity in the MHC (Van Den Bussche *et al.*, 1999, 2002). Moreover, large animals at high latitudes are vulnerable to catastrophic events which decimate their populations, as illustrated by the storms in late 1995 that killed 80% of the caribou and muskoxen (*Ovibos moschatus*) on Bathurst Island in the Canadian Arctic (Struzik, 1996). Similar events may have reduced the caribou and muskox populations elsewhere in Alaska and Canada (Miller, 1982; Groves, 1997), leading to genetic bottlenecks and reduced MHC variability.

Population fragmentation of caribou hosts might also be paralleled by differentiation among their parasites and pathogens. The helminth fauna in North American caribou and muskoxen are a complex mosaic of species (Hoberg *et al.*, 1999) and that complexity may reflect a history of repeated drastic fluctuations in the herds of their hosts. Two lines of evidence could reveal whether these herds are epidemiologically isolated: direct observations of the present-day animal exchanges, and genetic evaluations of individuals from each herd.

Since caribou are a significant hunting resource, their movements are closely monitored via radio and satellite telemetry by wildlife biologists in Alaska. All the herds we sampled are allopatric to one another. However, limited gene flow might occur stepwise between Porcupine and Western Arctic through the smaller Central Arctic herd (Fig. 1). Brad Griffith at the University of Alaska Fairbanks placed radio collars on females of Porcupine and Central Arctic herds and tracked their movements. In the late 1980s (but not since), the fringes of these herds came in contact after calving season in late summer. A few radio collared animals wandered south to wintering grounds with a different herd. After the northward spring migration,

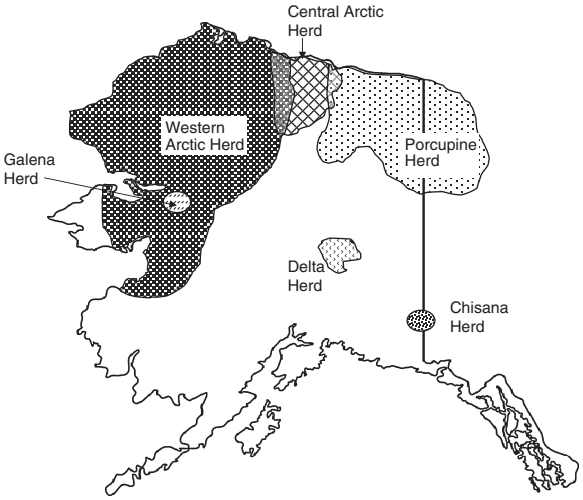


Figure 1. Location of the different caribou herds in Alaska.

the wanderers returned to their original herd, but they might have been bred by a male from the different herd. Of 167 Central Arctic females followed, only one switched herds and gave birth to a live calf. Dr Griffith believes that the exchange between Western Arctic and Porcupine herds would be several orders of magnitude smaller than between Central Arctic and Porcupine herds which have overlapping ranges (B. Griffith, personal communication). Data from females collared by the Alaska Department of Fish and Game show overlapping of the Central Arctic with Porcupine herds in recent years. Although the potential exists for gene flow between the Porcupine and Western Arctic herds through intermediates, we suspect that significant genetic exchange is unlikely at the present.

Some MHC class II studies have been performed on animals from high latitudes, including caribou (Cronin *et al.*, 1995), moose (Mikko & Andersson, 1995; Ellegren *et al.*, 1996) and muskoxen (Mikko *et al.*, 1999), but these have all investigated single MHC class II genes, and again involved using primers designed for other related species. Cronin *et al.* (2003) investigated genetic variation in reindeer and caribou using seven microsatellites. They concluded that there were no significant differences in allele frequencies between three Alaskan caribou herds (Western Arctic, Porcupine and Delta) for all seven loci combined, although there was a significant difference in allele frequencies for one locus, (IGF1). Earlier, Cronin *et al.* (1995) investigated DQA in Alaskan caribou, but the small sample size did not allow firm conclusions to be drawn about inter herd variation.

We have collected samples from cohorts of caribou in the same three herds in Alaska (Western Arctic, Porcupine and Delta), along with individuals from two other herds (Chisana and Galena), and have investigated four different MHC class II genes in these caribou: DRA, DRB, DQA and DQB. We wished to

investigate the level of MHC variation in caribou in general, and whether the herds had different MHC allele profiles. We complement this data on MHC class II genes with data from nine microsatellite loci from the three large herds. Our study does not include any investigation of MHC class I genes, alleles or haplotypes.

Materials and methods

Animals

Blood samples were taken from five Alaska caribou (*Rangifer tarandus grantii*) herds by Alaska Department of Fish and Game biologists during 2000 and 2003. These were the Porcupine, Western Arctic, Delta, Galena Mountain and Chisana herds.

Both the Porcupine herd of 130 000 animals (located in the northeast region of the state and the northwest region of Canada) and the Western Arctic herd of 460 000 animals (located in the northwest region of the state) migrate hundreds of kilometres each year between summer calving grounds north of the Brooks Range to wintering grounds south of the mountains (Fig. 1). The Delta herd of 4500 animals is limited to a fixed range in interior Alaska (and theoretically isolated from the Western Arctic and Porcupine herds). The Galena Mountain herd is much smaller and while it has previously been estimated at 300–500 animals, it has been in decline for several years, and the herd was down to possibly <100 animals by late 2004, which prompted banning of all hunting on the herd (Taylor, 2004). Since 1989, the isolated Chisana herd has declined rapidly from a steady population of 1800 animals to <360 in 2001 (Oakley & Mead-Robins, 2004). Both the Galena and Chisana are small isolated herds located in the central mountain regions of Alaska, although the range of the Galena Mountain herd may overlap with the Western Arctic herd.

Our samples included 32 female and two male caribou from the Porcupine herd, 23 female and 27 male from the Western Arctic herd and 43 females from the Delta herd. We had four caribou from the Chisana Mountain herd and five from the Galena Mountain herd, all of unknown gender.

Genomic DNA was extracted from the blood samples using Qiagen DNEasy blood extraction kit (Qiagen, Valencia, CA, USA) or MOBIO UltraClean™ BloodSpin™ DNA Isolation kits (MO-BIO Laboratories, Carlsbad, CA, USA). DNA concentrations were analysed using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microsatellite genotyping

Microsatellite genotypes were determined using ten loci previously described for caribou (RT1, RT6, RT7, RT9, RT10, RT13, RT25, (Wilson *et al.*, 1997) and NVHRT22, NVHRT63, NVHRT76, (Røed & Midthjell, 1998). PCR amplifications were performed in a final reaction volume of 10 μL containing 6.9–7.1 μL water, 1.0 μL Eppendorf 10× buffer, 0.2 μL 10 mM DNP mix, 0.2–0.4 μL 25 mM $MgCl_2$, 0.4 μL BSA (10 mg mL^{-1}), 0.5 μL of each 10 μM primer and 0.1 μL of Eppendorf Taq (Eppendorf, Westbury, NY, USA). The loci were amplified using a MJ Research PTC-225 thermal cycler. The cycling conditions were 1 min at 94°C, followed by three cycles of 30 s at 94°C, 20 s at 54°C and 5 s at 72°C, followed by 33 cycles of 15 s at 94°C, 20 s at 54°C and 1 s at 72°C, ending with 30 min at 72°C. All PCR products were visualized with UV light in agarose gels stained with ethidium bromide. Successful amplifications were run on an ABI 3100 Genetic Analyzer with GS500 ROX (ABI) size standard. Genotypes were determined using GeneMapper V3.7 software (Applied Biosystems, Foster City, CA, USA).

MHC genotyping

Polymerase chain reaction was used to amplify four MHC class II loci: DRA, DRB, DQA and DQB. ABI AmpliTaq, Qiagen HotStar Taq and Qiagen HotStarPlus Taq were all used during this research with the best results and cleanest sequencing from the latter (Qiagen, Valencia, CA, USA).

PCR products were purified using either Qiagen Gel Purification or Qiagen PCR purification kits. After purification, samples were sequenced with ABI BigDye v3.1 sequencing kit, according to the manufacturers instructions, and cleaned up using Sephadex columns or micro-filtration plates. Samples were sequenced using ABI 3100 or 3130 automated sequencing machines.

Primers

Several different primer sets were tried for each locus except DRA. We list in the methods all those that were tried and indicate which were successful. Table 1 lists all the primers that were assessed during this research, including those which did not work with caribou.

DRA

Exonic primers DRA-F and DRA-R which were designed for goat DRA (Takada *et al.*, 1998) generate an incomplete exon 2 sequence that is missing some bases at both the 5′ and 3′ ends. The PCR protocol for DRA using the Qiagen PCR kit was: 27.75 μL of nuclease free water, 5 μL of 10× PCR buffer, 2 μL 25 mM $MgCl_2$, 1 μL 10 mM each DNTP, 2 μL of each 10 pmol μL^{-1} primer, 0.25 μL HotStarPlus Taq and 10–50 ng μL^{-1} DNA in a 50-μL reaction. Thermocycling conditions were 95°C for 5 min, 35 cycles of

Table 1. Primers used in this study

Locus	Primer name	F/R	Primer sequence	Length (bp)	Tm	Original species reference
DRA	DRA-F	F	AATCATGTGATCATCCAAGCTG	22	58.9	Goat (Takada *et al.*, 1998)
DRA	DRA-R	R	CATTGGTGTTTGGGGTGTTGTTG	23	62.9	Goat (Takada *et al.*, 1998)
DRB	DRB 1.1	F	TATCCCGTCTCTGCAGCACATTTC	24	64.6	Goat (Amills *et al.*, 1995)
DRB	DRB 1.2	R	TCGCCGCTGCACACTGAAACTCTC	24	68.0	Goat (Amills *et al.*, 1995)
DRB	LA 31	F	GATGGATCCTCTCTCTGCAGCACATTTCCT	30	68.7	Moose (Ellegren *et al.*, 1996)
DRB	LA 32	R	CTTGAATTCGCGTCACCTCGCCGCTG	26	70.9	Moose (Ellegren *et al.*, 1996)
DQA	CBDQA-F	F	GACCACATTGGCACCTATG	19	60.2	Sheep (Snibson *et al.*, 1998)
DQA	CBDQA-R	R	GTTGGTAGCAGCAGTAGAGTT	21	60.6	Sheep (Snibson *et al.*, 1998)
DQA	JM08	F	ACCCTGACTCAGCTGACC	18	62.2	Sheep (Snibson *et al.*, 1998)
DQA	JM09	R	CATACTGTTGGTAGCAGC	18	57.6	Sheep (Snibson *et al.*, 1998)
DQA	JM10	F	CACTTATCAGCTGACCAC	18	57.6	Sheep (Snibson *et al.*, 1998)
DQA	JM11	R	GGTGGACACTTACCATTG	18	57.6	Sheep (Snibson *et al.*, 1998)
DQA	CBDQA2-R5	R	TAGAGAAAAAGGGAGTGA	18	53.1	AM/LJK*
DQB	JM05	F	TCCCCGCAGAGGATTTCGTG	20	64.5	Sheep (van Oorschot *et al.*, 1994)
DQB	JM06	R	TCCGCCGCTGCCAGGTGAAG	20	68.6	Sheep (van Oorschot *et al.*, 1994)
DQB	JM07	R	TCCGCTGCAAGGAGGTGATG	20	64.5	Sheep (van Oorschot *et al.*, 1994)
DQB	JM05m	F	TCCCCGCAGAGGATTTC	17	59.6	AM/LJK*
DQB	CB-DQBR1	R	ACACCGTGTCCACCTCGGC	19	66.6	AM/LJK*
DQB	CBDQB3-1R	R	TGTCCTGGATGGGGAGA	17	59.6	AM/LJK*
DQB	CBDQB3-2R	R	GCAGGTTGTGGTGGTTTAGAGCCT	24	66.3	AM/LJK*
DQB	CBDQB3-3R	R	AGGTTGTGGTGGTTTAGAGCCT	22	62.7	AM/LJK*
DQB	CBDQB3-4R	R	CAGCAGGTTGTGGTGGTTTAGA	22	62.7	AM/LJK*
DQB	CBDQB3-5R	R	GGCCTGGATAGAAATCTGTCA	21	60.6	AM/LJK*

*Primer designed by Angela Modrell and Lorna Kennedy.

94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min and 4°C hold. See Table 1 for primer sequence information.

DRB

Primers LA31 and LA32 which were designed for cattle (Sigurdardottir *et al.*, 1991) generate a sequence which is 8 bp short at the 5′ and 9 bp short at the 3′ end of exon 2. The PCR protocol for DRB using Qiagen PCR kit was: 27.5 μL of nuclease free H_2O, 5 μL of 10× PCR buffer, 2 μL of 25 mM $MgCl_2$, 1.25 μL of 10 mM each DNTP, 2 μL of each 10 pmol μL^{-1} primer, 0.25 μL HotStarTaq Plus, and 10–50 ng μL^{-1} DNA in a 50-μL reaction. Thermocycling conditions for LA31/32 primer pair 95°C for 5 min, 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min and 4°C hold. We also tested DRB1.1 and DRB1.2 which were designed for goat DRB (Amills *et al.*, 1995) but we generated poor sequences and the results were inconsistent. See Table 1 for primer sequence information.

DQA

Forward primers JM08 and JM10 which were designed for sheep (Snibson *et al.*, 1998) were used in combination with CBDQA2-R5m reverse primer to obtain 'A' and 'C' sequences from most samples. A second combination of JM08 and JM09 were used to obtain 'B' sequences. (See results for definition of A, B and C sequences). CBDQA2-R5m is an intronic primer designed after sequencing from exon 2 to exon 3 using primers designed by Cronin *et al.* (1995). The JM primers were designed to amplify sheep DQA, with JM08/09 amplifying DQA1 and JM10/11 amplifying DQA2 (Snibson *et al.*, 1998).

It was found that the most successful and consistent PCR results were obtained using Qiagen HotStarTaq. The PCR protocol for all primer combinations was: 26.5 μL nuclease free water, 5 μL 10× buffer, 2 μL 25 mM $MgCl_2$, 1.25 μL 10 mM each DNTP, 2.5–4 μL of each 10 pmol μL^{-1} primer, 0.25 μL HotStarTaq, and 10–50 ng μL^{-1} of DNA for a 50-μL reaction. Thermocycling conditions were 95°C for 5 min, 35 cycles of 94°C for 30 s, (49°C for JM10-CBDQA2-R5m, 53°C for JM08-CBDQA2-R5m, 55°C for JM08-JM09) for 30 s, and 72°C for 1 min, followed by 72°C for 10 min, and 4°C hold.

Other primers which were tested included CBDQA-F with CBDQA-R (Snibson *et al.*, 1998) which sequenced within the exon, and JM09m with JM11 which were selective in amplification (only amplified some alleles). We designed several other primers in exon 3 and used these with the forward primer from Cronin *et al.* (1995) to amplify intron 2. The resulting sequencing data confirmed that the intron/exon sequences for A alleles are very similar to each other and quite different to the intron/exon sequences obtained for C alleles, which are also very similar to each other. In some cases it was only possible to assign an allele to a group, i.e. A, B or C, rather than actual alleles. See Table 1 for primer sequence information.

DQB

Primers JM05m and CBDQB-R1 gave the most consistent results of the DQB primer combinations, after attempts to design intronic primers for this locus were unsuccessful. The exon 2 sequence generated by these primers lacks base pairs at both the 5′ and 3′ ends. The PCR protocol for DQB was: 27.5 μL nuclease free H_2O, 5 μL 10× PCR buffer, 2 μL 25 mM $MgCl_2$, 1.25 μL 10 mM each DNTPs, 2 μL of each 10 pmol μL^{-1} primer, 0.25 μL HotStarPlus Taq, and 10–50 ng μL^{-1} of DNA for a 50-μL reaction. Thermocycling conditions were 95°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 10 min and 4°C hold.

Other primer combinations that were tried included JM05 with JM06, JM05 with JM07 (van Oorschot *et al.*, 1994) and JM05m with CBDQB-R2. However, JM05m2 as a forward primer was not successful. An exon 2 to exon 3 forward primer CBDQB2-F was designed from a nonpolymorphic position in exon 2, the reverse primers CBDQB3-R1, R2, R3, R4, and R5 were designed from exon 3 sequences of equine and ovine sequences found on NCBI with the intent of sequencing for an intronic primer. These reverse primers proved to be either selective or unsuccessful. See Table 1 for primer sequence information.

RSCA

Reference strand-mediated conformational analysis (RSCA) based on methods developed for the canine MHC (Kennedy *et al.*, 2005) was used to confirm sequencing results for DRA. RSCA results for the other three loci were not definitive. The forward primers had a fluorescent label attached at the 5′ end. We used FAM as the fluorescent label in all cases.

Nomenclature

All the exon 2 sequences generated were not complete, so convention (Ellis *et al.*, 2006) says that we should not assign official allele numbers to any alleles we identify. However, for Rata-DRB, the sequences were 252 bases and are only missing ten bases at each end, and from other species, there are no polymorphic positions in those regions. Since some alleles have already been named with four digit numbers (Mikko *et al.*, 1999), we decided to name our DRB alleles in a similar way. Alleles with more than four amino acid differences received a new number in the series. Alleles with four or less amino acid differences were named as subtypes, e.g. 0901, 0902 and 0903. Alleles that were only identified in three or less animals received local names, e.g. ak09 and ak12.

Rata-DRA, DQA and DQB sequences were shorter than the full exon 2 length, and therefore local names were assigned to all alleles. An additional lower case 'a' or 'b' indicates alleles with synonymous changes only.

DRA sequences were 198 bases with 30 bases missing at each end. DQA sequences were 242 bases with only four bases missing at the start and none at the end. No polymorphic positions are located in these missing sections of DRA and DQA in other species.

DQB sequences were 195 bases with six missing at the start and 66 bases at the end. However, several polymorphic positions are likely to occur in the missing region of the exon.

Thus allele assignments are fairly certain for DRA, DRB and DQA, but it is very possible that DQB alleles could be further divided by missing polymorphisms.

Statistical methods

Allele frequencies were calculated by direct gene counting. Hardy–Weinberg equilibrium (HWE) was tested using the Monte Carlo version of the exact test of Guo & Thompson (1992) as implemented in PyPop (Lancaster *et al.*, 2003, 2007). An exact test of population differentiation (Raymond & Rousset, 1995) was used for three pairs of populations for both microsatellite and MHC loci. A Bonferroni correction for the number of microsatellite or MHC loci was applied.

Results

Microsatellites

One microsatellite locus, NVHRT63, (Røed & Midthjell, 1998) was monomorphic in the first nine samples run from each herd so was eliminated from subsequent analyses. Genotypes for nine microsatellite loci were generated for 23, 24 and 33 individual caribou from the Delta, Porcupine and Western Arctic herds respectively. All loci were quite variable with between 7 and 15 alleles. The mean number of alleles per locus did not differ significantly between populations. Among all the loci, there was one private allele for the Delta, seven for the Porcupine and 14 for the Western Arctic herd. These numbers might change if the sample size increased.

Table 2 shows the Hardy–Weinberg values for the microsatellite results for three of the caribou herds. Only three loci were not in HWE (RT01 in Delta herd, and RT10 and N76 in the Porcupine herd). Comparison of microsatellite data between herds (Table 3) suggests that there is very little difference between the herds.

MHC

We generated MHC data for all four loci on 86 of 136 caribou, and thus 50 caribou had one or more loci missing. However, no caribou failed to amplify at

Table 2. Tests of Hardy–Weinberg equilibrium for microsatellites

Population	Locus	2*n*	No. alleles	HWE, *P*-value
Delta	RT01	46	12	**0.003***
Delta	RT06	46	9	0.671
Delta	RT07	46	7	0.753
Delta	RT09	46	11	0.821
Delta	RT10	46	11	0.610
Delta	RT13	46	11	0.272
Delta	RT25	46	8	0.794
Delta	N22	46	10	0.481
Delta	N76	46	8	0.695
Porcupine	RT01	48	13	0.518
Porcupine	RT06	48	9	0.924
Porcupine	RT07	48	9	0.232
Porcupine	RT09	48	14	0.636
Porcupine	RT10	48	10	**0.004***
Porcupine	RT13	48	12	0.164
Porcupine	RT25	48	7	0.206
Porcupine	N22	48	12	0.572
Porcupine	N76	48	7	**0.032**
WAH	RT01	66	13	0.082
WAH	RT06	66	12	0.925
WAH	RT07	66	8	0.809
WAH	RT09	66	15	0.288
WAH	RT10	66	15	0.741
WAH	RT13	66	11	0.280
WAH	RT25	66	9	0.743
WAH	N22	66	11	0.065
WAH	N76	66	13	0.844

HWE, Hardy–Weinberg equilibrium; WAH, Western Arctic herd.
*Significant after correction for nine comparisons. *P*-values listed are uncorrected.
Bold values are indicated as low *P*-values.

all four loci; 31 were missing one locus only, and nine were missing two loci. In total, seven lacked DRA, 22 lacked DRB, three lacked DQA and 27 lacked DQB. All failed samples were re-amplified several times without success.

DRA

The DRA primers worked well, and results were obtained for 129 of 136 caribou of which 99 (76.7%) were homozygous. Three alleles were identified, called DRA1a, DRA2a and DRA2b. DRA2a and DRA2b differed by a single synonymous base change. The commonest allele was DRA2a, with a frequency varying from 75% to 100%. DRA2b was found in four Porcupine and one Western Arctic caribou. Table 4 shows the allele frequencies for the different herds.

DRB

Using primers LA31 and LA 32, we generated data for 114 caribou for DRB, of which 14 (12.3%) were homozygous. Nine alleles identified in caribou have been previously published by Mikko *et al.* (1999), and we found all but one of these within our group of caribou. Twelve other unpublished alleles have been deposited in Genbank from some earlier work from Z. Wei (using DNA cloning and sequencing, but a different set of primers), but these have two deletions compared to the Mikko sequences. We identified six of these alleles without the deletions in this cohort of caribou, and therefore presume that the deletions were some kind of sequencing artefact. We did not find the other six alleles. We also found 11 new alleles. Some of these were found in many animals, but others were limited to one or two. Table 5 shows the allele frequencies for the different herds.

DQA

Previously Cronin *et al.* (1995) identified three DQA alleles by sequencing, which they called A, B and C. They also identified two other alleles, D and E, using restriction fragment length polymorphism. The D allele was similar to the A allele, and the E allele was similar to the C allele.

Our data identified 14 DQA alleles, and these could be grouped into alleles similar to each of the Cronin alleles. So we named our alleles A1, A2, C1, C2 etc. We found five different A alleles, two different B alleles and seven different C alleles. Sometimes we could only say which group the allele was from, rather than a subtype, so we called these alleles A or C. The two B alleles only differed by a single synonymous base change.

We tried several different primer combinations, and found that JM08/JM09 only amplified B alleles, whereas JM08/JM09m amplified B and C alleles. JM10/JM11 and JM08/JM11 only amplified A alleles. CBDQA-F and CBDQA-R only amplified C alleles.

None of the reverse primers we designed in intron 2 amplified the B alleles, whereas they all amplified A and C alleles, whichever forward primer was used.

Table 3. Exact test of population differentiation based on microsatellite markers

Population pair	RT01	RT06	RT07	RT09	RT10	RT13	RT25	N22	N76
Delta–Porcupine	0.444	0.123	0.751	1.000	1.000	1.000	0.388	0.685	1.000
Delta–WAH	0.651	0.757	1.000	0.507	1.000	0.338	1.000	0.209	1.000
Porcupine–WAH	1.000	1.000	1.000	1.000	1.000	0.409	1.000	1.000	1.000

WAH, Western Arctic herd.
P-values listed are corrected for nine comparisons.

Table 4. DRA allele frequencies in the different herds

DRA	Porc, *n* = 30	Delta, *n* = 41	WAH, *n* = 50	Chisana, *n* = 4	Galena, *n* = 4
1a	16.7	6.1	10.0	0.0	25.0
2a	76.7	93.9	89.0	100.0	75.0
2b	6.7		1.0		

Porc, Porcupine; WAH, Western Arctic herd.

Table 5. DRB allele frequencies in the different herds

DRB	Porc, *n* = 27	Delta, *n* = 38	WAH, *n* = 44	Chisana, *n* = 4	Galena, *n* = 1
0101	7.4	8.0	5.7	25.0	
0102	14.8	10.7	27.3	12.5	50.0
0103					
0104	5.6		3.4		
0201	3.7	1.3	5.7		
0301	3.7	4.0	12.5		
0401	3.7	10.7	3.4		
04v*			2.3		
0501	1.9				
0601	1.9	2.7	1.1		
06v*	1.9	2.7	1.1	12.5	
0701	1.9				
0801	1.9	2.7	8.0		
0901	18.5	21.3	8.0	12.5	
0902	1.9		3.4	12.5	50.0
0903	16.7	25.3	13.6	12.5	
1001	3.7	5.3	1.1		
ak01	1.9		2.3		
ak09	1.9		1.1		
ak12		6.7			
ak20	3.7				
Other	3.8			12.5	

Porc, Porcupine; WAH, Western Arctic herd.
*04v and 06v are local names indicating alleles that are 1–2 bp different to 0401 and 0601, respectively.

All the caribou were tested with several primer combinations, and we were able to assign alleles to 133 of 136 animals. However, four animals had three DQA alleles, which indicated the presence of at least two DQA genes on some haplotypes.

If we assigned the A alleles to one locus and the B and C alleles to a second locus, then there were no triplets for either locus, whereas if we put the B alleles with the A alleles, then we did find triplets. Thus we suggest that the caribou has two DQA loci, one of which carries all the A alleles, and another which has all the B and C alleles.

However, some animals had only A alleles or only B/C alleles, suggesting that some haplotypes have only the DQA 'A' locus, some haplotypes have only the DQA 'B/C' locus and some haplotypes have both. Variable numbers of genes on different haplotypes have now been identified in many species.

If DQA was considered to be one locus then 35 of 133 (26.3%) were homozygous, but if DQA was considered to be two loci, then many more were homozygous: 74 (55.6%) for one locus and 43 (32.3%) for the other. The relatively high homozygosity compared to DRB, plus the fact that many animals were missing one or other DQA locus suggests that the primers may not be identifying all alleles at the DQA loci. Table 6 shows the allele frequencies for the different herds.

Table 6. DQA allele frequencies in the different herds

DQA	Porc, *n* = 34	Delta, *n* = 42	WAH, *n* = 48	Chisana, *n* = 4	Galena, *n* = 5
A1	16.2	11.9	22.9	12.5	
A2	7.4	3.6	1.0		10.0
A3	2.9	7.1	5.2	12.5	
A4	4.4	7.1	4.2		
A5	1.5	2.4			10.0
B1a	10.3	6.0	9.4		
B1b	2.9	4.8	2.1		
C1	19.1	19.0	26.0	25.0	10.0
C2	2.9	9.5	2.1		20.0
C3		3.6	3.1		
C4	2.9		1.0		
C5	1.5	4.8		12.5	
C6	1.5	6.0			
C7	5.9	3.6	1.0		10.0
Other	5.9	8.3	7.3	12.5	40.0

Porc, Porcupine; WAH, Western Arctic herd.

DQB

We tried using DQB primers designed for sheep (van Oorschot *et al.*, 1994) without success. We modified the forward primer (now called JM05m) and used it in combination with CBDQB-R1. This primer pair gave results for 109 of 136 animals but 74 (54.4%) were homozygous. This high percentage of homozygotes, together with the fact that 27 animals did not amplify with these primers suggests that not all alleles are amplified with these primers. Table 7 shows the allele frequencies for the different herds.

Table 7. DQB allele frequencies in the different herds

DQB	Porc, *n* = 31	Delta, *n* = 36	WAH, *n* = 35	Chisana, *n* = 2	Galena, *n* = 4
b01	21.0	26.4	32.9		50.0
b02	12.9	30.6	24.3	100.0	25.0
b03	19.4	18.1	8.6		
b04	4.8	1.4	10.0		
b05	4.8	4.2	5.7		
b06	1.6		1.4		
b07	9.7	6.9	2.9		
b08			5.7		
b09		4.2			
b10			1.4		
b11	4.8	8.3	4.3		25.0
b12	4.8		2.9		
b13	3.2				
Other	12.9				

Porc, Porcupine; WAH, Western Arctic herd.

Table 8. Possible haplotypes identified in the caribou

DRA	DRB	DQA1	DQA2	DQB	No caribou
–	0301	–	A1	–	6
–	0801	C1	–	–	7
–	0901	C7	–	b07	6
–	0903	C1	–	b02	37
1a	–	C6	–	b11	4
–	–	B1a	–	b03	11

Haplotypes are only shown where the alleles were always found together in these combinations.

Haplotype analysis

We would predict that the caribou MHC class II genes are close together in the genome, as in most other mammalian species. Thus, it should be possible to identify haplotypes. Only two animals out of 86 that were typed for all four loci were homozygous at all four loci, and four others were homozygous at DRA, DRB and DQA, but with DQB not typed. This allowed us to identify some haplotypes. Other haplotypes were identified by looking to see which alleles always occurred together. Thus for some alleles, it was possible to identify alleles at other loci that were always in combination with that allele, see Table 8. However, no haplotypes were identified that had an allele at all four loci. It was also not possible to assign two haplotypes to every animal, which again suggests that the DQ primer pairs were not identifying all alleles.

Hardy–Weinberg equilibrium

Table 2 shows HWE results for the nine microsatellite markers. After correction for the number of tests in each herd there were only two significant deviations from HWE. The RT01 marker in the Delta herd and the RT10 marker in the Porcupine herd had significant deviations from HWE. Table 9 shows the HWE data for each MHC locus and each herd. Unsurprisingly, DQA and DQB are not in HWE for any of the herds. This adds weight to the argument that not all alleles are being detected at these loci. However, DRA and DRB (except for Porcupine uncorrected for multiple tests) are in HWE, which suggests we are identifying most alleles at these loci.

Differences between herds

There was little evidence of population differentiation between herds based on the microsatellite markers (Table 3). There was substantially more evidence of population differentiation based on the MHC loci. While many of the MHC alleles were found in more than one herd, some alleles were restricted, see Tables 4–7. There were differences in the allele frequencies in each herd. The greatest degree and most consistent differentiation between herds were seen for the DQB locus. Table 10 shows that the Delta herd is significantly different from the WAH at DRB, DQA1, DQA2, and nearly so at DQB. The Delta and Porcupine herds showed significant differentiation for the DRA and DQB loci. Differentiation between the Porcupine and WAH herds was only significant for the DQB locus.

The most frequent DRB allele was different in each herd: Porcupine (DRB*0901), Delta (DRB*0903) and Western Arctic (DRB*0102). DQA*AK04 was the most common DQA allele in all three herds. DQB*AK01 was most frequent DQB allele in Porcupine and Western Arctic, while DQB*AK07 was most frequent in the Delta herd. There were no differences relating to gender.

Table 9. Tests of Hardy–Weinberg equilibrium for MHC

Population	Locus	2*n*	No. alleles	HWE, *P*-value
Delta	DRA	82	2	1.000
Delta	DRB	76	12	0.147
Delta	DQA1	70	8	**<0.001***
Delta	DQA2	56	6	**<0.001***
Delta	DQB	72	8	**<0.001***
Porcupine	DRA	60	3	1.000
Porcupine	DRB	54	20	**0.022**
Porcupine	DQA1	56	8	**<0.001***
Porcupine	DQA2	42	6	**<0.001***
Porcupine	DQB	62	11	**<0.001***
WAH	DRA	100	3	1.000
WAH	DRB	88	16	0.250
WAH	DQA1	70	8	**<0.001***
WAH	DQA2	60	5	**<0.001***
WAH	DQB	70	11	**<0.001***

HWE, Hardy–Weinberg Equilibrium; WAH, Western Arctic herd.
*Significant after correction for five comparisons. *P*-values listed are uncorrected.
Bold values are indicated as low *P*-values.

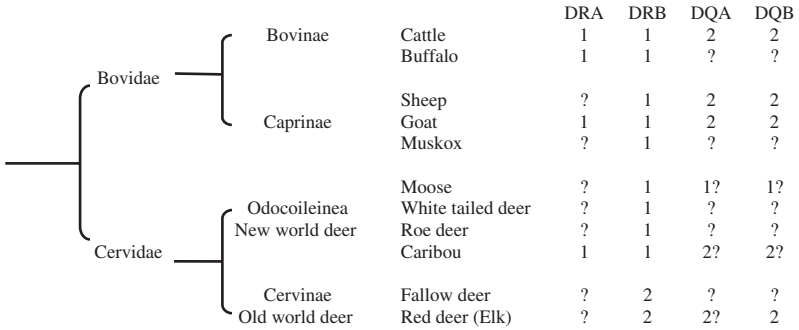
Table 10. Exact test of population differentiation based on MHC loci

Population pair	DRA	DRB	DQA1	DQA2	DQB
Delta–Porcupine	**0.022**	0.526	0.273	0.560	**0.009**
Delta–WAH	1.000	**<0.001**	**0.001**	**0.012**	0.066
Porcupine–WAH	0.234	0.800	0.106	0.063	**0.019**

WAH, Western Arctic herd.
P-values listed are corrected for five comparisons.
Bold values are indicated as low *P*-values.

Discussion

The characterization of the caribou MHC has been difficult due to the lack of sequence information for the species. We have had to use primers designed for other related species, with varying degrees of success.



		DRA	DRB	DQA	DQB
Bovinae	Cattle	1	1	2	2
	Buffalo	1	1	?	?
Caprinae	Sheep	?	1	2	2
	Goat	1	1	2	2
	Muskox	?	1	?	?
Odocoileinea	Moose	?	1	1?	1?
New world deer	White tailed deer	?	1	?	?
	Roe deer	?	1	?	?
	Caribou	1	1	2?	2?
Cervinae	Fallow deer	?	2	?	?
Old world deer	Red deer (Elk)	?	2	2?	2

Figure 2. Major expressed MHC genes in species related to the caribou. References: cattle (Davies *et al.*, 1997; Russell *et al.*, 1997), buffalo (Sena *et al.*, 2003), sheep (Snibson *et al.*, 1998; Dukkipati *et al.*, 2006), goat, muskox (Mikko *et al.*, 1999), moose (Mikko & Andersson, 1995; Ellegren *et al.*, 1996), white tailed deer (Van Den Bussche *et al.*, 1999), Roe deer (Mikko *et al.*, 1999), caribou (Olsaker & Roed, 1990; Cronin *et al.*, 1995), fallow deer (Mikko *et al.*, 1999), red deer (Swarbrick *et al.*, 1995; Swarbrick & Crawford, 1997).

Figure 2 shows the current information for the major expressed MHC genes for species related to the caribou. These data suggest that we could expect to find single DRA and DRB genes in the caribou, and our data is consistent with this premise. However, all of the bovidae and cervidae that have been characterized to date for DQA and DQB have two copies of each gene. It is therefore likely that the caribou will also have two copies of each of these genes. Our data for DQA is consistent with the presence of two genes, while our data for DQB is inconclusive.

While it is clear that the primers we used are not amplifying all possible alleles for DQA and DQB, nevertheless, we have demonstrated a considerable degree of variability and polymorphism within three of the four MHC class II loci investigated.

We can conclude from our small sampling of three different caribou herds that there is no lack of MHC class II diversity within these herds. Even the handful of samples from each of the smaller Galena and Chisana herds show considerable diversity. There is also some evidence of population differentiation based on the MHC class II data, even if we exclude DQA and DQB, where we are fairly certain that the primers are not amplifying all alleles. However, the microsatellite data show very little differentiation between the herds.

Previously Van Den Bussche *et al.*, (1999, 2002) have suggested that animals living at high latitudes may be exposed to fewer pathogens, and therefore need less diversity at the MHC. Some MHC studies in moose (Mikko & Andersson, 1995; Ellegren *et al.*, 1996), musk ox (Mikko *et al.*, 1999) and mountain goat (Mainguy *et al.*, 2007) have also revealed low MHC diversity in those species, but as indeed Mainguy *et al.* (2007) pointed out, all these studies investigated single MHC genes, and there may be unrevealed diversity at other loci.

However, it is clear from our caribou studies that using primers from related species may mean that not all alleles are amplified from a locus, which leads to an underestimate of the MHC diversity. Thus, it is possible that the previous studies that showed low diversity, may be underestimating that diversity by using sub-optimal primers.

Although Van Den Bussche *et al.*, 1999, 2002 were investigating ungulates, the implication is that this theory would apply to all species. Clearly, from our data, it does not apply to caribou in Alaska, nor to grey wolves in northern Canada (Kennedy *et al.*, 2007) and Finland (LJ Kennedy and H Lohi, unpublished data).

The two largest herds that were sampled, Porcupine and Western Arctic, clearly shared MHC alleles, but there were also unique alleles within each herd. Unsurprisingly, the isolated Delta herd was significantly different from the other two, but still had plenty of diversity. Interestingly, the two small declining herds, Chisana and Galena, showed reasonable diversity from only four or five animals sampled. The Chisana possibly had unique DRB alleles, otherwise all the alleles found in these two herds were also found in at least one of the other herds, indicating some not-so-distant common ancestry.

As has been so succinctly summarized by (Piertney & Oliver, 2006; Knapp, 2007) recently, one of the shortcomings of the recent explosion of molecular studies on the MHC in natural populations of non-model animals is the lack of information about the expression of these genes. There are no data as to which of these genes and alleles are expressed in the caribou, therefore are our conclusions valid with regard to functional diversity? There is no easy answer to this question, since it is difficult to obtain the appropriate samples for collecting RNA from animals in their natural environment. In the future, collection of blood samples in Paxgene (Qiagen, Valencia, CA, USA) (or other RNA compatible collection systems) would allow better characterization of the class II exon 2, by using cDNA and sequencing from exon 1 through to exon 3.

As molecular techniques progress, we will no doubt be able to surmise more about expression from

studying DNA, and may be less reliant on RNA for expression data. It is therefore worthwhile archiving DNA samples from all such studies.

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