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Detecting the signature of selection on immune genes in highly structured populations of wild sheep (*Ovis dalli*)

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Abstract

The confounding effects of population structure complicate efforts to identify regions of the genome under the influence of selection in natural populations. Here we test for evidence of selection in three genes involved in vertebrate immune function — the major histocompatibility complex (MHC), interferon gamma (IFNG) and natural resistance associated macrophage polymorphism (NRAMP) — in highly structured populations of wild thinhorn sheep (*Ovis dalli*). We examined patterns of variation at microsatellite loci linked to these gene regions and at the DNA sequence level. Simple Watterson's tests indicated balancing selection at all three gene regions. However, evidence for selection was confounded by population structure, as the Watterson's test statistics from linked markers were not outside of the range of values from unlinked and presumably neutral microsatellites. The translated coding sequences of thinhorn IFNG and NRAMP are fixed and identical to those of domestic sheep (*Ovis aries*). In contrast, the thinhorn MHC DRB locus shows significant evidence of overdominance through both an excess of nonsynonymous substitution and trans-species polymorphism. The failure to detect balancing selection at microsatellite loci linked to the MHC is likely the result of recombination between the markers and expressed gene regions.

Keywords: major histocompatibility complex, microsatellite, neutrality tests, *Ovis*, selection

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Introduction

Understanding processes responsible for the production and maintenance of genetic variation is a central aim in evolutionary biology. Levels of variation exhibiting deviation from neutral expectations of the mutation–drift equilibrium can result from both neutral demographic and selective processes. The task of separating these processes and identifying genes under the influence of selection can be challenging (Beaumont & Balding 2004). As a result, efforts to identify genes affected by selection have concentrated on species with well-characterized genomes, such as *Drosophila* and humans (Wall *et al*. 2002; Glinka *et al*. 2003; Stajich & Hahn 2005), where many coding regions have been characterized and are available for study. It remains important to examine patterns of genetic variation in nonhuman natural populations to gain a more general understanding of how selection shapes genetic variation. For this to be possible we must advance current techniques available for identifying selection.

Evidence for selection at the molecular level is most directly obtained by applying statistical tests of neutrality to patterns of sequence variation in coding regions (Tajima 1989; Kreitman & Akashi 1995; Fu 1997; Kreitman 2000; Nielsen 2001). However, it requires a great deal of gene isolation and *de novo* sequencing to apply this approach to multiple genes in nonmodel taxa. A solution lies in the use of neutral linked markers such as microsatellites which are also affected by selection through genetic hitch-hiking (Maynard Smith & Haigh 1974; Slatkin 1995). Recently, this process has been implemented in large-scale genome scans of many loci to identify regions of high linkage disequilibrium reflecting the presence of selection (Payseur *et al*. 2002; Vigouroux *et al*. 2002; Kauer *et al*. 2003). As selection is locus specific, only those markers linked to genes under

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selection are affected. In contrast, genetic drift and population-level effects on variation such as inbreeding and fluctuating population size are likely to affect all loci equally due to shared demographic history. Selective hitch-hiking can therefore be differentiated from neutral variation via the identification of markers with levels of variation outside neutral expectations. This can be accomplished in nonmodel taxa using heterologous markers such as cross-amplifying microsatellites linked to functional genes.

The idea of identifying outlier loci under the influence of selection originated with the F_{ST}-based test of Lewontin & Krakauer (1973). Methods for differentiating between neutrality and selection are not without problems. Lewontin and Krakauers' original method has been criticized due to its sensitivity to population structure and history (Nei & Maruyama 1975; Robertson 1975), which recent adaptations have attempted to improve upon (Bowcock *et al*. 1991; Beaumont & Nichols 1996; Vitalis *et al*. 2001; Schlotterer 2002). These are useful tools in species where many microsatellite loci are available. An alternative concept of examining selection is to test for neutrality (Watterson 1978) on a smaller number of loci where selection is presumed likely based on a prior knowledge of the functional significance of the linked gene. Genes involved in the immune response are excellent candidates for this type of analysis because of known associations with fitness.

Here we employ microsatellites linked to three regions involved in the vertebrate immune response: the major histocompatibility complex (MHC), interferon gamma (IFNG) and natural resistance macrophage protein (NRAMP) to examine genetic variation in natural populations of thinhorn sheep (*Ovis dalli*). The northerly and largely remote range of thinhorn in northwestern North America has prevented contact with domestic livestock. This is a major cause of population decline in the southerly sister species bighorn (*Ovis canadensis*) due to the transmission of novel pathogens (Gutierrez-Espeleta *et al*. 2001), especially the bacterium *Pasteurella haemolytica* (Foreyt 1989; Luikart & Allendorf 1996; Gutierrez-Espeleta *et al*. 2001). Selection may have changed the level of genetic variation at loci associated with immune genes in postepizootic bighorn sheep populations relative to disease-free thinhorn sheep populations. However, we still expect natural selection to be acting on thinhorn immune genes as other pathogens are present in the species (Kutz *et al*. 2001). Thinhorn provide a model of a species with a natural range of pathogens, rather than the novel disease present in bighorn.

Genes of the MHC region encode cell surface glycoproteins whose role is in the recognition of foreign antigens and their subsequent presentation to T cells. The peptide-binding region associating with antigenic peptides comprises the most variable region within the vertebrate genome (Hedrick 1994). High levels of heterozygosity in the MHC suggest the predominance of selection over neutral processes in maintaining variation. Correspondingly, there is abundant evidence of balancing selection (Paterson 1998; Hedrick 1999; Aguilar *et al*. 2004) from an excess of nonsynonymous sequence substitutions, the presence of trans-species sequence haplotypes and from selection tests on linked noncoding repeat regions (Paterson 1998; Huang & Yu 2003; Aguilar *et al*. 2004). Furthermore, there are known associations between MHC variability and levels of natural parasitic infection in domestic sheep populations (Buitkamp *et al*. 1996).

In contrast to the large body of work into the role of selection in the MHC, relatively few studies have been conducted on variation at IFNG and NRAMP. IFNG production is induced by early release cytokines or interaction with T or natural killer cell receptors upon the detection of viral and extracellular pathogens (Boehm *et al*. 1997). The IFNG region has been identified as a likely region of disease resistance in humans (Davis *et al*. 2000; Jenkins *et al*. 2000; Pokorny *et al*. 2001), horses (Horin *et al*. 2004), and sheep (Coltman *et al*. 2001) among others. Moreover, at linked microsatellites there are allelic correlations to disease resistance in both sheep (Coltman *et al*. 2001) and goats (Obexer-Ruff *et al*. 2003).

NRAMP is expressed in the membranes of macrophages and monocytes where it plays a role in the immune response to several important intracellular pathogens (Canonne-Hergaux *et al*. 1999). After phagocytosis of invading pathogens, the NRAMP protein is directed towards the phagosome membrane where it effects microbial replication (Gruenheid *et al*. 1997), possibly by removing iron from the phagosome (Fleming & Andrews 1998). Resistance to mycobacterial diseases such as *Salmonella* and the protozoan *Leishmania* in mice is via a single amino acid change (Vidal *et al*. 1995) while the homologous human protein is associated with resistance to tuberculosis and leprosy (Canonne-Hergaux *et al*. 1999). Polymorphic sites have been described between cattle breeds (Ables *et al*. 2002), where there is an association between a 3′ noncoding region and disease resistance (Barthel *et al*. 2001). The nucleotide sequence of IFNG and NRAMP have not been characterized in a wild sheep species, nor do we know of any application of specific tests of selection at these regions in nonhuman natural populations.

Rather than the coalescent methods previously used to identify outlying loci under the influence of selection (Payseur *et al*. 2002; Storz *et al*. 2004; Stajich & Hahn 2005), we here use empirical data from presumably neutral, nonlinked microsatellite markers (Worley *et al*. 2004) to provide a background level of neutral genetic variation for comparison with immune linked markers. Specifically, we aim to test whether patterns of variation at microsatellites linked to thinhorn immune genes deviate from expectations of neutrality. Balancing selection may be expected in genes involved in pathogen resistance. Increased allelic

diversity can result in increased resistance to a more varied array of pathogens. This is especially well documented in the MHC region (Hedrick 1999), suggested in the human NRAMP region (Blackwell & Searle 1999) but unreported in IFNG. Selection can result in both a decrease or an increase in genetic variation compared to levels expected under neutrality (Maynard Smith & Haigh 1974; Watterson 1978; Hudson *et al*. 1987). However, we can make specific predictions regarding the expected levels of allelic diversity within and between populations relative to neutrality assuming balancing selection is acting on immune loci.

Balancing selection generates an increased level of heterozygosity than that expected under neutrality. Signatures of increased heterozygosity can be revealed using a variety of statistics. Balancing selection predicts a reduction in the degree of genetic differentiation (F_{ST}) between populations (Muirhead 2001). In addition, migrants carrying rare alleles are at a selective advantage, resulting in an increase of the effective migration rate (Schierup *et al*. 2001) via the homogenization of between-population allelic distributions. This is in opposition to expectations of positive directional selection, where population-specific selection against maladaptive alleles increases the degree of differentiation between populations (Lewontin & Krakauer 1973). The effect of balancing selection on F_{ST} includes a historical component; excess heterozygosity can result from both past selective events and current processes. This is not true of F_{IS} statistics, where deviations from neutrality reflect contemporary processes only. Even so, excess heterozygosity opposes the effects of both inbreeding and random mating. We may therefore predict a reduced F_{IS} and Watterson's statistic F_A in the current adult population at immune linked loci under the influence of balancing selection relative to values at nonlinked neutral loci. Conversely, an increased F_{IS} and reduced within-population diversity are expected immediately following positive directional selection, such as a selective sweep following disease outbreak (Tajima 1989; Barton 2000; Fay & Wu 2000).

Here we aim to test these predictions using patterns of variation at microsatellites linked to immune genes in wild thinhorn sheep. We also characterize DNA sequence variation in MHC, IFNG and NRAMP expressed gene regions and use molecular evolution analyses to test for selection. By comparing evidence of selection between linked microsatellites and gene sequences, we examine the robustness of tests of selection on linked neutral markers in populations with background genetic structure.

Materials and methods

Sample locations and collection

Horn and blood samples were taken from 922 sheep from 24 sample areas across the species' range including the Yukon and Northwest Territories and British Columbia, Canada, and Alaska, USA. Full details of sample collection methods and locations are given in Worley *et al*. (2004). Sample area boundaries were defined from game management zones or units in the Northwest Territories (NWT) and northern British Columbia (BC), and by groups of adjacent management units from the Yukon Territory (YK). Alaskan sample areas comprised individuals collected from the same mountain range with a maximum distance of 140 km separating samples. Previous analyses have revealed widespread population substructure to be present within each of these sample areas. In order to minimize the confounding effects of population substructure on locating natural selection, analyses of immune linked microsatellite data were performed at the level of sample area rather than the larger regions defined by a Bayesian population structure analysis using the program STRUCTURE (Pritchard *et al*. 2000). Due to the lower sample sizes required for sequence characterization and logistical constraints, we sequenced individuals that were representative of eight STRUCTURE regions. Genomic DNA was extracted from horn material using a tissue extraction kit (QIAGEN) and from blood using a phenol–chloroform technique (Sambrook *et al*. 1989).

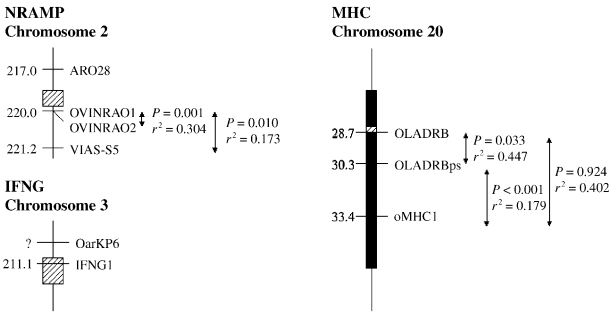

Fig. 1 A linkage map of gene regions examined in this study. Values to the left of the figures indicate the map distances of microsatellite markers along each chromosome and are taken from SMC sex-averaged maps of ARKdb (www.thearkdb.org). Approximate locations of coding regions in relation to microsatellite loci are indicated by hash filled rectangles. r^2 is the mean correlation coefficient of linkage between microsatellite pairs, while P is the overall probability of linkage. Only heterozygote genotypes were included when calculating these values for the null locus OLADRB. The associated correlation coefficients are moderate and variable over loci. Linkages for NRAMP loci except those shown were nonsignificant.

Microsatellite loci

Samples were genotyped at several microsatellite loci within or adjacent to the MHC, IFNG and NRAMP gene regions (Fig. 1). Loci were selected for their close proximity to coding regions and previous amplification success in related species (especially in the case of MHC linked loci).

The dinucleotide MHC microsatellites OLADRB (Schwaiger *et al*. 1993) and OLADRBps (Blattman & Beh 1992) are located within the class II region. Primer sequences for both were altered to improve the product quality from thinhorn samples (OLADRB-F, 5′-TGTGCAGCGGCGAGGTGAGCG-3′, OLADRB-R, 5′-CGTACCCAGA$^{T}/_{G}$TGAGTGAAGTATC-3′; OLADRBps-F, 5′-CATGGGTTTCATCCCTGAGT-3′, OLADRBps-R, 5′-CTCCTGTCTTGTCATCTCTACGA-3′). The dinucleotide microsatellite oMHC1 is located within the class I MHC region (Groth & Wetherall 1994). Two IFNG linked microsatellites were genotyped. The tetranucleotide microsatellite IFNG1 is situated in intron 1 of the IFNG gene (Schmidt *et al*. 1996). No recombination has been observed between this locus and the dinucleotide microsatellite KP6 (Paterson & Crawford 2000). Finally, the dinucleotide microsatellites OVINRAO1 and OVINRAO2 are positioned close to the 3′ end of the NRAMP gene (Pitel *et al*. 1996). ARO28 (Avraham *et al*. 1993) and VIAS-S5 (Primmer & Matthews 1993) flank the NRAMP coding region at the 5′ and 3′ ends, respectively.

Sheep included in this study have previously been genotyped over 12 presumably neutral dinucleotide microsatellite loci (Worley *et al*. 2004) developed in domestic sheep and cattle. None of the 12 loci are adjacent to any mapped gene involved in the immune response. Despite their presumed neutrality none of the loci were in Hardy–Weinberg equilibrium, a result of small-scale population substructure within sample regions leading to wide-scale Wahlund effects (Worley *et al*. 2004).

Each polymerase chain reaction (PCR) was carried out in 10-μL reactions containing 2 μL of DNA template, 80 μmol of each primer, 0.16 mM dNTPs, and 0.5 U *Taq* polymerase (Bioline, London). Concentrations of $MgCl_2$ varied with locus from 0.5 to 1.5 mM. The PCR profile comprised 35 cycles of 30 s at 94 °C, 30 s annealing at either 63 °C (OVINRAO1, OLADRBps), 60 °C (IFNG), 54 °C (OarKP6, ARO28, VIAS-S5) or 50 °C (OLADRB, oMHC1, OVINRAO2) followed by 40 s at 72 °C. Cycles were preceded by 5 min at 94 °C and terminated with 10 min at 72 °C. PCR products were genotyped using an ABI 3730 sequencer and analysed using the software GENEMAPPER (Applied Biosystems).

Initial observations of a high rate of excess homozygosity at OVINRAO1 in BC populations suggested a null allele at measurably high frequency. We therefore typed BC samples and a proportion of all others with the NRAMP1 primer set which amplifies the same microsatellite locus (Bussmann *et al*. 1998). Many OVINRAO1 genotypes in BC were therefore extrapolated from the NRAMP1 genotype. OVINRAO1 was initially chosen over the NRAMP1 primer because it amplified more reliably. As a result of using the less reliable NRAMP1 primer, sample sizes are low in some BC sample areas (BC751, BC752 and BC754) where null OVINRAO1 genotypes could not be verified using NRAMP1. Where this is the case, these regions are omitted from some analyses to avoid introducing bias in the data.

A high frequency null allele was also present in the locus OLADRB. We were not able to correct for this despite several attempts at redesigning the primer sequences.

DNA sequencing

We selected samples for sequencing each locus based on the genotype observed at the linked microsatellite and to be representative of the eight regions determined by a Bayesian analysis of population structure using the program STRUCTURE (Worley *et al*. 2004). All samples chosen for sequencing at IFNG and NRAMP regions were homozygous at the microsatellites KP6 and OVINRAO1, respectively. DRB samples were representative only of the STRUCTURE regions. Samples at all loci were selected that had a high confidence of belonging to a distinct STRUCTURE region ($P > 0.9$) to limit mixed ancestry. A total of 22 individuals were sequenced at the MHC locus DRB from each region (mean per sample area of 2.75). More individuals were sequenced for IFNG and NRAMP (36 and 43, respectively) because we were able to direct sequence these genes due to lower variability.

Cloning and sequencing exon 2 of the MHC gene DRB. Exon 2 of the class II MHC gene DRB was amplified in 22 thinhorn samples from across the species' range. Primers were shortened from those of Sigurdardottir *et al*. (1991) in order to achieve a product in this species (LA31-K, 5′-ATCCTCTCTCTGCAGCACATTTCCT-3′, LA32-K, 5′-TCACCTCGCCGCTGCACA-3′). For each sample, we performed five separate 10-μL PCRs. Larger volume reactions frequently failed due to low quality of DNA extracted from horn material. Each reaction comprised 2 μL of DNA template, 40 μmol of each primer, 0.16 mM dNTP's, 1.5 mM $MgCl_2$ and 0.5 U *Taq* polymerase (Bioline). The PCR profile comprised 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s. Cycles were preceded by 5 min at 94 °C and terminated with 10 min at 72 °C. PCR products of the same individual were pooled and cleaned using a standard gel purification kit (QIAGEN). The concentration of the purified PCR product was estimated by running 1 μL alongside a ladder of known concentration (Φ×174 RF DNA *Hae*III, ABgene) in 1.5% agarose. (Sigurdardottir *et al*. 1991).

Purified PCR product was ligated into pGEM-T vector (Promega) in 10-μL reactions containing approximately 10 ng insert DNA. Reactions were incubated overnight at 4 °C before transformation into JM109 *Escherichia coli* by heat shock. Cells were plated onto Amp/IPTG/X-Gal agar and cultured for 24 h before picking positive clones. Eight positive clones were grown further in LB glycerol overnight

for each sample before plasmid miniprep (Promega) and sequencing with both M13 (–21) forward and LA32-K reverse primers. Following precipitation, sequences were run on an ABI 3730 automatic sequencer.

IFNG and NRAMP sequencing. Two fragments of IFNG were amplified from 36 individuals (mean 4.5 per STRUCTURE cluster) using the primer pairs IFNG_F3 and IFNG_R3 and IFNG_F7 and IFNG_R7a (J. Gratten, personal communication). The first set amplifies a fragment encompassing exons II, III and surrounding partial intronic sequence. The second primer set amplifies exon IV and surrounding intronic regions. A total of 1256 base pairs of IFNG sequence were used for analyses.

A continuous 980-bp NRAMP fragment was amplified in 43 thinhorn by two primer sets designed from aligned GenBank sequences of the region (NRAMP-2F, 5′-CTCTCCTCTGGCTGACCATC-3′, NRAMP-2R, 5′-CACGATGGTGATGAGGACAC-3′, NRAMP-3F, 5′-GTGGGAGATCCAGACTCCTG-3′, NRAMP-3R, 5′-CCGAAGGTCAAAGCCATTAT-3′). The complete NRAMP amplicon comprised exons V, VI and VII and separating introns.

For each sample, we again amplified the required product in five different PCRs of 10 μL each. The final concentrations of reagents were the same as for DRB except $MgCl_2$ where 1 mM was used for most primer pairs (2 mM for IFNG_F7 and IFNG_R7a). PCR profiles for all reactions were again the same as for DRB with varying annealing temperatures (IFNG_F3 & IFNG_R3, 58 °C; IFNG_F7 & IFNG_R7a, 60 °C; NRAMP-2, 57 °C; NRAMP-3, 57 °C). PCR products were gel purified (Qiagen) and the DNA quantified before sequencing of gene regions. Direct sequencing was possible due to the low levels of polymorphism present in both genes.

Analyses of within-population diversity and tests of selection

Observed and expected heterozygosities were calculated for linked loci within each sample area using GENETIX version 4.01 (Belkhir 1996). We tested for Hardy–Weinberg equilibrium using exact tests as implemented by GENEPOP version 3.3 (Raymond & Rousset 1995) combining sample areas using Fisher's method. All statistics have previously been calculated for 12 nonlinked loci (Worley *et al*. 2004) enabling a comparison between marker types to be made. Linkage disequilibrium between linked loci was tested using the LINKDOS program (Garnier-Gere & Dillmann 1992); a part of GENEPOP. The program performs pairwise linkage disequilibria analyses in subdivided populations using the analysis of Black & Krafsur (1985). The level of correlation between locus pairs within each gene region was calculated in addition to the probability of linkage.

We calculated values of the statistic F_{IS} (using GENEPOP) at each locus both over all samples and within each sample area. Any difference between the values of F_{IS} per locus between linked and nonlinked loci, and hence putative evidence of contemporary selection, was examined by Mann–Whitney tests. (Black & Krafsur 1985).

We tested for selection using Watterson's homozygosity test of neutrality (Watterson 1978). Although this test was originally constructed using the assumption of an infinite alleles model, a mutation model from which microsatellites are thought to deviate, we use the statistic only to make relative comparisons between linked and nonlinked loci. Thereby the values calculated for linked loci are controlled by those reported from nonlinked markers. The test statistic F_A is the sum of the expected frequency of homozygotes under Hardy–Weinberg equilibrium, Σp_i^2, where i represents each allele. Significance is tested against distributions of values expected for populations under neutrality given a number of alleles (K) and population size (2n) (Ewens 1972). The distributions of expected values of F_A are available for up to $K = 40$ and 2n = 500 at http://allele5.biol.berkeley.edu/homozygosity/homozygosity.html. The test remains approximately valid for subdivided populations (Tillier & Golding 1988). The normalized deviate of Watterson's test statistic, Fnd_A, was calculated for each locus (Salamon *et al*. 1999) where

$$Fnd_A = \frac{F_{A\ observed} - F_{A\ expected}}{[Var(F_{A\ expected})]^{1/2}}$$

This allows loci to be compared across populations differing in allele number and population size. A positive value of Fnd_A is indicative of positive selection, a negative value results from balancing selection. The mean value of Fnd_A over m populations was calculated at all loci. The standard error of Fnd_A is represented by S_A, where

$$S_A^2 = \frac{\sum \left(Fnd_A - \overline{Fnd_A}\right)^2}{m - 1}$$

The overall test of selection at a locus comprises a two-tailed test on mean Fnd_A, using

$$z = \overline{Fnd_A}\left(\frac{\sqrt{m}}{S_A}\right)$$

Evidence of selection among populations

We compared overall mean genetic differentiation, as measured by locus F_{ST} (Weir & Cockerham 1984), between linked and nonlinked loci using Mann–Whitney tests. Under neutral expectations, we expect a homogeneous distribution of F_{ST} across all loci, as drift and gene flow

should affect all loci equally. An exception to this expectation may come from loci with different repeat motifs, as microsatellites with larger repeat units mutate at slower rates than those with smaller repeats (Chakraborty *et al.* 1997). As all analysed microsatellites were dinucleotides, this is not relevant here, and any differences between the F_{ST} values of neutral and putative selected loci can therefore be attributed to selection.

We examined isolation-by-distance relationships (Slatkin 1993) for individual linked loci and compared these with patterns observed for nonlinked markers. Pairwise geographic distances were calculated from linear distances between mean latitudinal and longitudinal locations of sample areas and plotted against the corrected pairwise F_{ST} value, $F_{ST}/(1 - F_{ST})$. Significance of the isolation-by-distance relationship was tested by Mantel tests as part of GENEPOP. The slope of the regression line of each plot was calculated, constraining the relationship through the origin. Values of the isolation-by-distance slope for linked loci were tested for differentiation from nonlinked loci using Mann–Whitney tests.

Examining sequence diversity

All sequences were edited and aligned using the software SEQSCAPE (Applied Biosystems). Polymorphic sites were identified in alleles from all three immune regions. Further analyses were conducted on the 16 putative peptide binding codons and 67 nonpeptide binding codons of DRB alleles. We estimated the rate of synonymous (d_S) and nonsynonymous (d_N) substitutions in MEGA (Kumar *et al.* 2001), using the distance model of Nei & Gojobori (1986) and applying a Jukes–Cantor correction for multiple substitutions at a site. Deviation from neutral expectations was tested by comparing the difference between these rates by using *t*-tests with an infinite number of degrees of freedom. All sequences were compared to those from other ungulates available in the GenBank database to look for alleles shared between species. Due to the low variability in IFNG and NRAMP, further analyses were only performed on DRB alleles. A bootstrapped (2000 replicates) neighbour-joining tree of DRB alleles was constructed in MEGA using Jukes–Cantor correction. In addition to thinhorn alleles, 34 sequences from eight other ungulate species were included in the tree (22 bighorn sheep, 6 domestic sheep, 1 goat, 1 bison, 2 cattle, 1 moose and 1 blesbok). (Nei & Gojobori 1986).

Results

Linkage disequilibrium

We observed significant linkage disequilibrium between the locus pairs OLADRBps and OLADRB and OLADRBps and oMHC1 within the MHC region of chromosome 20 and between the NRAMP markers OVINRAO1 and OVINRAO2 and OVINRAO1 and VIAS-S5 (Fig. 1). Due to the presence of null alleles in OLADRB, linkage to other loci was estimated using data from heterozygotes alone. Coefficients of linkage were moderate in all cases. Given the significant linkage between microsatellite loci, we would also expect linkage to be present between loci and adjacent coding regions. We were not able to assess the linkage present at IFNG due to fixation of the microsatellite IFNG1 (Table 1).

Genetic variation and evidence for selection within sample areas

Microsatellites contained between 1 (IFNG) and 23 (OLADRBps) alleles (Table 1). Expected heterozygosities of the polymorphic loci ranged from 0.300 (ARO28) to 0.811 (KP6). At nonlinked neutral markers, we previously observed widespread overall heterozygote deficit, a result of sampling over genetically differentiated subpopulations

Table 1 Characteristics of the linked microsatellite loci used in this study. Heterozygosities are given as the mean over sample regions. Hardy–Weinberg probabilities (as calculated by exact test in GENEPOP), F_{IS} and F_{ST} values are combined for populations using Fisher's method (*significant F_{IS})

Locus	*N*	Allele size range	*K*	Mean *K*	H_O	H_E	HWE prob.	F_{IS}	F_{ST}
OLADRB	892	148–238	14	4.17	0.311	0.443	< 0.001	0.302*	0.353
OLADRBps	900	238–286	23	8.92	0.741	0.755	< 0.001	0.020	0.118
oMHC1	838	178–200	11	5.08	0.574	0.588	0.025	0.028	0.185
IFNG1	200	122	1	n/a	n/a	n/a	n/a	n/a	n/a
KP6	897	182–212	16	8.71	0.751	0.811	< 0.001	0.076*	0.108
OVINRAO1†	734	218–238	9	4.00	0.560	0.585	0.787	0.046*	0.124
OVINRAO2	836	300–306	3	2.04	0.344	0.353	0.999	0.021	0.157
ARO28	914	239–245	3	2.08	0.308	0.300	0.980	− 0.010	0.171
VIAS-S5	912	106–114	5	2.75	0.382	0.374	0.065	− 0.012	0.297
Nonlinked loci	921				0.570	0.597	< 0.001	0.066*	0.160

†OVINRAO1 genotypes are corrected for a null allele by additional genotyping at NRAMP1 and conversion to the equivalent OVINRAO1 genotype.

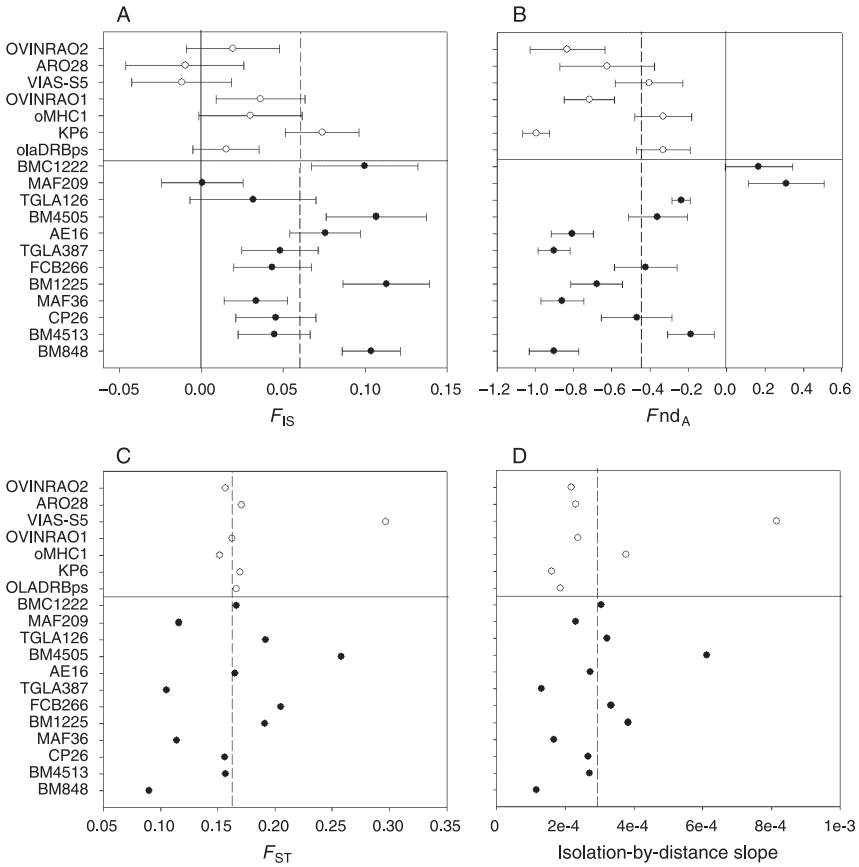

Fig. 2 Comparisons of the genetic characteristics at all linked and nonlinked loci. In all plots linked loci are shown in white above the horizontal line and nonlinked loci are shown in black below it. Vertical dashed lines indicate overall values for nonlinked loci. Loci are ordered from low to high variability (as measured by mean number of alleles per sample area), with OLADRBps being the most heterozygous of the linked loci. Standard errors for point values of F_{IS} and Fnd_A were calculated from values from all sample areas. Errors were negligible for F_{ST} and isolation-by-distance slopes and were therefore not included.

within each sample area (Worley *et al.* 2004). Here we observed a similar pattern, with deviations from Hardy–Weinberg expectation present at all but NRAMP loci (Table 1). The majority of F_{IS} values were positive, significantly so for some loci (Table 1). The mean F_{IS} for the MHC and NRAMP linked microsatellites was lower than the overall average for the unlinked markers (Table 1). Indeed linked loci showed a significant reduction in mean F_{IS} compared to nonlinked loci (Fig. 2A, Mann–Whitney $U = 13$, $P = 0.013$). However, most values fell within the range observed at individual nonlinked loci (Fig. 2A). We therefore find no difference between the values of F_{IS} between linked and nonlinked loci.

The locus OLADRB was omitted after preliminary analyses due to several lines of evidence that suggested null alleles. First, the levels of heterozygosity observed here were much lower than previously reported in domestic sheep (Paterson 1998). Second, although deviations from Hardy–Weinberg equilibrium were reported in other nonlinked loci, the magnitude of this deviation was much greater in OLADRB. Third, the overall value of F_{IS} was more than 10 times greater than that for other MHC linked loci (Table 1). This great deficit of heterozygotes in the data set strongly implicates nonamplified alleles.

Watterson's test results for linked loci are summarized in Table 2. There was significant deviation from neutrality in at least one sample area for each locus. The greatest deviation was observed at the IFNG linked locus KP6, at which 7 out of 24 populations deviated from neutrality. At all loci deviations from neutrality were in the direction of increased heterozygosity (lower F statistics) resulting from more equal allele frequency distributions than expected under neutrality. There were many more negative values of Fnd_A than positive, and the overall mean value of Fnd_A from all linked loci was significantly negative. Watterson's tests were also conducted on allele frequencies of all nonlinked markers. As with the linked markers most loci had negative values of Fnd_A (Fig. 2B). Moreover, there is a background trend for loci with higher levels of variability (as measured by the mean number of alleles, K) to show reduced values of mean Fnd_A ($r = -0.63$, $P = 0.03$). It is possible that NRAMP linked markers showed some evidence for lower values of Fnd_A than those observed in nonlinked loci with comparable diversity, but other linked markers show values in line with expectations.

Evidence for selection from sample area differentiation

We have previously reported a moderate level of genetic differentiation among sample areas included in this study (overall mean population $F_{ST} = 0.16$). Overall genetic differentiation calculated from polymorphic linked microsatellites

Table 2 Watterson's test results from linked microsatellite markers. Geographic location of sample areas can be found in (Worley *et al*. 2004). Values are given for the number of alleles per locus in each sample area (*k*), sample size of alleles (2n), the homozygosity statistic F_A (Watterson 1978), and the normalized deviate of this statistic (Fnd_A) amended for comparison over sample populations (Salamon *et al*. 1999). Significance of deviation from neutrality (*P*) is given for each value of F_A and for the overall mean Fnd_A for all seven polymorphic loci

Sample area	OLADRBps					oMHC1					KP6					OVINRAO1					OVINRAO2					ARO28					VIAS-S5				
	K	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*	*K*	2n	F_A	Fnd_A	*P*
CAR	9	50	0.175	−0.966		5	50	0.215	−1.630	<0.01	8	50	0.208	−0.846		3	50	0.382	−1.476		2	50	0.520	−1.609		2	50	0.507	−1.688	0.05	3	50	0.449	−1.078	
GAAR	9	68	0.297	0.253		4	68	0.565	0.134		9	68	0.187	−0.958		3	68	0.391	−1.480		2	64	0.805	0.080		2	68	0.943	0.901		2	68	0.673	−0.737	
NK	5	50	0.395	−0.321		4	50	0.466	−0.354		5	50	0.438	−0.007		2	32	0.695	−0.406		2	48	0.920	0.869		1	50	1	NA		2	50	0.635	−0.900	
YUCH	8	70	0.372	0.598		5	66	0.424	−0.249		7	70	0.252	−0.839		5	68	0.368	−0.640		2	66	0.537	−1.556		2	70	0.632	−0.991		4	70	0.522	−0.143	
D/OT/01	10	76	0.358	1.226		6	74	0.322	−0.616		9	78	0.182	−1.055		5	74	0.458	−0.075		2	78	0.521	−1.685		2	78	0.796	−0.024		2	80	0.928	0.767	
D/OT/02	7	84	0.307	−0.503		3	76	0.876	1.241		10	80	0.119	−1.580	<0.01	6	74	0.462	0.415		2	74	0.500	−1.800	<0.05	2	80	0.840	0.238		2	80	0.883	0.495	
G/OT/01	9	78	0.294	0.082		6	68	0.370	−0.221		9	80	0.182	−1.070		7	78	0.432	0.601		2	70	0.515	−1.704		2	78	0.837	0.222		2	78	0.975	1.057	
S/OT/01	7	80	0.230	−1.064		5	68	0.471	0.059		10	80	0.158	−1.142	<0.05	5	64	0.345	−0.776		2	80	0.508	−1.771	<0.05	3	78	0.731	0.410		2	72	0.895	0.596	
S/OT/02	9	76	0.251	−0.362		4	68	0.435	−0.659		8	76	0.175	−1.317	<0.05	5	74	0.317	−1.010		2	64	0.504	−1.772	<0.05	3	80	0.742	0.461		3	76	0.852	1.106	
S/OT/03	10	80	0.269	0.134		5	76	0.439	−0.212		9	80	0.159	−1.307	<0.05	6	76	0.350	−0.420		2	72	0.519	−1.683		2	80	0.561	−1.448		3	80	0.501	−0.895	
S/OT/04	10	78	0.217	−0.443		5	62	0.327	−0.887		10	76	0.188	−0.768		5	78	0.354	−0.779		2	66	0.502	−1.771	<0.05	2	82	0.567	−1.423		2	82	0.501	−1.821	<0.05
S/OT/05	9	80	0.252	−0.351		4	80	0.487	−0.401		10	78	0.166	−1.040		5	82	0.289	−1.216		2	78	0.512	−1.742		2	80	0.501	−1.810	<0.05	3	80	0.546	−0.639	
Y2E	10	78	0.249	−0.075		8	72	0.304	−0.076		9	80	0.169	−1.202	<0.05	4	78	0.309	−1.460	<0.05	2	70	0.500	−1.793	<0.05	2	80	0.500	−1.816	<0.025	2	80	0.638	−0.985	
Y2W	7	76	0.312	−0.370		4	74	0.379	−1.026		10	74	0.158	−1.108		4	78	0.499	−0.322		2	66	0.566	−1.380		2	78	0.501	−1.805	<0.05	4	78	0.630	0.459	
Y4	11	76	0.211	−0.238		6	64	0.361	−0.209		10	72	0.133	−1.400	<0.01	3	78	0.531	−0.719		2	74	0.829	0.187		2	80	0.525	−1.665		2	80	0.553	−1.499	
Y5	10	80	0.340	0.951		4	68	0.499	−0.273		10	80	0.124	−1.522	<0.001	4	80	0.478	−0.454		2	76	0.900	0.614		2	78	0.521	−1.686		3	80	0.509	−0.849	
Y7	8	74	0.160	−1.455	<0.01	3	62	0.546	−0.567		9	74	0.195	−0.906		3	78	0.496	−0.914		2	80	0.951	0.909		2	82	0.500	−1.827	<0.025	3	82	0.543	−0.661	
Y8	8	80	0.201	−1.087		7	74	0.534	1.488		10	80	0.202	−0.633		2	80	0.570	−1.393		2	76	0.683	−0.700		2	80	0.520	−1.697		4	80	0.718	0.972	
Y10	7	80	0.266	−0.776		7	78	0.470	0.657		10	78	0.143	−1.298	<0.05	4	70	0.526	−0.118		2	80	0.711	−0.541		2	76	0.772	−0.165		3	80	0.455	−1.153	
BC742	12	74	0.226	0.296		7	76	0.351	−0.053		7	76	0.261	−0.791		3	44	0.437	−1.115		2	58	0.715	−0.447		2	80	0.728	−0.442		3	78	0.553	−0.592	
BC750	11	80	0.215	−0.221		5	78	0.323	−0.984		7	80	0.281	−0.649		3	44	0.408	−1.289		2	70	0.680	−0.702		2	80	0.840	0.238		3	80	0.448	−1.194	
BC751	10	80	0.164	−1.064		5	72	0.280	−1.249		8	78	0.251	−0.621		3	12	0.431	NA		2	62	0.775	−0.094		2	80	0.861	0.365		3	80	0.613	−0.261	
BC752	8	72	0.189	−1.169		5	76	0.339	−0.872		7	76	0.223	−1.112		3	6	0.389	NA		2	74	0.829	0.187		2	80	0.975	1.054		3	80	0.454	−1.159	
BC754	10	80	0.166	−1.046		5	76	0.315	−1.025		8	80	0.243	−0.707		1	2	1.000	NA		3	76	0.642	−0.081		2	80	0.840	0.238		3	80	0.550	−0.620	
			mean	−0.332	0.018			mean	−0.333	0.024			mean	−0.995	<0.01			mean	−0.717	<0.001			mean	−0.833	<0.001			mean	−0.624	0.011			mean	−0.406	0.021
			$S_A/\sqrt{m}$	0.141				$S_A/\sqrt{m}$	0.148				$S_A/\sqrt{m}$	0.071				$S_A/\sqrt{m}$	0.131				$S_A/\sqrt{m}$	0.196				$S_A/\sqrt{m}$	0.247				$S_A/\sqrt{m}$	0.176	

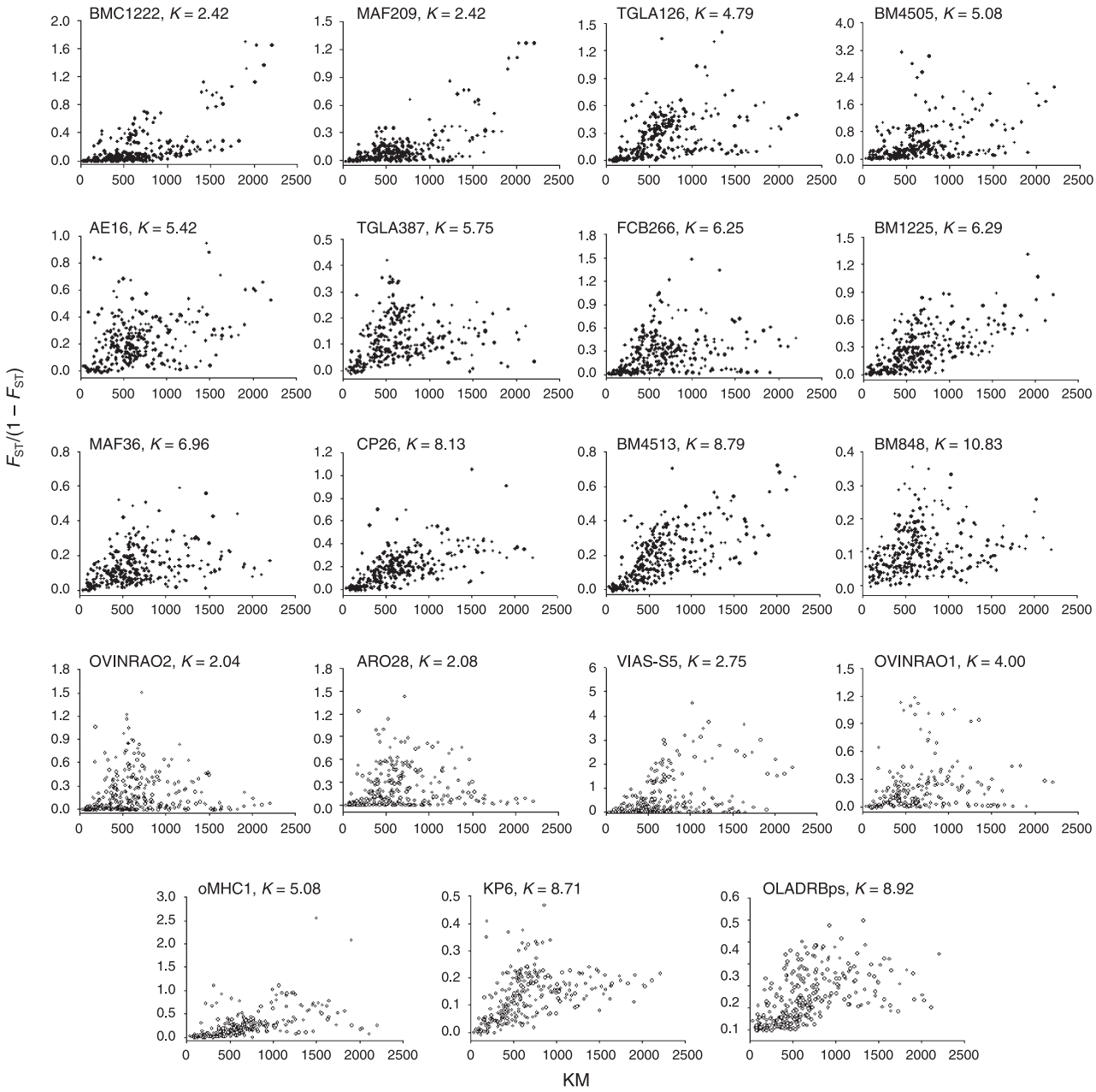

Fig. 3 Isolation-by-distance plots of all 19 loci included in this study. For each locus the with mean number of alleles per sample area is given as *K*. Plots reveal the bias to report increased genetic distance when using markers with low variability. The patterns present in linked markers do not appear to differ from the general trend seen in nonlinked loci.

showed less variation but was of a similar magnitude (Table 1). F_{ST} values for individual linked loci fell within the range of values observed for unlinked loci (Fig. 2C). We find no correlation between locus variability and F_{ST} ($r = -0.28$, $P = 0.38$).

The highly significant isolation-by-distance relationship observed for nonlinked microsatellites (Worley *et al*. 2004) was also observed here for linked loci (Fig. 3; all $P < 0.01$). When slopes of isolation-by-distance relationships were constrained through the origin, values for linked loci fell within the range of those observed for nonlinked loci (Fig. 2D).

Sequence polymorphism and correlation to microsatellite alleles

DRB exon 2. Seventeen thinhorn DRB sequence alleles were characterized from the 22 samples (11 homozygotes and 11 heterozygotes) with 57 out of the 249 bases showing polymorphism. Alleles have been deposited in GenBank

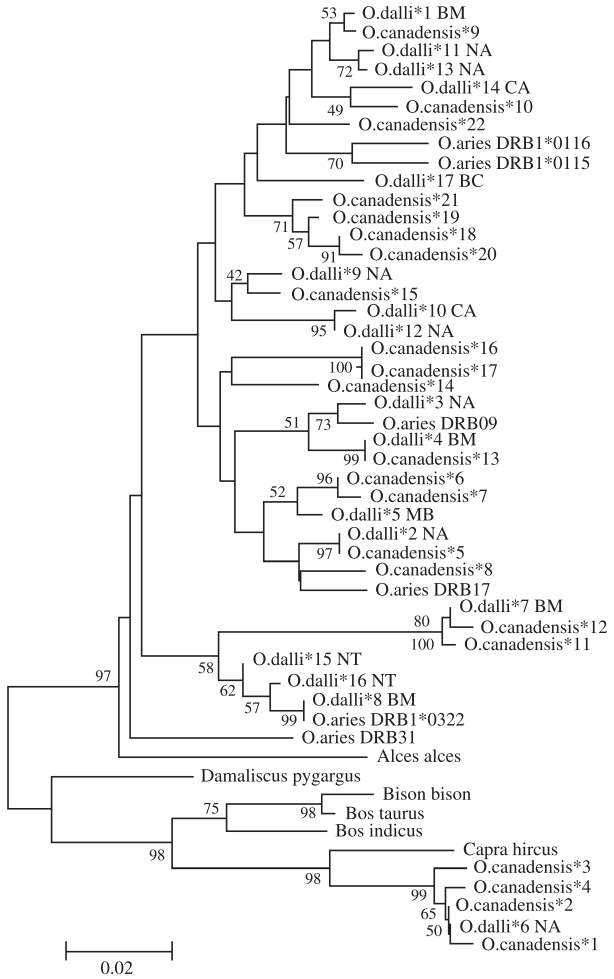

Fig. 4 Neighbour-joining tree of thinhorn and selected other ungulate DRB alleles. The scale refers to Jukes–Cantor distance and values indicate bootstrap significance (from 2000 replicates). Text following domestic and bighorn sheep alleles reflect those given to alleles as submitted in GenBank. Letters following thinhorn alleles encode the geographic locations of the respective sequences: NA, Northern Alaska; CA, Central Alaska; BC, British Columbia; NT, Northern Territories; BM, alleles found between mountain ranges.

under accession numbers AJ920396–AJ920412. Alignment with other GenBank sequences showed that 3 of the 17 thinhorn alleles, Ovda-DRB*2, *4 and *6, correspond to alleles 20, 18 and 2 (Gutierrez-Espeleta *et al.* 2001) from bighorn sheep. An additional thinhorn allele (Ovda-DRB*8) is shared with the *Ovis aries* allele DRB1*0322 (Kostia *et al.* 1998). Figure 4 shows the extent of trans-species polymorphisms between thinhorn and other ungulates. The 17 thinhorn alleles predict 16 distinct amino acid sequences in the translated protein containing a total of 30 variable sites. Correspondingly, there were a greater number of nonsynonymous relative to synonymous substitutions (Table 3). The excess of nonsynonymous

Table 3 Rate of nonsynonymous (d_N) and synonymous (d_S) substitutions at the MHC gene DRB

Position	Codon no.	d_N	d_S	d_N/d_S	P
Antigen binding	16	0.312	0.048	6.456	<0.001
Non-antigen binding	67	0.042	0.032	1.296	0.801
All	83	0.083	0.035	2.370	0.075

Table 4 Distribution of sequence alleles throughout each sample region. Regions are based on clusters from STRUCTURE analyses of neutral microsatellites: NA, northern Alaska; CA, central Alaska; OG, Ogilvie range; NT-N, Northwest Territories (north); NT-S, Northwest Territories (south); PY, Pelly range; SW, Southwest Yukon; BC, British Columbia. Sample region identification in parentheses represent codes shown in Worley *et al.* (2004; Fig. 4). Values in the table represent the number of alleles found within each sample region

	Sample region							
Allele	NA (A)	CA (B)	OG (C)	NT-N (D)	NT-S (E)	PY (F)	SW (G)	BC (H)
*DRB*1*		1	1	3		1		1
*DRB*2*	2							
*DRB*3*	2							
*DRB*4*						2	1	3
*DRB*5*		2	1				1	
*DRB*6*	2							
*DRB*7*		2						1
*DRB*8*				1	6	1		
*DRB*9*	1							
*DRB*10*		2						
*DRB*11*	1							
*DRB*12*	1							
*DRB*13*	1							
*DRB*14*		1						
*DRB*15*				1				
*DRB*16*				1				
*DRB*17*								1
*IFNG*1*	8							
*IFNG*2*	20	4			4	1	4	6
*IFNG*3*	2	8	2	4	2	3	2	2
*NRAMP*1*	10	10	8	6	8	2	5	4
*NRAMP*2*						8	2	15
*NRAMP*3*						2	1	5

mutations was even greater in the putative protein binding region in comparison to codons not involved in protein binding.

We observed some geographic structure in the distribution of sequence alleles (Table 4). Seven unique alleles were observed in the five northern Alaska samples. Of the remaining 10 alleles, two were unique to central Alaska, two to the Mackenzie ranges of the Northern Territories

and one to British Columbia. The remaining five alleles were widely distributed across the species range.

Interferon gamma. The combined 1256-bp amplified regions of thinhorn IFNG contained only two polymorphic sites: a rare synonymous substitution in exon IV restricted to northern Alaska and a polymorphism in intron 3 common throughout sample areas (Table 4). The fixed sequence that includes exons II and III has been deposited in GenBank under accession number AJ920413, while alleles of the intron 3–exon IV region were deposited under AJ920414–AJ920416. The common coding sequence is identical to that of a domestic sheep haplotype (GenBank Accession number X52640) whereas the northern Alaskan nucleotide sequence variant is novel. We found no evidence of geographic structure to the IFNG allele distribution (Table 4).

NRAMP. The thinhorn NRAMP exonic sequence is fixed and differs from that of domestic sheep at one synonymous nucleotide position in exon VII. There are four intronic polymorphic base positions resulting in three haplotypes. NRAMP alleles have been deposited in GenBank under the accession numbers AJ920417–AJ920419. There appears to be some evidence of population structure, with greater diversity present in the southern species range. Alaskan, northern Yukon and NWT samples shared the same fixed sequence while all three haplotypes were found in the southern Yukon and BC (Table 4).

Discussion

Of the recent studies identifying adaptive variation in natural populations, most have concentrated on sequence variation (Gutierrez-Espeleta *et al*. 2001; Landry & Bernatchez 2001; Huang & Yu 2003; Aguilar *et al*. 2004; Jarvi *et al*. 2004) and within-population allele frequencies (Paterson 1998). Studies of selection within a single population do not face some of the confounding effects of population genetic structure. Here we tested for selection at genes involved in thinhorn immune function in a structured network of subpopulations. We aimed to account for demographic history by comparing levels of genetic variation at linked microsatellites to a background of genetic structure empirically estimated from presumably neutral markers. We found widespread deviation from Hardy–Weinberg equilibrium and significant evidence of population genetic differentiation at immune linked microsatellite loci, a pattern that closely mirrors that found using nonlinked and presumably neutral loci. If we had conducted these analyses without reference to a background level of population structure, standard tests of neutrality would have indicated balancing selection at all immune regions under study. However, we found no evidence of the actions of current adaptive variation at any region after accounting for the level of population substructure. This includes the MHC, despite convincing evidence to the contrary from sequence data.

Evidence of non-neutral variation within sample areas

In common with most mammals (Greenwood 1980), thinhorn sheep exhibit strong female natal philopatry (Festa-Bianchet 1991). Microsatellite analyses further suggest low levels of gene flow and widespread Wahlund effects at the level of the sample area (Worley *et al*. 2004). We therefore examined diversity within sample areas by comparing F_{IS} between linked and nonlinked loci. At first glance, there did appear to be reduced F_{IS} for MHC linked loci compared to the mean for nonlinked loci (Table 1). However, when viewed against the distribution of estimates from individual loci (Fig. 2A), there is no evidence for deviation from neutrality at any linked locus.

Watterson's tests indicate deviation from neutrality at microsatellites linked to all three immune regions that are consistent with expectations of balancing selection (Table 2). However, evidence for selection only becomes widely significant when the global statistic Fnd_A is used in preference to the traditional sample area approach using F_A. Most previous studies reporting evidence of selection from Watterson's tests are limited to human populations. The few exceptions have concentrated on locating balancing selection within the MHC region using both sequence (Landry & Bernatchez 2001; Hambuch & Lacey 2002) and linked microsatellite alleles (Paterson 1998; Huang & Yu 2003). Of these, only Huang and Yu's study made comparisons to results from nonlinked loci, where in common with thinhorn sheep, the authors reported significant evidence of balancing selection. These results highlight the importance of characterizing the magnitude of neutral variation before trying to infer selection. It is clear that in some cases balancing selection can be inferred incorrectly where interpretations of non-neutrality are made against nonrepresentative background variation.

The identification of outlying loci, those that are presumably affected by selection, remains highly subjective. This issue is further complicated by underlying correlations between allelic diversity, and the probability of outlying a general trend, such as those present in Fnd_A. The relationship between Fnd_A and allelic diversity is perhaps intuitive, as the standardized Watterson's statistic Fnd_A is calculated using locus variation (see Materials and methods). Underlying correlations could lead to problems in accurately identifying selection in comparative studies of neutral and linked loci (such is the case here) when there is no overlap in diversity between the chosen neutral and linked loci. In these cases there may be an inherent bias in the incorrect reporting of selection.

Evidence of non-neutral variation from comparisons between sample areas

Balancing selection can lead to a reduction in genetic differentiation between populations (Fay & Wu 2000; Schierup *et al.* 2000). Compared to the overall level of genetic differentiation at nonlinked loci, F_{ST} was lower in the class II MHC linked marker OLADRBps and the IFNG locus KP6 (Table 1), but higher in the MHC class I linked marker oMHC1. The apparent difference between MHC linked microsatellites may be expected due to their association with different regions of the MHC. If significant this may reflect reduced levels of selection in class I MHC genes relative to those from the class II region. However, there was no overall significant difference between levels of F_{ST} at nonlinked and linked loci and therefore a lack of evidence for selection.

Unlike a previous study (Paetkau *et al*. 1997), we found no evidence for a negative relationship between genetic distance (F_{ST}) and locus diversity. Values of F_{ST} are most affected by extreme low levels of diversity, a category that none of our markers fall into. There does however, remain some variation in relative differentiation between nonlinked loci (Fig. 2C). It would be extremely difficult to recognize any putatively selected loci showing only slight deviations from neutrality against such a background of variability. In many cases where outlying loci have been identified, much higher levels of differentiation were present than those expected under neutrality, resulting in relatively easy identification of loci under selection. In this study any overall putative evidence of balancing selection between sample areas is lost upon comparison with individual nonlinked loci. This reflects the pattern present in several other mammalian species where little evidence of selection has been found from comparisons of between population diversity (Boyce *et al*. 1997; Bernatchez & Landry 2003).

Regional adaptive variation can leave signals in isolation-by-distance relationships (Storz & Dubach 2004). The presence of significant isolation by distance at all thinhorn loci irrespective of immune linkage (Fig. 3) again reflects the predominance of nonselective forces over selection in maintaining genetic variation at all three immune regions.

Sequence variation and congruence with microsatellite variation

The major histocompatibility complex. The allelic diversity present in the thinhorn DRB gene is of a similar magnitude to the bighorn (Gutierrez-Espeleta *et al*. 2001) and domestic sheep (Schwaiger *et al*. 1994). In common with many other studies of the MHC, the null hypothesis of neutrality was rejected at the DRB locus (Table 3, Fig. 4). Due to the rejection of neutrality at DRB together with significant linkage disequilibrium within the MHC (Fig. 1), we might have also expected to find evidence of balancing selection in the class II linked microsatellite marker OLADRBps. The correlation coefficients associated with linkage disequilibrium between MHC loci are moderate. As a result of this imperfect linkage, the effects of balancing selection on the DRB locus do not extend to other MHC loci.

An alternative explanation for the lack of congruence between sequence and microsatellite alleles is that evidence of selection from DRB sequences may not reflect current processes, but result from historical selection. Given the similar levels of DRB diversity between thinhorn and bighorn, species with very different disease histories, there would appear to be no correlation between current DRB diversity and disease resistance. This is in agreement with the hypothesis of ancient rather than present-day selection. Under these conditions, variation in the quickly evolving linked microsatellites is the result of present neutral processes and therefore mirrors the characteristics of nonlinked markers. In addition, simulation studies have shown that tests of selection cannot distinguish between neutrality and balancing selection in cases where the selection coefficient is low with respect to migration rate (Beaumont & Balding 2004). Although thinhorn exhibit low rates of migration between populations, the presence of several biological subpopulations within each sample area effectively increases the migration rate. This could lead to the lack of evidence of balancing selection present in MHC linked microsatellites.

We did find evidence of population genetic structure in DRB sequence alleles (Table 4). For example, alleles present in BC are often seen in the neighbouring region of southern Yukon (PY). Evidence of allele sharing between samples from adjacent regions compares well with patterns observed from neutral DNA (Worley *et al*. 2004). A larger magnitude of allelic diversity was present within the more isolated populations of the far north of Alaska. We have no data to compare the magnitude of allelic diversity to incidences of disease, but would hypothesize that there is no more disease present in this region than others across the species range. In addition, all alleles present in northern Alaska are absent in all other regions. This may well reflect the geographic isolation of sheep from this region, preventing gene flow to other mountain ranges. Low levels of allelic diversity, such as that observed in the southern ranges of the Mackenzie Mountains (Table 4; NT-S) could reflect population bottleneck. Indeed, this region has been identified a region of putative rapid population growth from analyses of the mitochondrial control region (K. Worley, unpublished data). However, the DRB-allele-rich region of northern Alaska also showed some evidence of rapid growth from mtDNA.

Interferon gamma. The nucleotide diversity of IFNG is the lowest of the three gene regions, with only two

polymorphic bases along the sequenced 1256 bp. Given that the polymorphic microsatellite provides no evidence to discount the null model of neutrality, we can conclude that sequence variation in thinhorn IFNG reflects background rather than adaptive selection. In addition there does not appear to be any population structure within IFNG. The two common alleles were found throughout the species' range in approximately equal numbers. The existence of a rare allele in northern Alaska (as with novel DRB alleles) reflects the genetic isolation of sheep in this region.

NRAMP. This study represents the first work to test for evidence of selection at the NRAMP gene. Homologous intronic bovid sequences revealed 13 polymorphic sites (Ables *et al*. 2002) compared to the four present within the thinhorn. The greatest number of polymorphisms from all bovine species and the thinhorn can be found in intron 5. This is also the case in other mammals, such as the rhesus macaque where intronic polymorphisms can be correlated to TB resistance (Deinard *et al*. 2002). A relationship between intronic variation and disease raises a potentially interesting point in the thinhorn. We discovered increased NRAMP intronic diversity in the south of the species' range (Table 4). It is in these regions that sheep habitat is most influenced by anthropogenic factors. The low levels of neutral microsatellite diversity in BC (Worley *et al*. 2004) are mirrored in this study by a reduced level of heterozygosity at OVINRAO1 in southern sample areas and by evidence of a population bottleneck from mtDNA (K. Worley, unpublished data). It remains to be tested whether increased intronic variation within the thinhorn NRAMP, in opposition to the decrease in variability in other regions, is related to pathogen resistance.

Conclusions

Examining evidence of selection in a substructured population is always problematic. Our microsatellite data from MHC, IFNG and NRAMP show empirically the extent of these difficulties. Without prior knowledge of neutral variation traditional tests of neutrality suggest the presence of balancing selection in all regions under study. While this is clearly the case within the MHC (as indicated in DRB sequences), linked microsatellite data alone are misleading. After accounting for the effects of population structure all tests of selection are rejected. This is not surprising as the approach of using microsatellites to identify regions of selection has low statistical power. There remain difficulties in the appropriate statistical analyses of data such as we have in this study, leading to highly conservative and often subjective tests which could result in the loss of evidence of weak selection.

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This study forms part of Kirsty Worley's PhD thesis, supervised by Dave Coltman, on evolutionary genetics neutral and immune linked loci in thinhorn sheep. Dave Coltman is an associate professor at the University of Alberta who is interested in the evolutionary genetics of mountain ungulates and other handsome critters. Jean Carey is the Yukon's sheep and goat management biologist. Alasdair Veitch is a senior wildlife biologist with the Government of the Northwest Territories and is responsible for research and management on a wide range of species; primarily ungulates, carnivores, and birds.
