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Source: Wildlife Biology, 9(4) : 251-259

Published By: Nordic Board for Wildlife Research

URL: <https://doi.org/10.2981/wlb.2003.012>

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Major histocompatibility complex B-LB gene variation in red grouse *Lagopus lagopus scoticus*

Stuart B. Piertney

Piertney, S.B. 2003: Major histocompatibility complex B-LB gene variation in red grouse *Lagopus lagopus scoticus*. - Wildl. Biol. 9: 251-259.

Major histocompatibility complex (MHC) variation at the class II β (B-LB) gene was surveyed both within and among populations of red grouse *Lagopus lagopus scoticus* from northeastern Scotland. Variation among individuals was assayed by direct DNA sequencing, with 13 unique alleles being resolved among 10 unrelated individuals. All nucleotide substitutions were non-synonymous (amino acid changing) substitutions. Variation among five populations was screened using single stranded conformational polymorphism (SSCP). Twelve B-LB alleles were resolved, with low levels of genetic structure between the populations. This is in contrast to the considerable genetic differentiation observed among the same populations determined from microsatellite DNA analysis. Both the patterns of nucleotide substitution within individuals, and the discrepancy between MHC and microsatellite derived patterns of population genetic structure highlight that balancing selection is acting to maintain variation in the grouse MHC. The significance of MHC dynamics and diversity is discussed in the context of grouse ecology and conservation.

Key words: conservation, grouse, *Lagopus*, MHC, microsatellite, selection

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The major histocompatibility complex (MHC) is fundamental to the vertebrate immune system. The MHC is a multigene family whose products are recognised as key molecules in binding foreign peptides for presentation to T-cells and subsequent initiation of an immune response (Klein 1986). An exceptional feature of the MHC is the remarkable level of allelic variability observed in regions responsible for binding antigen. There is considerable evidence that these high levels of variation are maintained by balancing natural selection: 1) the rate of non-synonymous (amino acid changing) nucleotide substitutions in the peptide binding region exceeds the rate of synonymous (silent) substitutions (Hughes & Nei 1992); 2) MHC gene phylogenies pre-date the species phylogenies from which they are derived (Klein, Satta, Ohuigin & Takahata 1993); and 3) the distribution of allele frequencies is more platykurtic than would be expected under neutral theory (Hedrick & Thompson 1983). There is, however, little consensus of the precise mechanisms underlying this balancing

selection (Apanius, Penn, Slev, Ruff & Potts 1997). There are generally two schools of thought. The first states that MHC polymorphism is a result of selection for new MHC molecules in response to a large and ever-evolving group of pathogens and parasites (Doherty & Zinkernagel 1975, Hedrick & Kim 1998, Hamilton & Zuk 1982). The second suggests that high polymorphism is maintained by reproductive mechanisms such as mate choice, selective abortion and selective fertilisation (Potts, Manning & Wakeland 1991, 1994, Wade-kind, Chapuisat, Macas & Rulicke 1996).

An intimate association between MHC polymorphism, resistance to parasites and mate choice, means that MHC genes may play a role in a range of phenomena that have traditionally been the domain of evolutionary biologists and behavioural ecologists (Edwards, Grahn & Potts 1995), affecting individual and population-level responses to parasite burden, behavioural interaction between individuals, levels of inbreeding and the demographics of natural populations. However, these asso-

ciations between MHC genotype and individual phenotype have primarily been observed in a small number of model species for which we have sufficient information on the genetic architecture of the MHC to properly assess diversity and facilitate appropriate analyses. A major challenge now is to extend these studies to include non-model species in natural habitats.

The main avian MHC model is the chicken *Gallus gallus domesticus* (Kaufman, Milne, Göbel, Walker, Jacob, Auffray, Zoorob & Beck 1999), which provides a useful template for understanding the genetic architecture of other galliform bird species (see Fig. 1). The chicken genome is parcelled into five pairs of large chromosomes and 34 pairs of microchromosomes, and the MHC is on the 16th chromosome. Two regions encoding MHC genes have been characterised, the B-locus (or B-F/B-L region) and the Y-locus. The B-F/B-L region contains highly polymorphic class I (termed B-F) and class II (termed B-L) genes, which encode classical MHC molecules. There are a number of differences between the chicken and the mammalian MHC, primarily in terms of size, gene order and genic complexity. The B-F/B-L region is relatively small (approximately 92 kilobase pairs), with small introns and intergenic regions. Also, whilst the mammalian MHC encodes for multi-gene families of both class I and class II molecules, the chicken B-F/B-L codes for only two class I (B-F) and two class II (B-L) molecules, of which only one of each is polymorphic and expressed at high levels (Kaufman & Solomonsen 1997).

B-L molecules are found on antigen presenting cells and present exogenous antigens to CD4+ T-helper cells. They are composed of two chains, α and β , which are encoded by different genes but combine to form the peptide binding region of the mature molecule. The second exon of the β -encoding gene (B-LB) has been shown to contain a number of highly polymorphic subdomains that diversify by point mutations and gene conversion (She, Boehme, Wang, Bonhomme, Wakeland 1990), and, in chicken, have the highest ratio of non-synonymous:synonymous nucleotide substitution.

In the present study, I characterise B-LB gene diversity in red grouse *Lagopus lagopus scoticus*. Red grouse represent an intuitively attractive species for understanding MHC dynamics and evolution in the natural environment as the demography and dynamics of grouse population has been shown to be influenced both by individual interaction (MacColl, Piertney, Moss & Lambin 2000) and parasite prevalence (Hudson, Dobson & Newborn 1998). As previously highlighted, both are considered as potential processes that may affect MHC diversity. A necessary prerequisite for understanding the

role of parasites and mating behaviour in shaping MHC diversity is to confirm that the patterns of polymorphism are indeed consistent with the effects of selection. Here I examine the levels of balancing selection in the peptide binding regions using a dual approach. Firstly, by determining the relative frequencies of synonymous and non-synonymous nucleotide substitutions within individuals. Secondly, by determining levels of population differentiation calculated from MHC loci among populations previously shown to be diverged by microsatellite DNA markers (Piertney & Dallas 1997, Piertney, MacColl, Bacon & Dallas 1998, Piertney, MacColl, Lambin, Moss & Dallas 1999). Given that patterns of gene flow and stochastic processes such as random genetic drift are predicted to affect neutral loci equally, but selection is more likely to be locus specific (Lewontin & Krakauer 1973), any discrepancy between neutral markers and putative selected loci is evidence that selection is actually operating (Landry & Bernatchez 2001).

Material and methods

Direct DNA sequencing of the B-LB gene

Total genomic DNA was extracted from 10 unrelated grouse from Glas Choille, northeastern Scotland (57°07'N, 3°19'W) using sampling and extraction procedures outlined in Piertney et al. (1999). The second exon, the 3' portion of the third exon, and the intervening intron of the B-LB gene (Fig. 1) were PCR amplified as a single product with the primers Ex2a (5'-GAGT-GCCACTACCTGAACGGCACCGAGCGG-3') and Ex3b (5'-CGTCACGTAGCACGCCAGACGGTC-3'; Zoorob, Bernot, Renoir, Choukri, Auffray 1993) in 50 μ l reactions using an MJ Research PTC-100 thermal cycler. Each reaction mix contained 25 ng of template DNA, 2.5 mM MgCl₂, 75 mM Tris-HCl (pH 9.0), 20 mM (NH₄)₂SO₄, 0.01% (v/v) Tween-20, 0.2 mM of each nucleotide, 5 pmoles of each primer and 0.5 units of a proof-reading Taq polymerase (Bio-X-Act; Bioline Ltd). PCR profiles commenced with an initial denaturation step of three minutes at 90°C, then 30 cycles of 30 seconds denaturation at 92°C, 30 seconds annealing at 65°C and 30 seconds extension at 72°C. An extra two minutes extension step at 72°C followed the final cycle.

All PCR reactions were then incubated at 72°C for 15 minutes in the presence of 1 unit of a non-proof reading Taq polymerase (BioTaq; Bioline Ltd) to add a single deoxyadenosine overhang to the 3' end of all fragments. PCR products were then cloned into TA cloning vector (Invitrogen Ltd) according to the manufacturer's instructions. Vector molecules were heat transformed into

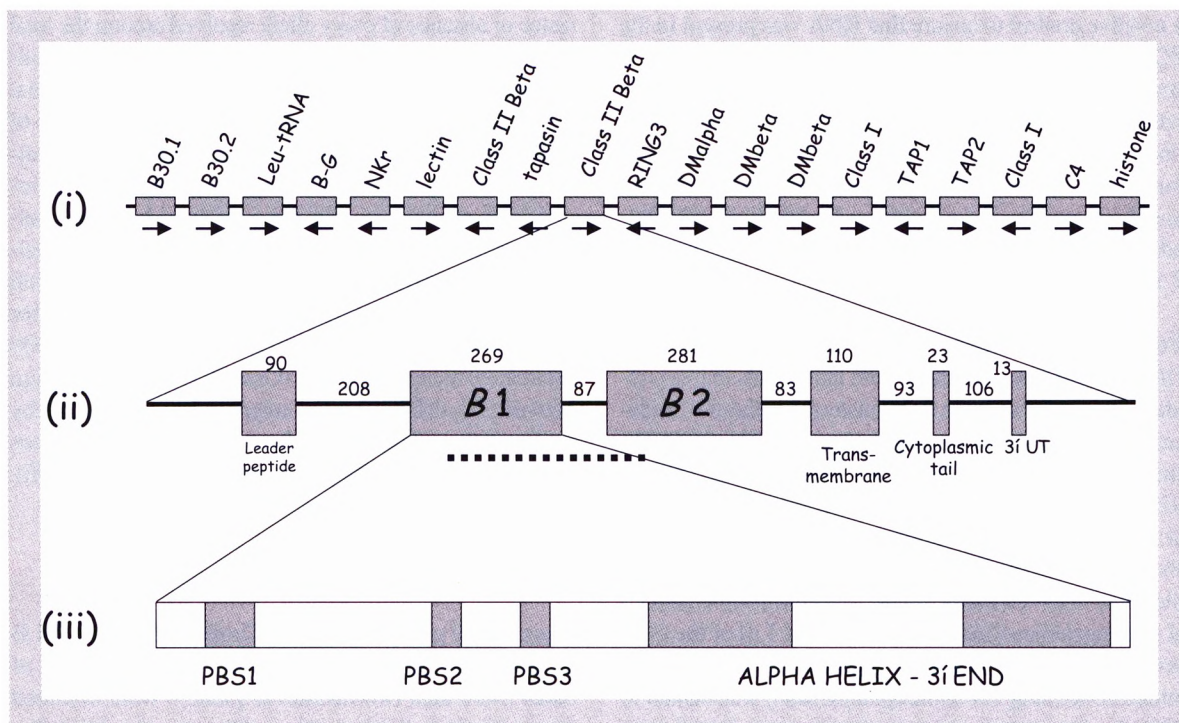


Figure 1. Schematic representation of (i) the B-F/B-L region, (ii) the B-LB gene, and (iii) the second exon of the B-LB gene in chicken. In (i) the arrows represent transcriptional orientation of each of the constituent genes. In (ii) the numbers represent the sizes (in base pairs) of each intron and exon. The dashed line highlights the region amplified by the Ex2a and Ex3b primers. In (iii) the dotted and non-dotted areas represent the polymorphic and conserved subdomains, respectively.

INV α F' One-Shot™ *Escherichia coli*, then grown overnight at 37°C on LB medium containing 50 μ g ml⁻¹ ampicillin and surface-streaked with 40 μ g of 40 mg ml⁻¹ X-Gal. Five white colonies were picked at random for each original PCR reaction, then grown overnight at 37°C in 5 ml of LB broth containing 50 μ g ml⁻¹ ampicillin. The plasmids were subsequently purified using a Qiaprep Spin Miniprep Kit (Qiagen Ltd) according to the manufacturer's instructions, then sequenced using an ABI377 automated sequencer (Perkin Elmer).

Unique sequences were aligned using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997), with confirmation by eye. Sequences were annotated to delimit introns, exons and reading frames according to Brown, Jardetzky, Gorga, Stern, Urban, Strominger & Wiley (1993) and Edwards et al. (1995). The genetic relationships among the unique MHC sequences were ascertained by constructing a phylogenetic tree using maximum parsimony (branch and bound search) with Paup*v10b (Swofford 1993). The tree was rooted using chicken (B12 haplotype; Zoorob et al. 1993).

The relative number of synonymous (dS) and non-synonymous (dN) substitutions per site among sequences at the putative peptide binding region and across the en-

tire second exon were determined according to Nei & Jobori (1986) using a Jukes & Cantor (1969) correction for multiple substitutions using MEGA (version 2.1; Kumar, Tamura, Jakobsen & Nei 2000). To test whether selection was operating, a Z-test was performed among all sequences by comparing dN and dS, as detailed in Nei & Kumar (2000).

Expression profiling

To ensure that the primers Ex2a and Ex3b amplified expressed MHC molecules, semi-quantitative reverse transcription PCR (rtPCR) was performed. Two grouse were sacrificed (under licence to Redpath & Hudson) and samples of testes, muscle, liver, heart, Bursa of Fabricius, pancreas, spleen, kidney, caecum and brain immediately snap-frozen in liquid nitrogen. Total RNA was extracted using an Rneasy Mini Kit (Qiagen Ltd) according to the manufacturer's instructions. For each tissue type, paired rtPCR amplifications were performed using: 1) primers Ex2a and Ex3b, and 2) β -actin F 5'-TACCACAATGTACCCTGGC-3' and β -actin R 5'-CTCGTCTTGTGTTTATGCGC-3' in separate 50 μ l reactions using Ready-to-go RT-PCR Beads (Amersham Pharmacia Biotech). The β -actin primers were used as

a positive control to ensure that RNA was present in the PCR reactions. PCR profiles commenced with a 95°C denaturing step, then 23 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 30 seconds extension at 72°C. Next, 5 µl aliquots were run-out on 1% agarose gels containing ethidium bromide, and the intensity of the bands under UV light calculated using Gelworks 1D Advanced software (version 2.5.1; UVP Ltd).

Population structure

DNA obtained from feathers from individuals in five populations of grouse from northeastern Scotland (Candacraig 57°05'N, 03°04'W; Mar Lodge 56°59'N, 03°28'W; Invercauld 57°00'N, 03°20'W; Tillypronie 57°10'N, 02°54'W; Allargue 57°09'N, 03°20'W; see Piernthey et al. 1998) was PCR amplified using primers Ex2a and Ex3b as described above. Polymorphism among the samples was resolved using single stranded conformational polymorphism (SSCP). A mixture of 5 µl of the PCR product and 5 µl of a denaturing loading buffer (formamide containing 1% bromophenol blue) was heated to 95°C for one minute, and then placed on ice. Of this mix, 5 µl was loaded on a non-denaturing acrylamide gel (MDE gel mix; BioWhittaker Molecular Applications Ltd), and run at 20 W for 15 hours at 4°C. Gels were then silver-stained using a Sterling Silver Staining Kit (National Diagnostics) according to the manufacturer's instructions. Individual profiles were scored by the pre-

sence or absence of every allele resolved among the total sample, and allele frequencies and F_{ST} analogues calculated using Popgene 1.32 (<http://www.ualberta.ca/~fyeh/index.htm>). F_{ST} values obtained from analysis of MHC variability were compared to the equivalent values previously obtained from genotyping the same individuals at seven tri- and tetra-nucleotide microsatellite loci (see Piernthey et al. 1998). MHC derived F_{ST} values were considered significantly different if they fell outside the 95% confidence intervals calculated for microsatellite F_{ST} by bootstrapping over loci (1,000 replicates) using F_{STAT} v2.8 (Goudet 1998). Correlation between F_{ST}-MHC and F_{ST}-microsatellite was tested by performing a Mantel test between the two matrices using GENETPOP software (Raymond & Rousset 1995).

Results

From the 10 grouse from Glas Choille, 13 unique B-LB sequences were obtained (Table 1). In a number of cases more than two distinct sequences were obtained from a single individual. Given that a proof-reading Taq polymerase was used in the PCR, and as such any polymorphism due to Taq error can be discounted, the Ex2a and Ex3b primers must be amplifying more than a single locus in the grouse MHC.

The relationships among the sequences are given as a 50% majority rule consensus tree (based on six equal-

Table 1. Alignment of 13 MHC unique sequences obtained using the primers Ex2a and Ex3b. Sequences are given in relation to Lasc-1, where a dot represents identity. Exons are delimited by the black line and separated into triplet codons. The codons delimited by grey shading represent the locations of the polymorphic subdomains (BS2, BS3 and α-helix from 5' to 3', respectively) and highlighted in bold are the 14 antigen binding site codons (as determined by Brown et al. 1993).

Lasc-1	GAG	TGC	CAC	TAC	CTG	AAC	GGC	ACC	GAG	CGG	ACG	AGG	TTC	GTG	GTG	AGG	CAC	GTC	TAC	AAC	60
Lasc-2	C..	
Lasc-3	C..	
Lasc-4	C..	C..	...	
Lasc-5	C..	
Lasc-6	C..	
Lasc-7	C..	
Lasc-8	C..	
Lasc-9	C..	A..	
Lasc-10T.	C..	
Lasc-11	C..	
Lasc-12T.	C..	
Lasc-13	C..	
Lasc-1	CGG	CAG	CAG	TAC	GTG	CAC	TTC	GAC	AGC	GAT	GTC	GGT	CTC	TTC	GTG	GCC	GAC	GCG	GTG	CTG	120
Lasc-2	
Lasc-3G.	
Lasc-4	
Lasc-5	
Lasc-6	
Lasc-7	
Lasc-8	
Lasc-9	
Lasc-10	
Lasc-11	
Lasc-12	
Lasc-13	

[illegible]

ly parsimonious trees) in Figure 2. The topology highlights some grouping of sequences into clades, with sequences 11 and 13 appearing most distinct.

The rate of non-synonymous nucleotide substitution was greater than the level of synonymous substitution both within the second exon as a whole ($dN = 0.009 \pm$

0.004; $dS = 0.00 \pm 0.00$) and among the nucleotides deemed to constitute the peptide binding site ($dN = 0.010 \pm 0.007$; $dS = 0.00 \pm 0.00$). The null hypothesis of neutrality ($dN = dS$) of the sequences can be rejected ($P < 0.01$).

At least one of the MHC genes amplified by Ex2a and

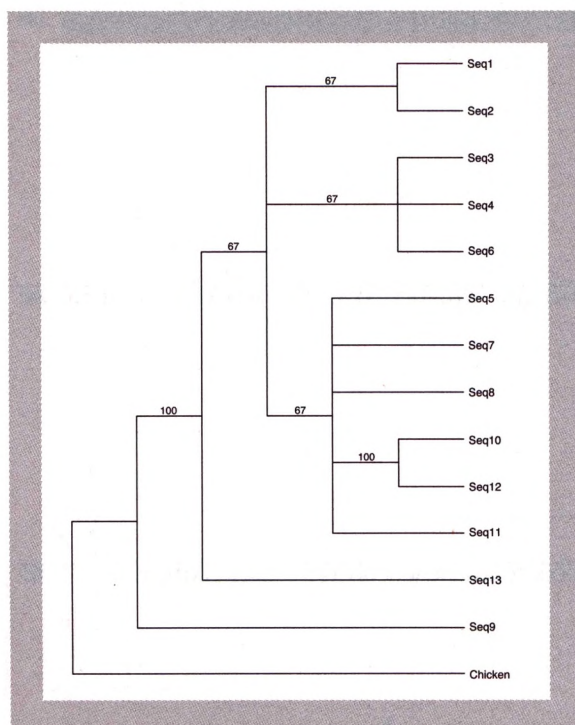


Figure 2. The 50% majority rule consensus tree of the six most parsimonious trees showing the relationship between the 13 unique B-LB DNA sequences obtained using primers Ex2a and Ex3b. The values on branches represent the proportion of trees in which the taxa above the indicated node are together.

Ex3b is expressed. MHC and β -actin amplifications are presented for each tissue type in alternate lanes in Figure 3. Given that β -actin is constitutively expressed by tissues, one of three patterns are expected for such analysis. If neither a β -actin nor an MHC band is present then the rtPCR has failed, probably due to RNA degradation prior to extraction from tissues. In this case no conclusion about MHC expression can be made. If a β -actin band is present, but no MHC band, then it can be concluded that the MHC was not being expressed in that tissue at the time of sampling. If both a β -actin and an MHC band is present then it can be concluded that MHC expression is occurring, and comparison of the ratio of band intensity between β -actin and MHC among

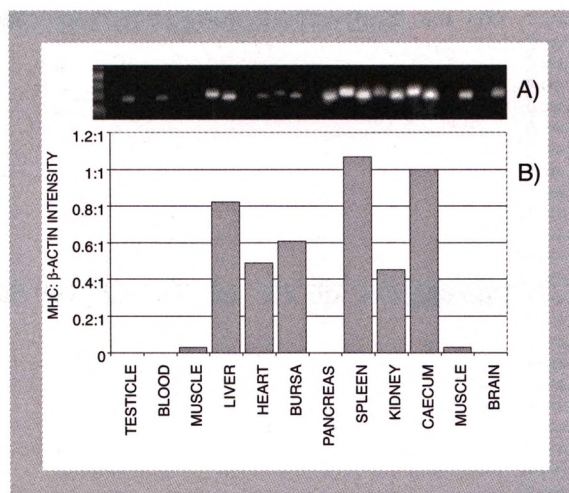


Figure 3. Agarose gel showing expression profiles of B-LB (320 b.p.) and β -actin (290 b.p.) for 12 different tissues from red grouse (A). The far-left lane is a marker of 200, 300, 400, 500 and 600 b.p. In (B), the ratios of intensity between the β -LB and β -actin bands for each of the 12 different tissues are shown.

tissues can provide indications of the relative levels of MHC expression. In this case, Figure 3 highlights that MHC expression is absent (or is sufficiently low as to be undetectable) in the testes, the blood and the brain, but occurring in the other tissues. The highest levels of expression are found in the spleen, the caecum and the liver (the MHC fragment has 105, 100 and 82% intensity of the β -actin fragment, respectively).

SSCP analysis resolved 12 different MHC alleles among the five populations of red grouse surveyed. Allele frequencies for each population are given in Table 2. Pairwise population values of F_{ST} derived from the SSCP analysis are plotted against equivalent F_{ST} values obtained microsatellite DNA analysis in Figure 4. The F_{ST} values for microsatellites are, in every case, higher than those derived from the MHC analysis. In 7/10 (70%) cases the microsatellite derived F_{ST} was significantly greater than the MHC derived F_{ST} . The two F_{ST} estimates were not significantly correlated (Mantel test: $P > 0.05$).

Table 2. Allele frequencies of 12 resolved MHC B-LB alleles among samples of red grouse from five populations in northeastern Scotland. Sample sizes (N) are given in parentheses.

Population	Lasc-a	Lasc-b	Lasc-c	Lasc-d	Lasc-e	Lasc-f	Lasc-g	Lasc-h	Lasc-i	Lasc-j	Lasc-k	Lasc-l
Candacraig (31)	0.14	0.16	0.26	-	0.12	0.07	0.01	-	0.09	0.12	0.03	-
Mar Lodge (30)	0.20	0.11	0.19	0.11	0.03	0.08	-	-	0.16	0.13	-	-
Invercauld (28)	0.14	0.21	0.23	-	0.18	-	-	-	0.04	0.18	-	0.04
Tillypronie (24)	0.14	-	0.21	-	0.13	0.17	-	-	0.14	0.17	0.03	-
Allargue (28)	0.13	0.13	0.17	0.01	0.12	0.12	0.04	0.01	0.07	0.12	0.06	0.01

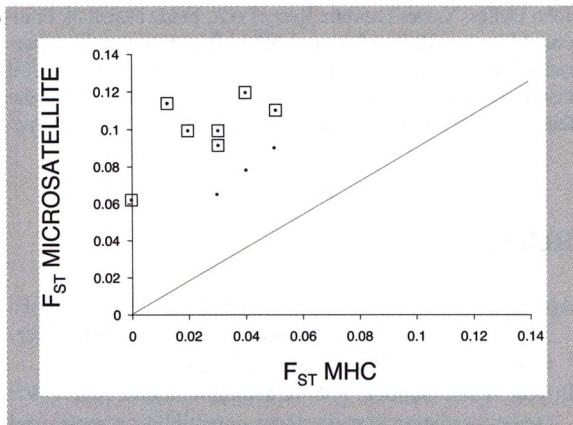


Figure 4. Correlation between pairwise genetic differentiation (F_{ST}) between five populations of red grouse measured from MHC and microsatellite loci. The points delimited by squares are the cases where microsatellite F_{ST} is significantly greater than MHC F_{ST} . The dotted line indicates MHC F_{ST} = microsatellite F_{ST} .

Discussion

This study has provided the first characterisation of the MHC in red grouse, and has highlighted that the levels of diversity and the strength of balancing selection are comparable with those observed in mammalian species.

The PCR primers utilised are amplifying at least two loci, of which at least one is expressed across a range of tissues. Several B-LB genes have been isolated and characterised in chicken, and mapped to the B-F/B-L region (see Fig. 1), the Y-locus (an unlinked region on the same chromosome encoding MHC genes), and from a third unknown location (Jacob, Milne, Beck & Kaufman 2000). The classical B-LB genes with high sequence polymorphism and high expression levels are those found in the B-F/B-L region, which would suggest that the primers utilised here are amplifying the B-F/B-L genes. That said however, if both of the B-F/B-L genes were heterozygous then SSCP would detect a maximum of four alleles. In a number of cases in the population level survey of MHC variation, more than four alleles were detected from a single individual, indicating that the Ex2a and Ex3b primers are also amplifying class II loci outwith the B-F/B-L region.

An inability to assign allelic or locus relationships among the sequences does not alter the conclusion that balancing selection is acting to maintain the observed levels of variation. A high ratio of non-synonymous to synonymous substitutions will be manifest in inter-locus as well as interallelic comparisons of MHC genes provided that the frequency of non-synonymous substitutions is not too high (Hughes & Nei 1989). Indeed,

inclusion of alleles from loci exposed to less selection will act to reduce the average non-synonymous:synonymous substitution ratio, and therefore underestimate the strength of selection at the B-F/B-L B-LB genes. That aside, a goal must be to design new primers that amplify the dominantly expressed B-LB gene that lies between the tapasin and RING 3 genes in the B-F/B-L region (Jacob et al. 2000) to compare orthologous sequences among individuals and more precisely measure the magnitude of this selection. A potential approach to this end would be to use long-PCR and site primers in the class II beta gene and a neighbouring gene, and then amplify across the intervening intron.

The major challenge now is to understand the processes that underpin the balancing selection that clearly operates at the grouse MHC. The two major factors thought to maintain diversity, antagonistic co-evolution between host and parasites, and behavioural interaction leading to disassortative mating, certainly play a fundamental role in shaping the ecology, demographics, dynamics and behaviour of red grouse (Hudson & Dobson 1989, 1997, Hudson et al. 1998, Moss, Watson, Trenholm & Parr 1993, MacColl et al. 2000), and so must represent potent selective forces in natural populations. Preliminary surveys (S. Pierniey, pers. obs.) indicate an excess of heterozygote MHC genotypes within populations, though it is as yet unclear whether this results from disassortative mating or a selective advantage of heterozygote genotypes to parasite insult. What is clear is that the detection of balancing selection in an oviparous species indicates that the postulated maternal-foetal interaction hypothesis, which suggests that MHC diversity is maintained by a lower frequency of spontaneous abortion between MHC dissimilar than between MHC similar mates, cannot totally explain all MHC diversity.

The signature of balancing selection detected from the patterns of nucleotide substitution within populations is mirrored by the discrepancies in the levels of genetic differentiation among populations calculated from MHC and microsatellite DNA. Previous studies have shown that grouse populations surveyed in northeastern Scotland show significant differences in microsatellite allele frequency (Pierniey et al. 1998), with a strong signature of isolation by distance. Such patterns were attributed to reduced gene flow among populations, which coupled with social structure (Pierniey et al. 1999) prevents the complete admixture of microsatellite alleles, even over short distances. In contrast, reduced differentiation at the MHC loci reflects balancing selection maintaining a platykurtic distribution of allele frequencies despite restricted gene flow. Other studies that have compared patterns of population differentiation

among MHC and other markers have not necessarily found the same pattern. Both in bighorn sheep *Ovis canadensis* (Boyce, Hedrick, Muggli-Cockett 1997) and in Atlantic salmon *Salmo salar* (Landry & Bernatchez 2001) F_{ST} from MHC and microsatellite markers were generally correlated, indicating that random drift was defining allele frequencies for both types of marker. Discrepancies between markers tended to occur across environmental gradients, indicating that the patterns of variation reflect adaptive differences between populations that are brought about by current or historic selection regimes. In the present study, the MHC markers did not highlight a complete lack of population differentiation among the populations, which may reflect either local differences in selective pressure, or indicate that stochastic processes are influencing MHC diversity.

Frequently, markers such as microsatellites are utilised to highlight populations with reduced genetic variability with an implicit assumption that a population depauperate in microsatellite variation also shows a proportionate reduction in the genetic variation associated with the quantitative traits that underpin reproductive fitness and adaptive potential (Reed & Frankham 2001). The differences in the patterns of genetic structure resolved from microsatellites and MHC markers, such as those seen here, highlight that direct comparison between markers is inappropriate. Indeed, it has been considered that populations with low MHC variation, especially endangered populations, are particularly susceptible to infectious diseases and parasites (Hedrick & Kim 1998). As such, the maintenance of MHC diversity is important for population persistence, and should be a priority in captive breeding programmes and management of endangered species (Hughes 1991). Moreover, identification of populations that have increased risk of extinction through reduced genetic variation and require management could be undertaken with markers such as the MHC. Progress in this direction will be hampered because of a limited knowledge of MHC structure and evolution in certain focal species, but advances such as degenerate or 'universal' PCR primers have been developed to amplify certain target MHC regions for subsequent analysis across a range of taxa (e.g. Edwards et al. 1995) will help. However, as highlighted by my study, some caution needs to be exercised and efforts made to ensure that the number of loci amplified, and their levels of expression, are ascertained.

Acknowledgements - this study continues a collaboration on grouse population genetics and dynamics between the Centre for Ecology and Hydrology at Banchory and the NERC-funded Molecular Genetics in Ecology Initiative at the University of Aberdeen. The input of Steve Albon, Philip Bacon,

John Dallas, Xavier Lambin, Kerry Lock, Freda Marshall, Francois Meugeot, Robert Moss and Paul Racey is greatly appreciated. Steve Redpath, Francois Meugeot and Andrew MacColl also provided samples. This study was carried out under the tenure of a NERC Advanced Postdoctoral Research Fellowship.

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