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Isolation and characterization of a MHC class II *DRB* locus in the European water vole (*Arvicola terrestris*)

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Abstract In so-called model species, such as human and mouse, genes of the major histocompatibility complex (MHC) are characterized by extremely high levels of polymorphism, and it is considered that such diversity is maintained by balancing selection. There is now a recognized need to expand studies into nonmodel species to examine whether high MHC diversity is mirrored in natural populations, and to determine the ecological, ethological, and evolutionary processes that underpin balancing selection. To address such issues, a necessary prerequisite is the ability to characterize diversity at a single, expressed, polymorphic MHC locus on which selection may be acting. Here, we provide the first description of allelic diversity at exon 2 of an MHC class II *DRB* locus in the European water vole (*Arvicola terrestris*), characterize variation across four natural populations, and test whether the patterns of variation are consistent with the effects of balancing selection. Using single-strand conformation polymorphism analysis and subsequent DNA sequencing of gel excisions, five *DRB* alleles were resolved, each with a unique amino acid sequence, among 100 individuals from four geographically distinct populations. Reverse transcription polymerase chain reaction confirmed that the alleles were products from an expressed locus. Intra-allelic amino acid differences were high (10.5–33.3%), and the nonsynonymous substitution rate exceeded the synonymous substitution rate for the functional peptide-binding region ($d_N:d_S=3.91$ and $P<0.005$). Phylogenetic comparison of resolved alleles with closely related homologues indicated that each allele represented a unique lineage preserved across speciation events. These results indicate that balancing selection has maintained diversity of *DRB* allelic lineages and amino acid function over evolu-

tionary time scales, but may be less effective at preserving alleles in contemporary populations where stochastic micro-evolutionary processes may dominate.

Keywords MHC class II · Polymorphism · Evolution · Balancing selection · *Arvicola*

Introduction

Genes of the major histocompatibility complex (MHC) encode proteins that primarily function to recognize and present foreign peptides to the vertebrate immune system (Klein 1986). MHC class II molecules are mainly expressed on specialized antigen-presenting cells and bind endogenously derived antigens that are subsequently presented to CD4⁺ T cells to initiate an immune response.

A prominent feature of the mammalian MHC is incredibly high genetic diversity, both in terms of number of alleles and nucleotide differences. In humans, *HLA-DRB* genes exhibit levels of both nucleotide and allelic polymorphism that place them among the most variable of all known loci (Garrigan and Hedrick 2003; Gaudieri et al. 2000). The function of MHC molecules in pathogen recognition has led to the consensus that balancing selection maintains the high levels of polymorphism at MHC loci. However, the exact nature of the selective processes acting on MHC loci remains largely unresolved (Bernatchez and Landry 2003). Both parasite-mediated and sexual selection have been implicated, and the challenge for empirical studies is to identify, understand, and disentangle the selective mechanisms that give rise to MHC diversity. Inbred or congenic strains of laboratory model species, primarily mice, have been used to identify explicit relationships between MHC alleles and a variety of parasite taxa, intraspecific strains (Apanius et al. 1997 and references therein), and patterns of MHC-based sexual selection (Egid and Brown 1989; Yamazaki et al. 1976). However, while model system studies have greatly enhanced our understanding of how selection can act upon MHC genes, the significance of these findings in a

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broader evolutionary context is not clear. From an evolutionary perspective, working with natural populations has the potential to enhance the understanding gained from laboratory studies by examining how ecological factors influence the dynamics of MHC evolution and how MHC variability affects individual fitness and population level processes (Piertney and Oliver 2006).

A prerequisite to studying the effects of selection on MHC diversity in natural populations is the ability to examine variation at individual, expressed, polymorphic loci (Edwards and Hedrick 1998). Isolation and characterization of individual genes has proven something of an impediment for several studies, which have then focused on effects of selection on patterns of diversity across multiple, simultaneously amplified loci. The potential occurrence of multiple pseudogenes can confound studies that then wish to compare allelic diversity with factors associated with fitness and reproductive success. Such problems dictate that species-specific characterization of MHC loci is required.

Here, we examine diversity at a single, expressed, polymorphic MHC class II *DRB* locus in the water vole (*Arvicola terrestris*) and test whether patterns of MHC variation are consistent with the effects of balancing selection. In northern Scotland, water vole populations approximate metapopulations, colonizing small “islands” of suitable habitat that are each prone to extinction and are linked by occasional dispersal (Lambin et al. 2004). Within population fragments, there is considerable variance in the prevalence and abundance of arthropod, helminth, and microbial parasites that will each interact with the MHC as immune insults. Given that these fragmented populations vary in size and parasite load, water voles represent an ideal model with which to examine the relative contributions of stochastic and deterministic processes in defining MHC diversity.

Materials and methods

To target maximum diversity, water voles ($n=100$) were trapped live across a broad geographic range in northern Scotland: one island population (Coiresa 56°8.6' N, 5°37.7' W) and three mainland metapopulations (Assynt 58°8' N, 5°1' W; Grampian 56°56' N, 3°27' W; and Ythan 57°23' N, 2°17' W). Twenty-five individuals were sampled per population. Ear punch samples were collected and stored in 70% ethanol. Genomic DNA was then extracted using the salt–chloroform method of Mullenbach et al. (1989).

Oligonucleotide primer sequences JS1 5'-AGTGT CATTCTACAACGGGACG-3' and JS2 5'-GATCCCG TAGTTGTGTCTGCA-3' (Schad et al. 2004) were used to amplify a 171-base pair fragment of the MHC class II *DRB* exon 2 that includes part of the functional peptide-binding region (PBR). These primers were originally designed for the lemur *Microcebus murinus* but were also used to amplify homologues in a number of rodent species (Froeschke and Sommer 2005; Harf and Sommer 2005; Meyer-Lucht and Sommer 2005). Polymerase chain

reactions (PCR) were performed in a total volume of 20 μ l, each consisting of 80 ng DNA, 1 \times PCR buffer, 2.5 mM MgCl₂, 2 mM deoxyribonucleotide triphosphate, 1 μ M each of forward and reverse primers, 1 \times High-Spec additive and 0.8 U of Bio-X-Act Short DNA Polymerase (Bioline). PCR conditions were optimized for maximum annealing temperature and minimum cycle number to reduce the production of erroneous PCR products (Zylstra et al. 1998). Thermocycling was carried out on a Hybaid PX2 thermocycler and comprised an initial denaturing step of 94°C for 3 min, followed by 23 cycles of 45 s of denaturing at 94°C, 45 s annealing at 61°C, and 60 s of extension at 72°C. A final 3-min extension at 72°C followed cycling.

Single-strand conformation polymorphism (SSCP) analysis was used to identify individual *DRB* alleles. Five microliters of each PCR reaction was added to 3 μ l of SSCP stop solution (Geneflow), heated to 95°C for 5 min, then immediately snap-cooled on ice. Five microliters of this mixture was loaded on a polyacrylamide gel mounted on a Gibco S2 sequencing gel apparatus. Gel composition was optimized for maximum band separation and resolution, and contained 35 ml SequaGel MD (National Diagnostics, Atlanta, USA), 4.2 ml 10 \times Tris–Borate–EDTA, 1 ml glycerol, and 30 ml H₂O. Running conditions for SSCP gels comprised 2 h at 860 V, followed by 18 h at 1 kV. SSCP patterns were visualized using Sterling Rapid Silver Stain Kit (National Diagnostics) according to the manufacturer's protocol. Standards for each unique band were used on each gel to ensure consistency in scoring between gels.

Individual bands were excised directly from SSCP gels using a scalpel and placed in 20 μ l of sterile water for 3 h. Fragments were reamplified by PCR using the same procedure as previously described, except that 5 μ l of dissolved fragment was used as a direct replacement for DNA, and the PCR comprised 33 cycles. PCR products were purified then sequenced bidirectionally using the original PCR primers on an ABI3700 automated DNA sequencer (Applied Biosystems, CA, USA) according to the manufacturer's instructions. To validate each allelic sequence, the above procedure was carried out for three separate PCR reactions per individual for at least three different individuals of the same putative allelic band.

To ensure that the *Arte-DRB* locus was expressed, reverse transcription PCR was performed on four individuals from which liver tissue was available. RNA was extracted using the Qiagen *Rneasy* kit according to the manufacturer's protocol. To remove contaminant genomic DNA, extracted RNA was treated with RQ-1 DNase (Promega, USA). First-strand cDNA synthesis was performed on the DNase-treated total RNA with Bioscript RNase Minus (Bioline) using an Oligo(dT)₁₈ primer with subsequent incubation at 42°C for 90 min. The final product was diluted to one part in three with analytical reagent grade water. PCR, SSCP, and sequencing of excised SSCP bands was then carried out for genomic DNA, except that 2 μ l of cDNA was used in place of genomic DNA in the initial PCR reaction. Multiple PCR reactions were carried out for each individual and 2 μ l of

the DNase-treated total RNA was used as a negative control against genomic DNA contamination for each cDNA PCR reaction.

Sequences were aligned using Clustal X (Thompson et al. 1997) with confirmation by eye. Mega 3.1 (Kumar et al. 2004) was used to calculate the relative rate of nonsynonymous (d_N) to synonymous (d_S) substitutions applying the method of Nei and Gojobori (1986) with Jukes–Cantor correction for multiple mutations at single sites. The probability that $d_N=d_S$ was determined using a Z-test (Nei and Kumar 2000). A National Center for Biotechnology Information BLAST search was carried out for all *Arte-DRB* sequences to confirm gene identity. The phylogenetic relationships among *Arte-DRB* sequences together with homologues from *Rattus norvegicus* (GenBank accession nos. AY626204 and AY626204) and the top four homologues obtained from a BLAST search (GenBank accession nos. AF516936, AJ490323, AY169006, and AY918086) were inferred using likelihood within phylogenetic analysis using parsimony (version 4.0b8; Swofford 1998). The program Modeltest (Posada and Crandall 1998) was applied to ascertain the optimal model of evolution from 56 different likelihood models using the Akaike's (1974) information criterion. This model was identified as base frequencies of $A=0.1836$, $C=0.2573$, $G=0.4078$, and $T=0.1513$; rate matrix of A to $C=3.3239$, A to $G=2.5749$, A to $T=9.5407$, C to $G=2.0262$, C to $T=2.5749$, and G to

$T=1.00$; and proportion of invariable sites (I)=0.4793. Confidence in the resultant topology was assessed with 1,000 bootstrap iterations (Felsenstein 1985). The tree was rooted through a *Homo sapiens* homologue (GenBank accession no. DQ141378).

Results

All individuals were successfully amplified by PCR. SSCP analysis of 100 individuals revealed 11 distinct patterns, incorporating five separate bands with one or two bands per individual. Sequencing of multiple representatives of each band confirmed five unique alleles (Fig. 1). These were labeled *Arte-DRB*01–05* (GenBank accession nos. DQ202212–DQ202216) according to the nomenclature of Klein et al. (1990). Alleles resolved from sequencing cDNA matched those from genomic DNA for those individuals where paired RNA and DNA samples were available. BLAST results showed close homology to rodent *DRB* alleles (range 91.2–95.3% nucleotide identity over the entire allele). The closest homology to a model species was found for *R. norvegicus RT1-Db* alleles (range 84.2–92.4%). The geographic distribution of alleles was variable; *Arte-DRB*01* was the only allele present in all four population samples; *Arte-DRB*02* occurred in the three mainland metapopulations, but was absent in the Coiresa

Fig. 1 Nucleotide and predicted amino acid sequences for the MHC class II *DRB* exon 2 alleles in *A. terrestris*. A dot indicates identity with the top sequence. Asterisks and numbers in bold identify sites in the putative peptide-binding region as predicted by Brown et al. (1993)

<i>Arte-DRB*01</i>	CAGCGCGT	GCGGTATCTAGAGAGACAGTTCTACAACCGGGAGGAGTACGTGCGCTTC
<i>Arte-DRB*02</i>CT.....T.CA.....T.....
<i>Arte-DRB*03</i>G.CC.....GTCA.....
<i>Arte-DRB*04</i>CT.....G.TC.....T.CA.....T.....
<i>Arte-DRB*05</i>G.CC.....GTCA.....C.....
PBR		* * * * * *
Position		26 28 30 32 37 38
<i>Arte-DRB*01</i>	Q R V R Y L E R Q F Y N R E E Y V R F	
<i>Arte-DRB*02</i> L . . . Y I F . . .	
<i>Arte-DRB*03</i> A . . V I	
<i>Arte-DRB*04</i> L . V . Y I F	
<i>Arte-DRB*05</i> A . V I A	
<i>Arte-DRB*01</i>	GACAGCGACGTGGGCGAGTTCGCGAGGTGACCGAGCAGGGGCGGGGCATAGCCGAG	
<i>Arte-DRB*02</i>CA.....C.....T.....
<i>Arte-DRB*03</i>C.....T.....CCGGAC.....
<i>Arte-DRB*04</i>C.....T.....CCGGAC.....A..
<i>Arte-DRB*05</i>A.....C.....T.....C..TC.....
PBR		* * * * * *
Position		47 56 58
<i>Arte-DRB*01</i>	D S D V G E F R E V T E Q G R G I A E	
<i>Arte-DRB*02</i> H . A . . . L	
<i>Arte-DRB*03</i> A L . . . P D	
<i>Arte-DRB*04</i> A L . . . P D . K	
<i>Arte-DRB*05</i> A L . . . R S	
<i>Arte-DRB*01</i>	AACTTGAACAGCCAGAAGGAGTCTCGAGCGGAAGCGGGCCGAGAAAGACCGGTG	
<i>Arte-DRB*02</i>C.....G.....AAC.G.....C.GTG.....
<i>Arte-DRB*03</i>	T...G.....CT..A.....C.GTG.....TAC
<i>Arte-DRB*04</i>	T...G.....CT..A.A...A.TT.....C.GTG.....
<i>Arte-DRB*05</i>	T...G.....A.....C.GTG.....TAC
PBR	* * * * * *	* * * *
Position	60 61 65 68 70 71 74 78	
<i>Arte-DRB*01</i>	N L N S Q K E L L E R K R A E K D T V	
<i>Arte-DRB*02</i>	. F . . R N R . . A V	
<i>Arte-DRB*03</i>	Y W D F M . Q . . . A V . . . Y	
<i>Arte-DRB*04</i>	Y . . . R . D F I . Q L . . A V	
<i>Arte-DRB*05</i>	Y W Q . . . A V . . . Y	

island population; *Arte-DRB*03* was found in the Assynt and Ythan metapopulations, but was absent from Coiresa and Grampian; *Arte-DRB*04* was present only in the Grampian population; whereas *Arte-DRB*05* was unique to the Coiresa island population.

A total of 43 of 171 (25.1%) nucleotides and 23 of 57 (40.4%) amino acids were variable. Sequences varied by an average of 13.4 (23.5%) amino acid sites (6–19, 10.5–33.3%). Seventeen of 57 (29.8%) amino acid sites belonged to the putative PBR described by Brown et al. (1993). Of these, 14 (82.4%) were polymorphic. In contrast, 9 out of 40 (22.5%) non-PBR sites were variable.

For codons within the PBR, the rate of nonsynonymous substitutions ($d_N=0.513\pm0.147$) exceeded that of synonymous substitutions ($d_S=0.131\pm0.065$) ($Z=3.04$ and $P\leq0.005$), whereas for non-PBR codons, d_N (0.071 ± 0.032) was comparable to d_S (0.060 ± 0.028) and did not vary significantly from parity (Table 1).

Phylogenetic analysis revealed that *Arte-DRB* alleles did not form a monophyletic clade when compared with other rodent *DRB* alleles (Fig. 2). The likelihood of the resolved polyphyletic topology was significantly greater than any topology constrained to be reciprocally monophyletic across species (Shimodaira–Hasegawa, $P\leq0.05$).

Discussion

This is the first study to examine MHC *DRB* variation in *A. terrestris*. From SSCP analysis of 100 individuals, we found five different *Arte-DRB* sequences, each with a distinct amino acid structure at the PBR. A GenBank BLAST search confirmed the homology to *DRB* for all alleles. In comparison to levels of *DRB* allelic variation reported for most other rodents, the number of alleles resolved here is low (Froeschke and Sommer 2005; Musolf et al. 2004). For the same 171-bp fragment in hairy-footed gerbils (*Gerbillurus paeba*), 34 alleles were described in 40 individuals (Harf and Sommer 2005). In yellow-necked mouse (*Apodemus flavicollis*), 27 *DRB* alleles were identified from 147 individuals (Meyer-Lucht and Sommer 2005). However, high levels of *DRB* polymorphism were not reported for all mammals. Those studies that have resolved comparable diversity to that observed here have been on species that are known to have experienced severe population bottlenecks (e.g., Ellegren et al. 1993; Ellegren et al. 1996; Smulders et al. 2003;

Sommer et al. 2002). *A. terrestris* has experienced an estimated 98% population decline in some regions of Britain since the 1950s through habitat loss and predation from the introduced American mink (*Mustela vison*) (Strachan et al. 2000). However, the Assynt population used in this study has remained largely unperturbed with concomitantly high levels of diversity at microsatellite loci (Lambin et al. 2004). It is thus likely that the low number of *DRB* alleles in *A. terrestris* is not the consequence of a population bottleneck.

Low inherent levels of diversity at a single MHC locus may be compensated for by increased diversity at other MHC (*DRB* or otherwise) paralogs, as observed in the naked mole rat (*Spalax ehrenbergi*) (Nizetic et al. 1987), feline MHC (Yuhki et al. 2003), rhesus macaques (Doxiadis et al. 2000; Doxiadis et al. 2001), and Californian sea lions (*Zalophus californianus*) (Bowen et al. 2004). It is therefore possible that diversity may be higher at another MHC class II locus or across multiple duplicate loci in *A. terrestris*.

Despite a low level of allelic diversity, a strong signal of balancing selection acting on the *Arte-DRB* locus was apparent in the observed pattern of sequence polymorphism. Analyses of variation among the five *Arte-DRB* sequences revealed compelling evidence for selection favoring functional diversity. Variation in terms of amino acid differences occurred disproportionately in the functional PBR compared to the non-PBR and the relative rate of d_N was significantly greater ($P\leq0.005$) than d_S for PBR but not non-PBR codons. In addition, d_N was 7.2 times higher in PBR than non-PBR sites. Such inequality in $d_N:d_S$ is characteristic of balancing selection acting to maintain functional polymorphism and was reported in a number of MHC studies (Ekblom et al. 2003; Garrigan and Hedrick 2001).

Comparative phylogenetic analysis of *Arte-DRB*01–05* with other *DRB* alleles showed that in each case, *Arte-DRB* alleles were more closely related to a *DRB* allele of a different species than to any other *Arte-DRB* allele. The retention of ancestral polymorphisms across speciation events can result in closer interspecific than intraspecific similarities between alleles. This phenomenon, known as transspecies polymorphism (or evolution; Klein 1980), was documented for MHC alleles from a number of species and is regarded as evidence for balancing selection, preserving allelic lineages over large time scales (Figuroa et al. 2000; Go et al. 2002). A criticism of using transspecies polymorphism as evidence for the nonneutral retention of alleles is that independently similar sequence motifs can repeatedly arise as a result of positive selection acting on functional codons within exons, thereby creating a misleading impression of the evolutionary relationship between alleles (Kriener et al. 2000a,b). To minimize the potential for such misleading relationships, we reconstructed a tree using the sequence data from Fig. 2 with the functional PBR codons removed (data not included). The general topology and bootstrap support for the tree without PBR codon sequence data was similar to that illustrated in Fig. 2, and the assertion that *Arte-DRB* alleles do not form a monophyletic group when compared to other *DRB* alleles was upheld.

Table 1 The relative rate of nonsynonymous (d_N) and synonymous (d_S) substitutions among alleles for codons in the functional peptide-binding region (PBR) and non-PBR of exon 2 of the *Arte-DRB* gene

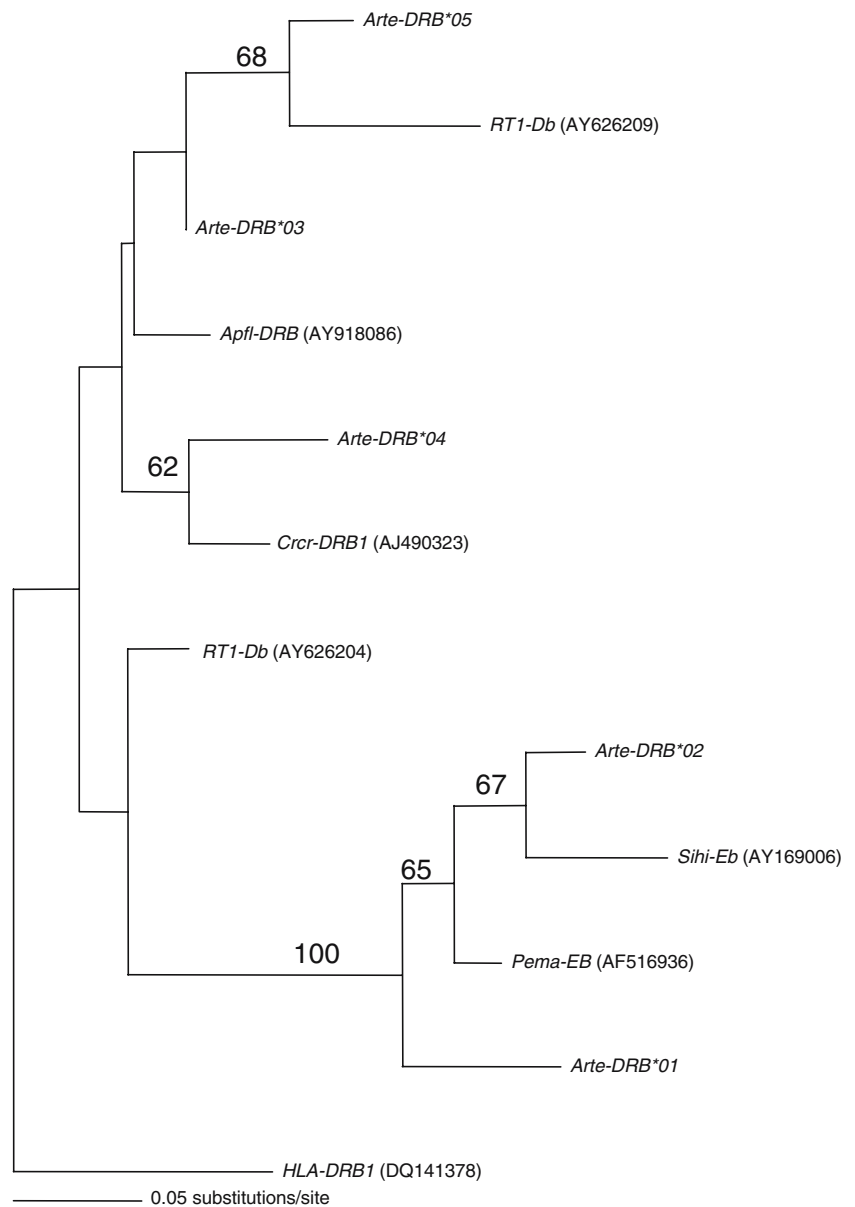
	<i>N</i>	d_N	d_S	$d_N:d_S$	<i>P</i>
PBR	17	0.513 (0.147)	0.131 (0.065)	3.91	<0.005
Non-PBR	40	0.071 (0.032)	0.060 (0.028)	1.18	0.75

Values in parentheses are standard errors based on 1,000 replicates.

P values are the probability of the null hypothesis of neutrality ($d_N=d_S$) (Nei and Kumar 2000)

N Number of codons used for the test

Fig. 2 Maximum likelihood phylogeny for five *A. terrestris* DRB exon 2 alleles. A human sequence *HLA-DRB1* sequence (GenBank accession no. DQ141378) was used as an outgroup. Numbers represent bootstrap values (%) >50 (1,000 replicates) and the scale bar represents genetic distance. A number of rodent MHC class II sequences were included for comparison. *Arte Arvicola terrestris*, *Apfl Apodemus flavicollis*, *Gepa Gerbillurus paeba*, *Peer Peromyscus maniculatus*, *RT1 Rat MHC*, and *Sihi Sigmodon Hispidus*, GenBank accession nos. are in parentheses



According to theory, the efficacy of selection in maintaining genetic diversity can vary substantially with the degree of population subdivision (Muirhead 2001). Furthermore, the significance of nonselective evolutionary forces, such as random genetic drift, is strongly related to population size, while the relative strength of drift in shaping allelic diversity is affected by the interconnectedness of populations by the dispersal of individuals. The low number of *Arte-DRB* alleles resolved here may reflect the action of random genetic drift acting in relatively small populations to reduce diversity. Alternatively, the complex population dynamics of water voles may alter the coevolutionary relationship between MHC genotypes and the parasite community in a way that favors a different pattern of polymorphism to that observed in other studies. While the observed patterns of sequence diversity imply a signature of balancing selection acting to maintain DRB functional diversity, the challenge now is to identify the

degree in which both deterministic and stochastic micro-evolutionary processes operate on MHC variation in contemporary water vole populations.

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