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ORIGINAL ARTICLE

Contrasting evolutionary histories of MHC class I and class II loci in grouse—effects of selection and gene conversion

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Genes of the major histocompatibility complex (MHC) encode receptor molecules that are responsible for recognition of intracellular and extracellular pathogens (class I and class II genes, respectively) in vertebrates. Given the different roles of class I and II MHC genes, one might expect the strength of selection to differ between these two classes. Different selective pressures may also promote different rates of gene conversion at each class. Despite these predictions, surprisingly few studies have looked at differences between class I and II genes in terms of both selection and gene conversion. Here, we investigated the molecular evolution of MHC class I and II genes in five closely related species of prairie grouse (*Centrocercus* and *Tympanuchus*) that possess one class I and two class II loci. We found striking differences in the strength of balancing selection acting on MHC class I versus class II genes. More than half of the putative antigen-binding sites (ABS) of class II were under positive or episodic diversifying selection, compared with only 10% at class I. We also found that gene conversion had a stronger role in shaping the evolution of MHC class II than class I. Overall, the combination of strong positive (balancing) selection and frequent gene conversion has maintained higher diversity of MHC class II than class I in prairie grouse. This is one of the first studies clearly demonstrating that macroevolutionary mechanisms can act differently on genes involved in the immune response against intracellular and extracellular pathogens.
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INTRODUCTION

The major histocompatibility complex (MHC) has an essential role in the immune response of vertebrates. MHC genes encode receptor molecules that bind small fragments of peptides derived from the processing of pathogens and present them to immune cells, initiating a cascade of immune reactions (Janeway *et al.*, 2005). There are two major subgroups of MHC genes, class I and class II, which are primarily responsible for the recognition of intracellular and extracellular pathogens, respectively (Janeway *et al.*, 2005). MHC genes are considered the most polymorphic loci in the vertebrate nuclear genome (Hughes, 1999). In humans, over 8000 MHC allelic variants have been resolved worldwide with nearly 1800 allelic variants at the MHC DRB-1 locus alone (Robinson *et al.*, 2013), and nucleotide diversity in the MHC region is up to two orders of magnitude higher than the genomic average (Garrigan and Hedrick, 2003). In birds, hundreds of alleles in each class have been reported for some species, for example, 862 class I and 977 class II alleles were identified in the great tit *Parus major* (Sepil *et al.*, 2012) and common yellowthroat *Geothlypis trichas* (Bollmer *et al.*, 2012), respectively. MHC polymorphism at the species or population level is thought to be primarily driven by the immense diversity of parasites and pathogens (Spurgin and Richardson, 2010), and at least two major evolutionary mechanisms are responsible for maintaining this unusually high diversification of MHC alleles: positive (balancing) selection and recombination.

Balancing selection on the vertebrate MHC acts primarily through negative frequency-dependent, overdominant and fluctuating selection (Hughes and Nei, 1988; Takahata and Nei, 1990; Hedrick, 2002). Signatures of balancing selection acting on MHC genes are usually supported by three lines of evidence: (i) high allelic diversity, (ii) higher rates of non-synonymous (dN) to synonymous (dS) nucleotide substitutions than expected under neutral evolution and (iii) alleles retained between species following divergence for longer evolutionary periods than expected for non-selected loci (Kamath and Getz, 2011). If ancestral alleles are retained through speciation events, then some alleles will be more similar between species than within species (that is, trans-species polymorphism; Klein *et al.*, 1993, 1998; Cutrera and Lacey, 2007).

MHC haplotypes may arise via meiotic reciprocal recombination (crossing over) or gene conversion, and the latter process is thought to explain most MHC allelic diversity (Hosomichi *et al.*, 2008). There are two types of gene conversion commonly occurring in the MHC region: (i) allelic (intra-locus) gene conversion occurring during meiosis as a consequence of the repair of mismatched heteroduplexes (Galtier *et al.*, 2001) and (ii) non-allelic (inter-locus) gene conversion between duplicated (paralogous) DNA fragments (Ohta, 1999). While *de novo* MHC sequence variation is generated by point mutation, gene conversion can generate new haplotypes by transferring sections of DNA within and across duplicated MHC loci (Spurgin *et al.*, 2011). Gene conversion (either intra- or inter-locus) is now recognized to have a crucial role in generating and maintaining MHC diversity in many vertebrates (Reusch and Langefors, 2005; Schaschl *et al.*, 2006),

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including birds (Chaves *et al*., 2010; Spurgin *et al*., 2011; Promerová *et al*., 2013).

MHC evolution is also driven by gene duplication, which produces a varying number of MHC loci in different taxa (Kelley *et al*., 2005), with up to 20 expressed class II B genes per individual reported for some avian species (Bollmer *et al*., 2010). Despite the frequent duplication events at the MHC, the birth-and-death model of MHC evolution assumes that many duplicated genes may become dysfunctional or deleted (Nei *et al*., 1997; Nei and Rooney, 2005). Gene loss (death) seems a possible evolutionary scenario for the surprisingly compact structure of the MHC region in Galliformes. The 'minimal essential' MHC was first described in the domestic chicken *Gallus gallus*, which has two class I and two class II B genes encoding only one dominantly expressed locus at each class (Kaufmann *et al*., 1999). In chickens, the MHC is dense with short introns and there are few duplications or pseudogenes, which produces a simple genetic architecture (Kaufmann, 1999). Other galliform species, such as the ring-necked pheasant *Phasianus colchicus* (Wittzell *et al*., 1999a) and black grouse *Tetrao tetrix* (Strand *et al*., 2007; Wang *et al*., 2012), share the compact genetic organisation of the chicken MHC. Recent studies of the greater prairie chicken *Tympanuchus cupido* have revealed an even simpler MHC architecture, with only one class I locus and two class II loci, leading to the smallest core MHC-B of any bird species yet studied (Eimes *et al*., 2013).

Although the evolution of the MHC has been the subject of research for over five decades (Clarke and Kirby, 1966; Ejsmond *et al*., 2014), most studies have focused on the MHC of a single species and typically a single gene, either class I or II (reviewed in Bernatchez and Landry, 2003). Thus, comparisons of MHC evolution at both class I and II are restricted to relatively few vertebrates, mostly teleost fish (Shum *et al*., 2001; Kruiswijk *et al*., 2005; Schaschl and Wegner, 2007) where class I and class II genes are distributed on different chromosomes and not contained within a single complex. Given the different roles of class I and II MHC genes and the pathogens they recognize, one might expect selection to differ between these two classes. Furthermore, recombination is expected to be different depending on the number of loci. For example, species such as the greater prairie chicken with a single class I locus and two class II loci can only show signs of intra-locus gene conversion within the single class I locus, while both intra- and inter-locus gene conversion can occur at class II where there are two loci (Reusch and Langefors, 2005). In addition, the rate of intra-locus gene conversion may depend on the strength of selective pressures, as stronger balancing selection might favour new alleles produced by gene conversion (Wegner, 2008). So far, there is limited evidence for contrasting evolution of both MHC classes in higher vertebrates, where all MHC genes are linked in a cluster on the same chromosome (Klitz *et al*., 1986; Bonneaud *et al*., 2004; Kuduk *et al*., 2012). Here, we investigated the molecular evolution of MHC class I and class II sequences in five closely related species of *Centrocercus* and *Tympanuchus* grouse (subfamily Tetraoninae). To date, the arrangement and number of genes in the 61 kb MHC class II B region of these species has only been determined in *T. cupido* (Eimes *et al*., 2013), and was found to have a simple MHC architecture relative to most galliform and other bird species. In this study, we sought to determine whether a simple MHC structure extended to other grouse (Tetraoninae). We focused our study on exon 3 of MHC class I genes and exon 2 of MHC class II B genes, both corresponding to the antigen-binding regions of MHC molecules. The main goals of this paper were to: (i) characterize levels of polymorphism of both MHC classes; (ii) characterize key evolutionary mechanisms (balancing selection, gene conversion) acting on MHC genes; and (iii) compare the role of these mechanisms in shaping the evolution of MHC class I and class II in grouse.

MATERIALS AND METHODS

Sample collection

We sampled five species of North American prairie grouse: *Centrocercus minimus* (Gunnison sage-grouse), *C. urophasianus* (greater sage-grouse), *Tympanuchus phasianellus* (sharp-tailed grouse), *T. pallidicinctus* (lesser prairie chicken) and *T. cupido* (greater prairie chicken). *Tympanuchus* and *Centrocercus* are closely related and, together with *Dendragapus*, form a monophyletic clade within the Tetraoninae (Stein *et al*., 2015). Blood and muscle tissue samples were collected between 1997 and 2011 for 183 greater prairie chickens and for 20–22 individuals from each of the other grouse species. Sampling locations are summarized in Supplementary Table S1. Protocols used for DNA extraction are described in previous publications (Bellinger *et al*., 2003; Oyler-McCance *et al*., 2005; Galla and Johnson, 2015).

Amplification and 454 pyrosequencing

We genotyped MHC class I exon 3 and class II B exon 2 in 266 and 263 birds, respectively (Table 1). Although these exons correspond to only ~50% of the antigen-binding regions of MHC class I and II molecules, they are known to be hypervariable (Wittzell *et al*., 1999b) and most MHC studies have focused

Table 1 Allelic diversity of MHC class I and class II in five species of *Centrocercus* and *Tympanuchus* grouse, where *n* is the number of individuals genotyped, A is the number of alleles (private alleles in parentheses) and A/I is the mean (±s.d.) number of alleles per individual

Genus/species	*MHC class I*			*MHC class II*		
	n	*A*	*A/I (±s.d.)*	n	*A*	*A/I (±s.d.)*
Centrocercus						
C. minimus (Gunnison sage-grouse)	20	3 (0)	1.25±0.44	19	2 (1)	1.10±0.32
C. urophasianus (greater sage-grouse)	22	7 (4)	1.55±0.51	20	12 (10)	2.05±0.83
Tympanuchus						
T. phasianellus (sharp-tailed grouse)	21	11 (4)	1.67±0.48	21	15 (3)	2.24±0.89
T. pallidicinctus (lesser prairie chicken)	20	12 (2)	1.75±0.44	21	15 (0)	2.76±0.89
T. cupido (greater prairie chicken)	183	25 (12)	1.80±0.40	182	30 (11)	2.44±0.91
Resampled *T. cupido*	20	14.9	—	20	15.3	—
Total	266	37	1.61±0.22	263	46	2.12±0.63

Abbreviation: MHC, major histocompatibility complex. Number of alleles was also estimated for *T. cupido* using a resampling approach (5000 replicates) based on 20 individuals to allow comparison with the other sampled species with a similar sample size. Note that A values for particular species do not sum to the total A because of interspecific allele sharing.

exclusively on these regions (for example, Kruiswijk *et al*., 2005; Schaschl *et al*., 2006; Cutrera and Lacey, 2007; Alcaide *et al*., 2013,2014). For MHC class I, we used primers 1a2inExon3F and 1a2Raltintron2 originally developed for the greater prairie chicken (Bateson *et al*., 2014). As previously documented for this species (Bateson *et al*., 2014), we observed sequence length polymorphism at exon 3 of class I in all three *Tympanuchus* species. The most common sequence length was 258 bp, while sequences of 255 and 261 bp were generated by a 3-bp deletion at position 58 and a 3-bp insertion at position 40, respectively. There was no sequence length polymorphism within *Centrocercus* (all sequences were 255 bp). For MHC class II, we used a forward primer (Blex2F) developed for greater prairie chicken (Eimes *et al*., 2010) and a reverse primer (RNA R1a) developed for black grouse (*Tetrao tetrix*, Strand *et al*., 2007), which amplified a 237-bp fragment of the 270-bp exon 2. PCR protocols are described elsewhere (see Bateson *et al*., 2014 for class I, see Eimes *et al*., 2010 for class II).

We used 454 pyrosequencing for genotyping both MHC class I and class II in all individuals, except for nine *C. minimus* that were part of an initial set that was cloned and sequenced to test the primers (see Bateson *et al*., 2014 for cloning methods). Amplifications were completed using fusion primers containing Roche (Basel, Switzerland) 454 adapter sequences, an 8-bp barcode used to distinguish individuals and a pair of either the MHC class I or II primers. PCR products were purified, pooled and sequenced on a Roche 454 FLX Genome Sequencer using Titanium chemistry at Research and Testing Laboratory, LLC (Lubbock, TX, USA).

MHC allele validation

Reads containing the complete forward primer, complete barcode and at least 10 internal bases of the reverse primer were extracted from multifasta files and sorted according to barcodes with jMHC software (Stuglik *et al*., 2011). Barcodes, adapters and primers were trimmed and low quality reads were removed. True alleles were identified in several steps. First, we extracted sequence variants of expected exon sizes for MHC class I (255, 258, 261 bp; Bateson *et al*., 2014) and class II (237 bp; Eimes *et al*., 2010). We also extracted amplicons that showed minor sequence variation (1–3 bp) from a high copy number variant of the expected size (putative true allele). Repeatable putative errors believed to originate during 454 sequencing were identified using the criteria proposed by Lighten *et al*., (2014) and were clustered with similar high copy number sequences for further analyses. Next, we retained only those sequence variants that were found in at least three reads (following Radwan *et al*., 2012), as it is extremely unlikely that three identical sequences occur by chance owing to sequencing errors (Galan *et al*., 2010).

We used the degree of change and copy number variation approaches developed by Lighten *et al*. (2014) to identify true alleles and discard all other types of artefacts. The degree of change approach estimates a number of allelic copies of each individual by differentiating putative true sequences and artefacts on the basis of the observed sequencing depths of the 10 most common sequence variants (Lighten *et al*., 2014). The method relies on the assumption that there should be an obvious reduction in sequencing depths between putative alleles and artefacts, so the least amplified true allele is associated with an inflection point in a linear plot of cumulative sequencing depths. The copy number variation approach identifies the number of allelic copies by comparing the observed proportion of reads for each allele within individual to a theoretical genetic model that calculates expected read counts for a range of loci and allelic copies (Lighten *et al*., 2014). All putative true alleles identified with either of these two approaches were checked to determine whether they could be explained as chimeras that are generated repeatedly during PCR by recombination among pairs of true alleles (Lenz and Becker, 2008). The algorithm used to identify PCR-derived chimeras from true recombinants included two criteria: (i) PCR-derived chimeras should always co-occur with both parental sequences in the same amplicon, whereas true recombinants may or may not co-occur with one or both parental sequences; (ii) PCR-derived chimeras should generally be less abundant than true alleles (following Zagalska-Neubauer *et al*., 2010). Identification of true alleles was confirmed by their alignment with previously published greater prairie chicken alleles (Eimes *et al*., 2010,2011, 2013) in a neighbour-joining tree using Geneious v7.1.7 (http://www.geneious.com, Kearse *et al*., 2012).

After this processing, we had 155 807 class I and 264 253 class II reads for all individuals, with an average of 606 class I (range: 25–3124) and 1036 class II (range: 145–6197) reads per individual. On average (±s.e.), there were 347.8 ±15.1 and 439.7 ± 15.2 reads per true allele within individuals, at class I and II, respectively. Genotyping repeatability of MHC class I and II was high ($R = 0.93$; $F_{35, 36} = 29.13$; $P < 0.001$), based on 18 birds with rare alleles that were repeated using independent PCRs in two separate 454 pyrosequencing runs.

Identifying locus-specific MHC class II alleles

As our 454 pyrosequencing primers amplified both MHC class II loci in prairie grouse (BLB1 and BLB2, Eimes *et al*., 2013), we modified the locus-specific approach of Strand *et al*. (2013) to identify alleles occurring at BLB1 (see Supplementary Methods). This nested PCR approach consisted of an initial amplification (2068 bp) from Blec1 to the beginning of exon 2 in BLB1 (Eimes *et al*., 2013). The product from this initial PCR was used as template for a second PCR that amplified exon 2 of BLB1 (see Supplementary Methods for details). To determine whether a particular allele occurred at BLB1, we genotyped five individuals (or as many as possible if a smaller sample size was available) that carried each of the MHC class II alleles identified in our sample of prairie grouse; this included 75 of 263 individuals. We used Geneious to align the sequences from the nested PCR to the alleles previously found in the same individuals with 454 pyrosequencing. We were unable to amplify the second MHC class II locus (BLB2) across prairie grouse using the long-range primers of Strand *et al*. (2013) or redesigned primers on the basis of our map of the greater prairie chicken MHC (Eimes *et al*., 2013). However, we observed a maximum of two alleles per individual with the nested PCR approach, thereby providing no evidence to suggest that we amplified more than the BLB1 locus.

Analysis of polymorphism and selection

MHC class I and class II sequences were aligned in Geneious v7.1.7 and sequence polymorphism (number of segregating sites, total number of mutations, average number of nucleotide differences and average nucleotide diversity) across all five grouse species was analysed with DnaSP v5.0 (Librado and Rozas, 2009). To test whether positive selection historically operated on the MHC class I and class II sequences in grouse, we used two different approaches. First, we compared relative rates of non-synonymous (dN) and synonymous (dS) substitutions across the sequences (Hughes and Nei, 1988). Substitution rates were computed in MEGA v6.0 (Tamura *et al*., 2013) according to the modified Nei-Gojobori (mNG) method (Nei and Gojobori, 1986; Zhang *et al*., 1998) with the ratio of the numbers of transitions and transversions (R) = 2 and with the Jukes and Cantor (1969) correction for multiple substitutions at the same site. Calculations were carried out separately for the putative non-antigen-binging sites (non-ABS) and antigen-binding sites (ABS) at class I (Kaufman *et al*., 1992) and class II (Brown *et al*., 1993), including three additional ABS sites (codons 17, 48, 58) recognized by Bondinas *et al*. (2007) for MHC class II. A codon-based two-tailed *Z*-test of selection was performed separately on ABS and non-ABS regions, as well as on the entire sequence, to test for the null hypothesis of neutrality ($dN/dS = 1$). We also used one-tailed *Z*-tests to determine whether positive selection ($dN/dS > 1$) acted on ABS regions and on the entire sequence of MHC class I exon 3 and MHC class II exon 2.

Second, signatures of positive selection acting on ABS and non-ABS regions were detected with the partitioning approach for robust inference of selection (PARRIS; Scheffler *et al*., 2006), as implemented in the Datamonkey web server (Delport *et al*., 2010). The PARRIS method infers positive selection acting on nucleotide sequences by using likelihood ratio tests (LRT) to compare a null-model (M1, no-selection) where dN is restrained to be $\leqslant$ dS, against a full model (M2, selection) where dN is allowed to be $>$ dS. This method expands on other maximum likelihood methods for detecting positive selection by allowing substitution rates to vary across sites and accounting for recombination.

We also tested for codon-specific signatures of positive and negative selection across MHC class I and class II sequences using three different approaches implemented in the Datamonkey server: single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and random effects likelihood (REL; Kosakovsky Pond and Frost, 2005). We considered a codon to be evolving under selection when it was identified as such by at least two methods

(Winternitz and Wares, 2013). To visualize the magnitude of dN versus dS, we plotted normalized dN—dS values calculated with the FEL approach, which under most scenarios is intermediate between the SLAC and REL methods in terms of type I error (Kosakovsky Pond and Frost, 2005). We used mixed effects model of evolution (MEME; Murrell *et al.*, 2012) to detect evidence of episodic diversifying selection acting on individual residues. This method combines fixed effects on the level of a site with random effects at the level of branches and has superior performance over previous models under a broad range of scenarios (Murrell *et al.*, 2012).

Gene conversion

Although sequence analysis is unlikely to successfully distinguish between gene conversion and meiotic reciprocal recombination, the latter process is usually invoked to explain exchanges of large DNA segments between MHC regions (Cullen *et al.*, 2002; Otting *et al.*, 2005; Hosomichi *et al.*, 2008). In the chicken, large genomic segments (>40 kb) shared between MHC haplotypes were argued to originate from reciprocal recombination, while sharing of smaller segments (1–2 kb) was attributed to gene conversion (Hosomichi *et al.*, 2008). As we only analysed recombination at the intra-exonic level (within a 261-bp class I sequence and 239-bp class II sequence), it seems safe to assume that we were likely to detect mostly gene conversion events, and thus all recombination processes are hereafter referred to as gene conversion.

We assessed gene conversion at MHC class I and class II within and between *Centrocercus* and *Tympanuchus* with GENECONV v1.81a (Sawyer, 1989). The software detects gene conversion events by testing whether the nucleotide substitutions observed between a set of DNA sequences are randomly distributed along the sequences (Drouin *et al.*, 1999). This approach is considered one of the most powerful to infer gene conversion events correctly among several different recombination detection methods (Posada, 2002), although it might have limited power when the conversion rate is high (Mansai and Innan, 2010). Simulated global P values <0.05 based on 10 000 permutations were considered evidence of gene conversion. Mismatches within conversion tracts were not allowed by setting the g-scale parameter (mismatch penalty) to 0. The minimum number of recombination (gene conversion) events was also calculated with the four-gamete method of Hudson and Kaplan (1985) implemented in DnaSP software. Sequence alignments of class I exon 3 and class II exon 2 were also screened for gene conversion by testing whether the physical distance between sites correlated with the measures of linkage disequilibrium (LD). For this purpose, we used two indices of LD, r^2 of Hill and Robertson (1968) and D' of Lewontin (1964), which were calculated in DnaSP. Our null hypothesis predicted that, in the absence of gene conversion, adjacent sites should show similar levels of LD to more distant sites (Meunier and Eyre-Walker, 2001; Kiemnec-Tyburczy *et al.*, 2012), which was tested with the Pearson product-moment correlation coefficient.

Phylogenetic clustering

To assess the phylogenetical relationships of MHC class I and II in prairie grouse, we constructed phylogenetic trees using maximum likelihood (ML) in MEGA v6.0 (Tamura *et al.*, 2013). Evolutionary distance was computed with the Hasegawa-Kishino-Yano (HKY) model and clade support was tested with 1000 bootstrap replications. Sequences of the mallard *Anas platyrhynchos* (GeneBank accession nos.: JN810924 for class I, AF390589 for class II), chicken *Gallus gallus* (GeneBank accession nos.: KF032370 for class I, HQ203710 for class II) and helmeted guineafowl *Numida meleagris* (GeneBank accession nos.: EF643463 for class I, EU826064 for class II) were used as outgroups.

RESULTS

Allelic diversity

We found 37 class I and 46 class II alleles across all five grouse species (Table 1). There were more alleles in *Tympanuchus cupido* (25 class I and 30 class II alleles) than in the other grouse species (3–12 class I alleles; 2–15 class II alleles), but this was due to the much larger sample size for *T. cupido* ($n=182$–3) than the other species ($n=19$–22; Table 1). To produce comparable estimates of MHC diversity, we resampled MHC variation in *T. cupido* using the sample size comparable with that for the other species ($n=20$). Number of alleles at both class I and II was similar for all *Tympanuchus* species after resampling *T. cupido* (Table 1). There was a high rate of allele sharing within genera, with 52% ($n=13$) of class I and 60% ($n=18$) of class II alleles shared between *T. cupido* and the two other *Tympanuchus* species. Within *Centrocercus*, *C. minimus* shared all three class I alleles and one of two class II alleles with *C. urophasianus*. Only one class I (*Tycu-IA*33*) and one class II (*Tycu-BLB*14*) allele were shared between *Centrocercus* and *Tympanuchus*. In all species, we found one or two class I alleles per individual (mean 1.61 ± 0.22 (s.d.); Table 1), while there were one to four class II alleles per individual in all *Tympanuchus* and 1–3 class II alleles per individual within *Centrocercus* (see Table 1), confirming the presence of only one class I locus and two class II loci in both grouse genera.

Of the 46 alleles found at class II, we confirmed the presence of 35 alleles at the BLB1 locus by genotyping 75 individuals with BLB1-specific primers after 454 pyrosequencing. For this BLB1-specific analysis, we tested all 46 class II alleles and found an average of 4.02 ± 0.55 copies per allele across the 75 individuals. Of the 46 alleles, 24 were found only at BLB1, 11 alleles were shared between the two class II loci (BLB1 and BLB2; inferred by comparing sequences obtained with BLB1-specific primers and our previous 454 pyrosequencing results) and the other 11 alleles presumably belonged to the BLB2 locus because they were not observed in the nested BLB1 PCR results. Most of the alleles that were not found at BLB1 (9 out of 11 alleles) were relatively rare (present in <3% of individuals genotyped with 454 pyrosequencing, $n=263$). Below we present the results comparing class I and class II using all alleles for class II (that is, at both BLB1 and BLB2; $n=46$), as well as the alleles that occurred at the single BLB1 locus ($n=35$).

At class I, we found a lower proportion of segregating sites (39/261 segregating sites per total number of sites) and fewer mutations ($\eta=52$) than at class II (95/237 segregating sites and $\eta=128$ mutations at BLB1 alone; 96/237 segregating sites and $\eta=131$ mutations across all class II sequences (BLB1 and BLB2)). The average number of nucleotide differences was 12.97 ± 2.13 (s.d.) for class I and 33.43 ± 3.43 for BLB1 class II locus (31.36 ± 3.30 for all class II sequences). Consistent with the lower mutation rate, the average nucleotide diversity was more than two times lower for class I (0.051 ± 0.004) than class II (0.141 ± 0.008 for BLB1 sequences; 0.132 ± 0.008 for all class II sequences). ABS regions had much higher nucleotide diversity than non-ABS regions at both class I (ABS, 0.115 ± 0.011 versus non-ABS, 0.038 ± 0.003) and class II (ABS, 0.272 ± 0.023 versus non-ABS, 0.085 ± 0.009 for BLB1 sequences; ABS, 0.260 ± 0.013 versus non-ABS, 0.080 ± 0.008 for all class II sequences).

Tests of selection

There was strong support for positive selection (dN/dS>1) acting on ABS regions at both class I and II (Table 2). Although dN/dS was higher for the class I ABS region, this was mainly due to few synonymous mutations (dS), which greatly elevated the ratio despite a relatively low value of dN. By contrast, dN—dS indicated stronger selection acting on class II ABS region (Table 2). Separate analyses for each species confirmed that selection was stronger on the ABS region of class II than class I in three of four species (Supplementary Table S2; *C. minimus* was not analysed because of a low number of alleles at each class). Strong positive selection at ABS regions was confirmed using PARRIS as implemented in Datamonkey (LRT=14.08, $P<0.001$ for class I; LRT=16.77, $P<0.001$ for BLB1 sequences; LRT=18.07, $P<0.001$ for all class II sequences). A partitioning approach indicated

Table 2 Nonsynonymous (dN) and synonymous (dS) substitutions at ABS and non-ABS of MHC class I exon 3 and class II exon 2 of *Centrocercus* and *Tympanuchus* grouse

MHC class	*Region (no. of codons)*	*dN*	*dS*	*dN—dS*	*dN/dS*	P *values*	
						Departure from neutrality (dN/dS≠1)	*Positive selection (dN/dS> 1)*
Class I	All sites ($n=87$)	0.43	0.42	0.01	1.02	0.32	0.16
	ABS ($n=17$)	1.16	0.24	0.92	4.83	**0.016**	**0.006**
	Non-ABS ($n=70$)	0.20	0.35	−0.15	0.57	0.96	—
Class II (all sequences)	All sites ($n=79$)	1.50	1.07	0.43	1.40	**0.011**	**0.006**
	ABS ($n=25$)	3.26	1.89	1.37	1.72	**0.002**	**0.001**
	Non-ABS ($n=54$)	0.59	0.65	−0.06	0.91	0.92	—
Class II (BLB1 locus)	All sites ($n=79$)	1.28	1.02	0.26	1.25	**0.021**	**0.008**
	ABS ($n=25$)	2.99	1.61	1.38	1.86	**0.001**	**0.001**
	Non-ABS ($n=54$)	0.45	0.62	−0.17	0.73	0.73	—

Abbreviations: ABS, antigen-binding sites; MHC, MHC, major histocompatibility complex. The dN/dS ratio values and *Z*-test results for departures from neutrality (dN/dS≠1) and positive selection (dN/dS>1) are also shown. Significant *P* values (<0.05) are in bold.

no evidence for positive selection at non-ABS regions (LRT = 4.58, $P=0.10$ for class I; LRT = 0.00, $P=1.00$ for BLB1 sequences, LRT = 0.04, $P=0.97$ for all class II sequences). There was also evidence of positive selection on the entire class II exon 2 (dN/dS = 1.25 for BLB1 sequences, dN/dS = 1.40 for all class II sequences), but not for class I exon 3 (dN/dS = 0.94; Table 2).

The codon-based maximum likelihood approach detected signatures of positive and episodic diversifying selection acting on MHC class I less frequently than on MHC class II, regardless of comparing only BLB1 alleles or all class II sequences (Table 3; Figure 1). Fewer cases of positive or episodic diversifying selection were found at amino acid residues in MHC class I (3 of 87 residues, 3.4%) than class II (14 of 79 residues, 17.7%; G = 4.88, df = 1, $P=0.027$; Figure 1; Table 3). In general, signatures of positive or episodic diversifying selection were detected more frequently in ABS regions, when compared with non-ABS regions. There was evidence for these types of selection on 52.0% of all residues predicted to be in ABS regions of class II ($n=25$), in contrast to only one non-ABS site (1.9%, $n=54$; G = 14.79, df = 1, $P<0.001$; Figure 1, Table 3). This difference was much less pronounced and non-significant in MHC class I (G = 1.65, df = 1, $P=0.20$), with only two ABS codons (11.8%, $n=17$) and one non-ABS codon (1.4%, $n=70$) identified as under positive or episodic diversifying selection (Figure 1, Table 3). Signatures of negative (purifying) selection were more frequent in non-ABS regions, with zero or two ABS codons identified as under purifying selection at MHC class I and class II, respectively, compared with three non-ABS codons at each class (Table 3).

Table 3 Codons under different types of selection at ABS and non-ABS regions of MHC class I exon 3 and class II exon 2 of *Centrocercus* and *Tympanuchus* grouse (see also Figure 1)

MHC class	*Region*	*Type of selection*	*Codon*	*Models*	
					All sequences	BLB1 locus
Class I	ABS	P/ED	6	F, R, S, M	
		P/ED	22	F, R, S, M	
	non-ABS	P/ED	4	F, R, S, M	
		N	3	F, S	
		N	30	F, S	
		N	32	F, S	
Class II	ABS	P/ED	2	F, R, M	F, R, M
		P/ED	4	F, R, S, M	F, R, S, M
		P/ED	29	F, R, S, M	F, R, S, M
		P/ED	48	F, R, S, M	F, R, M
		P/ED	58	F, R, M	F, R, M
		P/ED	77	F, R, S, M	F, R, S, M
		P	79	F, R	F, R
		ED	17	M	M
		ED	19	—	M
		ED	21	—	M
		ED	28	M	—
		ED	47	M	M
		ED	52	M	—
		ED	59	M	M
		ED	62	M	M
		N	61	F, R, S	F, R, S
		N	76	—	F, R, S
	non-ABS	ED	9	—	M
		ED	64	M	—
		N	12	F, R, S	F, R
		N	16	F, R	F, R
		N	68	F, R, S	F, R, S

Abbreviations: ABS, antigen-binding sites; ED, episodic diversifying selection; F, fixed effects likelihood model (FEL); N, negative (purifying) selection; MHC, MHC, major histocompatibility complex; P, positive selection; R, random effects likelihood model (REL); S, single likelihood ancestor counting model (SLAC). The models that detected selection are also indicated for each codon. Episodic diversifying selection detected with mixed effects model evolution (MEME).

Gene conversion

We found little evidence for gene conversion at MHC class I. In *Tympanuchus*, only 13% (4/32) of class I alleles had signatures of gene conversion (four significant pairwise gene conversions recognized in total, see Supplementary Figure S1 and Supplementary Table S3) and a minimum of $\rho_M = 5$ gene conversion events were recognized within this genus with the four-gamete test. *Centrocercus* had no evidence for gene conversion at MHC class I.

The occurrence of gene conversion was much more frequent across all class II sequences. In *Centrocercus*, 67% (8/12) of class II alleles had signatures of gene conversion (six significant pairwise gene conversions; Supplementary Table S4), and a minimum of $\rho_M = 9$ conversion events were estimated ($n=12$ alleles). There were four different conversion tracts (26–171 bp) identified within *Centrocercus*. In *Tympanuchus*, 91% (32/35) of class II alleles were involved in at least one intra-generic gene conversion event and 22 different conversion tracts were identified (37–183 bp). In total, the substitution approach recognized 102 significant pairwise conversions among *Tympanuchus* MHC class II alleles (Supplementary Figure S1 and Supplementary Table S3). The four-gamete test identified a minimum

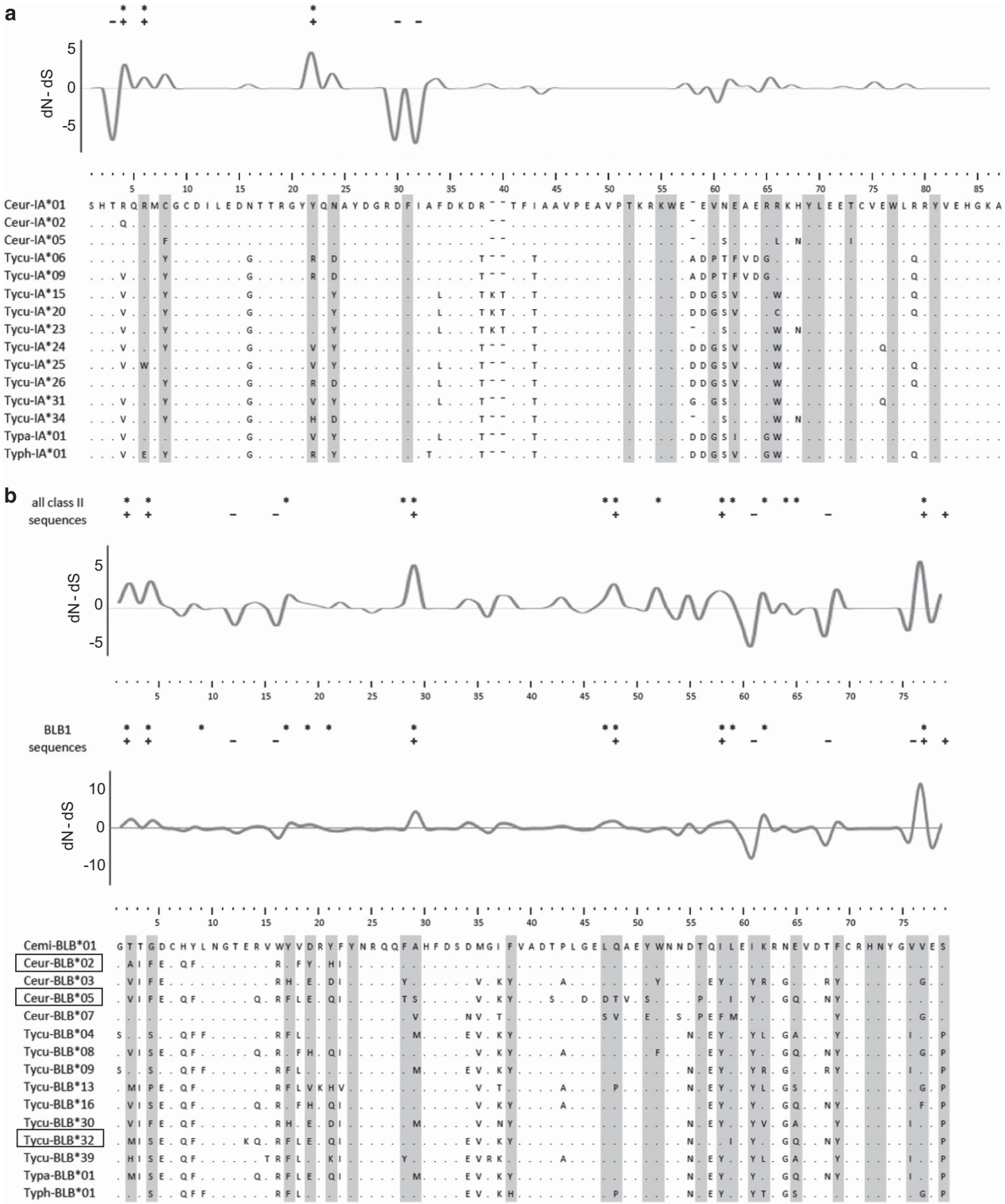

Figure 1 Alignments of amino acid sequences of MHC class I exon 3 (**a**) and MHC class II exon 2 (**b**) of *Centrocercus* and *Tympanuchus* grouse; subsets containing 15 representative alleles are shown for each MHC class. MHC class II alleles not found at BLB1 locus are in frames. Dots indicate the same amino acid with top sequence. Grey shading indicates putative ABS according to Kaufman *et al*. (1992) for MHC class I and according to Brown *et al*. (1993) and/or Bondinas *et al*. (2007) for MHC class II. Amino acid residues under positive selection are marked with a plus (+), under negative selection are marked with a dash (–) and under episodic diversifying selection are marked with an asterisk (*). Spatial variation in the selection parameter (normalized dN—dS) is shown at the top of each alignment.

of $\rho_M = 11$ conversion events. All alleles private to either *T. phasianellus* or *T. pallidicinctus* shared fragments with *T. cupido* alleles, and the only allele private to *C. minimus* shared a fragment with two *C. urophasianus* alleles suggesting pre-speciation conversion events. The only allele shared between *Tympanuchus* and *Centrocercus* (*Tycu-BLB*14*) showed evidence of conversion with one *C. urophasianus* allele (*Ceur-BLB*11*), but with no private *Tympanuchus* alleles, suggesting that the conversion event likely occurred after the two genera had diverged.

An analysis using only BLB1 alleles supported frequent gene conversion at MHC class II. In *Centrocercus*, 40% (4/10) of BLB1 alleles had signatures of gene conversion, while 88% (23/26) of BLB1 alleles were involved in at least one conversion event in *Tympanuchus* (2 and 62 significant pairwise gene conversions, respectively; Supplementary Table S5). Among BLB1 alleles, there were at least 8 and 11 gene conversion events (ρ_M) recognized with the four-gamete test in *Centrocercus* and *Tympanuchus*, respectively. After restricting the analysis to BLB1 alleles that were not shared with the BLB2 locus (7 *Centrocercus* alleles and 17 *Tympanuchus* alleles), no gene conversion events were found in *Centrocercus*, but there was evidence for frequent gene conversions in *Tympanuchus* (all 17 alleles involved in 53 significant pairwise gene conversions; Supplementary Table S6). A minimum number of gene conversions (ρ_M) among BLB1-specific alleles was estimated at 3 and 10 events in *Centrocercus* and *Tympanuchus*, respectively.

We also found considerable support for gene conversion at the inter-generic level, although it was less frequent in MHC class I than in class II. Gene conversion events between three private *Centrocercus* and four private *Tympanuchus* alleles (three different conversion tracts and six significant pairwise conversions, Supplementary Table S2) were detected for MHC class I, while there was evidence for gene conversion between eight *Centrocercus* and eight *Tympanuchus* private MHC class II alleles (seven different conversion tracts and 12 significant conversions; Supplementary Table S2).

These contrasting patterns of gene conversion at MHC class I and class II loci were supported by LD analysis. We found no correlation between LD statistics and physical distance between sites at MHC class I ($r = 0.03$, $P = 0.62$ for Lewontin D'; $r = -0.03$, $P = 0.54$ for Hill and Robertson r^2). By contrast, LD decreased with distance between sites across all class II sequences ($r = -0.07$, $P < 0.001$ for Lewontin D'; $r = -0.13$, $P < 0.001$ for Hill and Robertson r^2), as well as across BLB1 sequences only ($r = -0.06$, $P = 0.005$ for Lewontin D'; $r = -0.12$, $P < 0.001$ for Hill and Robertson r^2), indicating strong signatures of gene conversion.

Phylogenetic clustering

Phylogenetic trees of MHC class I and class II sequences revealed contrasting evolutionary histories (Figure 2). All MHC class I alleles of *Centrocercus* clustered within a single clade, yet paraphyletic with an allele shared with *T. cupido* (*Tycu-IA*33*) and one private allele of *T. phasianellus* (*Typh-IA*02*; Figure 2a). This result is consistent with the low signal of inter-generic gene conversion at MHC class I (see Figure 2a). All four private *Tympanuchus* alleles identified as having undergone gene conversion with *Centrocercus* alleles (*Tycu-IA*16*, *Tycu-IA*23*, *Tycu-IA*31, Typa-IA*02*) were placed in a neighbouring *Tympanuchus* clade (Figure 2a). By contrast, MHC class II alleles of *Centrocercus* grouse did not form a distinct clade and were located among other grouse species, most likely reflecting high rates of inter-generic gene conversion (Figure 2b). In fact, most *Centrocercus* class II alleles closely clustered with *Tympanuchus* alleles that were identified as having undergone inter-generic gene conversion (Figure 2b). Neither MHC class I and class II sequences formed monophyletic groups corresponding to species, which, in accordance with the high rate of allele sharing within genera, indicate trans-species evolution of this gene family in grouse.

DISCUSSION

We found striking differences in selection and gene conversion between MHC class I and class II genes within prairie grouse. Although we found positive selection acting on antigen-binding regions of both classes, only two putative ABS residues of class I (11.8%) were positively selected, compared with over half of putative class II ABS residues, which were under positive or episodic diversifying selection. Consequently, we found evidence of positive selection acting on the entire MHC class II exon 2, but no evidence of positive selection on the entire MHC class I exon 3. We also identified gene conversion as an important mechanism shaping the evolution of MHC class II in prairie grouse. By contrast, there was little evidence for gene conversion events occurring between class I alleles. Overall, the combination of strong positive (balancing) selection and frequent gene conversion has maintained higher diversity of MHC class II than class I in prairie grouse.

Surprisingly few studies have compared the relative importance of balancing selection on MHC class I and class II (Shum *et al*., 2001; Siddle *et al*., 2007; Kuduk *et al*., 2012), and only fragmentary information is available for birds. Previous studies on MHC variation in *T. cupido* suggested that the strength of balancing selection might be much less pronounced at class I than at class II (Eimes *et al*., 2013). However, this conclusion was based on the analysis of a severely bottlenecked population and it was argued that drift could have removed most evidence of selection at class I (Eimes *et al*., 2013). Although MHC class II variation in the same population was significantly reduced following the bottleneck, signals of historical positive selection on class II ABS codons were still retained (Eimes *et al*., 2011). This pattern suggests that persistence of divergent MHC class II alleles through the bottleneck was likely to be an adaptive process that preserved differences in pathogen-recognition capacity among haplotypes to support immune response, while the loss of variation at class I could be driven more by stochastic processes, such as genetic drift, rather than selection. Consistent with our results, substitution rates (dN/dS ratios) supported stronger selection for diverse MHC class II than for class I alleles in the domestic chicken (Hosomichi *et al*., 2008) and in a more distantly related non-passerine, the blue petrel *Halobaena caerulea* (Strandh *et al*., 2011).

A stronger signal of balancing selection at MHC class II genes is, however, unlikely to be a general pattern across avian species. Comparison of MHC class I and class II evolution within the genus *Falco* revealed that both classes accumulated synonymous and non-synonymous mutations at similar rates, indicating no differences in the intensity of adaptive evolution (Gangoso *et al*., 2012). The opposite pattern of selection can be inferred from multiple studies on two passerine species, the red-winged blackbird *Agelaius phoeniceus* and house finch *Carpodacus mexicanus*, which showed stronger signals of selection in the antigen-binding regions of MHC class I than of class II (Edwards *et al*., 1998; Jarvi *et al*., 2004; Alcaide *et al*., 2013). Differences in selection on the MHC class I of passerines and non-passerines may be related to their effective population sizes and exposure to pathogens (Alcaide *et al*., 2013), but these differences are unlikely to explain the stronger selection we found on class II than class I loci within the same set of species. Instead, our data suggest that differences in selection in prairie grouse species might be explained by stronger selection from extracellular (on class II) than intracellular (class I) pathogens.

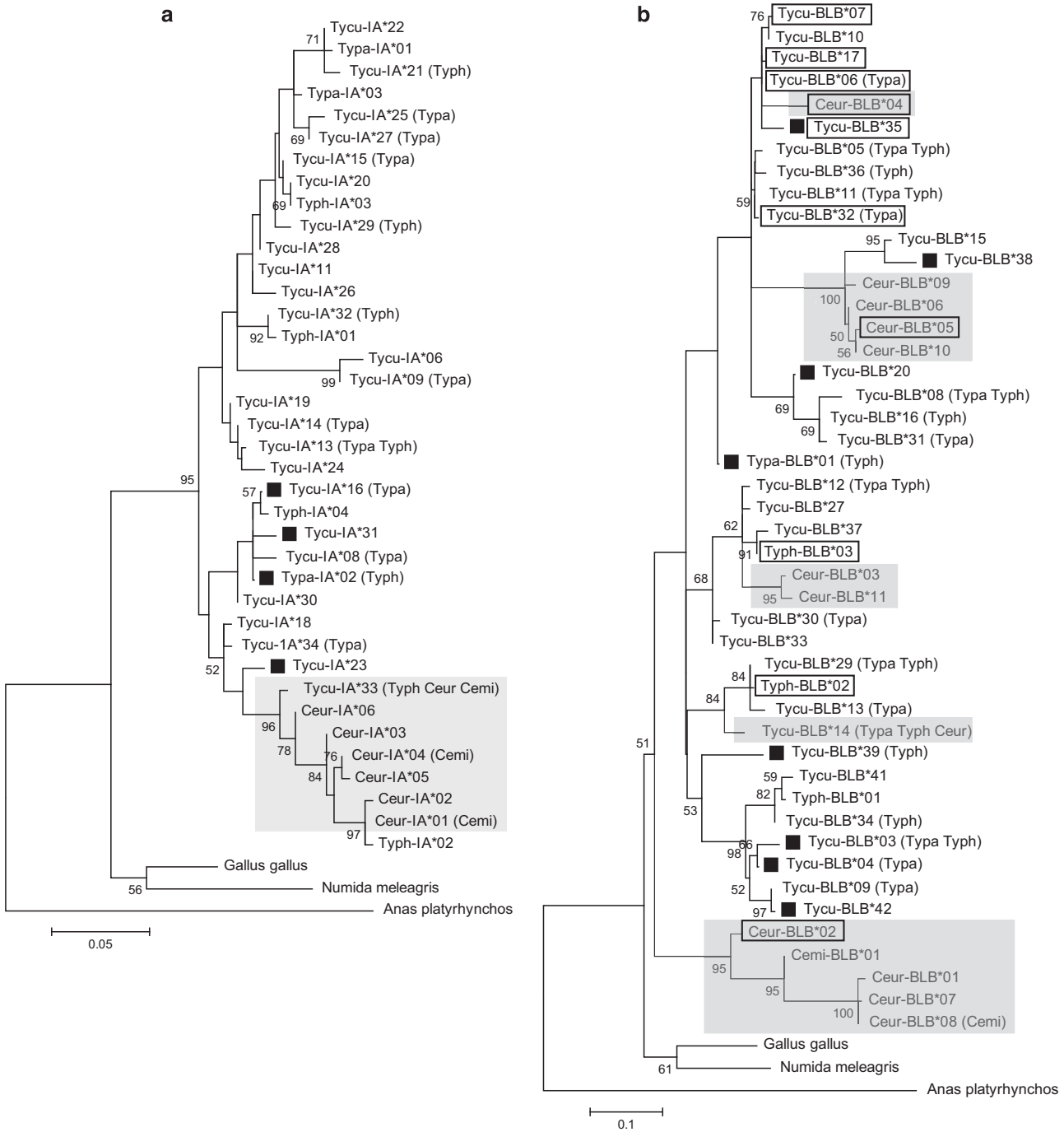

Figure 2 Maximum likelihood phylogenetic trees of MHC class I exon 3 (**a**) and MHC class II exon 2 (**b**) sequences from five *Centrocercus* and *Tympanuchus* grouse. Bootstrap values are provided for nodes with >50% support, and the scale bar indicates genetic distance in units of nucleotide substitutions per site. *Centrocercus* alleles are shaded in grey and alleles shared between species are indicated by acronyms given in brackets (Ceur = *C. urophasianus*; Cemi = *C. minimus*; Tycu = *T. cupido*; Typa = *T. pallidicinctus*; Typh = *T. phasianellus*). Private *Tympanuchus* alleles identified as having undergone intergeneric gene conversion are marked with black squares. The mallard *Anas platyrhynchos*, domestic chicken *Gallus gallus* and helmeted guineafowl *Numida meleagris* were used as the outgroups (for GenBank accession numbers and references see Methods).

Consistent with this hypothesis, field studies suggest that extracellular parasites are a strong selective force in wild grouse populations (reviewed in Peterson, 2004). The proventricular nematode *Dispharynx nasuta* causes significant mortality in the ruffed grouse *Bonasa umbellus* (Gross, 1925) and in the blue grouse *Dendrapagus obscurus* chicks (Bendell, 1955). It was also suggested to be an important factor in the extinction of the heath hen *T. c. cupido* (Gross, 1928). Two other nematodes, *Trichostrongylus cramae* and *T. tenuis*, cause caecal lesions and inflammation and have been associated with reduced fecundity and survival of grouse. For example, experimental reductions in the intensity of *Trichostrongylus* parasites led to increased body weight, adult survival, egg hatchability and nesting success in the red grouse *Lagopus lagopus scotica* (Shaw, 1990; Hudson *et al*., 1992). In prairie chickens, it has also been argued that

cestode (*Raillietina variabilis*) infections can affect host population dynamics (Leigh, 1941; Harper *et al*., 1967). Similarly, nearly 50% of sampled greater sage-grouse have been reported to be infected by *Raillietana* tapeworms, which were suggested to have a direct or indirect adverse impact on individual birds (Christiansen and Tate, 2011). Although such infectious agents as bronchitis and reticuloendotheliosis viruses are also known to be pathogenic for prairie grouse (Drew *et al*., 1998; Peterson *et al*., 2002), in general, there is much less empirical evidence for the detrimental effects of intracellular pathogens on wild grouse populations (Peterson, 2004).

There was also a much stronger signature of gene conversion among class II than among class I alleles. In *Tympanuchus* and *Centrocercus*, 91% and 67% of class II alleles, respectively, were involved in at least one intra-generic gene conversion event. By contrast, 13% of *Tympanuchus* class I alleles and no *Centrocercus* class I alleles had signatures of gene conversion. We also found a negative correlation between LD and physical distance between sites at MHC class II, but not at class I, indicating that recombination is important for increasing diversity of class II (Kiemnec-Tyburczy *et al*., 2012). These contrasting patterns of gene conversion at class I and II might be explained by the presence of gene duplication at class II. Our pyrosequencing results supported the presence of two class II loci in both *Centrocercus* and *Tympanuchus* grouse. As gene conversion may occur not only between alleles from the same locus, but also between loci (Ohta, 1999), recombination rate should increase with the number of duplicated loci, although it is expected to be inversely proportional to the distance between loci (Ezawa *et al*., 2006). While the unlinked MHC loci, such as B and Y, are less prone to inter-locus recombination (Burri *et al*., 2008), both class II B loci in grouse are separated by less than 7 kb (Wang *et al*., 2012; Eimes *et al*., 2013), which should facilitate a high gene conversion rate. Although the evidence for inter-locus recombination at MHC is now well-established in different vertebrate taxa (Hughes and Nei, 1989; Cardenas *et al*., 2005; Zagalska-Neubauer *et al*., 2010), including galliforms (Strand *et al*., 2013), the relative rates of inter- and intra-locus gene conversion at MHC are difficult to assess. The scarce available information suggests that both types of recombination may occur at a similar rate within class II B (Reusch and Langefors, 2005). In addition, high similarity among sequences derived from different MHC loci (apparent paralogs) suggests that inter-locus gene conversion may be an important evolutionary mechanism in fish (Reusch and Langefors, 2005; Aguilar and Garza, 2007) and birds (Edwards *et al*., 1995; Wittzell *et al*., 1999a, b; Burri *et al*., 2008). In grouse, we found that ca. 25% of class II alleles were shared between BLB1 and BLB2, indicating significant homogenisation of both loci. Our evidence for signatures of recombination between *Tympanuchus* and *Centrocercus* alleles suggests that class II loci may have been homogenized by inter-locus gene conversion in their ancestor and retained after species diversification. This appears to be rare empirical evidence for the concerted evolution of duplicated MHC genes, often invoked to explain why avian phylogenies based on MHC usually sort by species rather than by locus (Hess and Edwards, 2002).

We also found much more frequent intra-locus gene conversion at the BLB1 class II locus than at the class I locus, as all *Tympanuchus* BLB1-specific alleles ($n = 17$) were involved in gene conversions events compared with only four of 32 class I alleles. This higher rate of intra-locus gene conversion at class II could be due to stronger selective pressure acting on these genes, or it could be due to an interaction of gene conversion and selection. When gene conversion occurs, even if the rate is low, weak selection per amino acid site is effective for enhancing polymorphisms and may easily increase the number of MHC alleles in the population (Ohta, 1999). Thus, stronger balancing selection might favour new alleles produced by gene conversion leading to greater allelic polymorphism across evolutionary time. Indeed, a meta-analysis of teleost fish found that the dN/dS ratio was positively related to recombination rate (Wegner, 2008). Wegner (2008) suggested that recombination could increase the efficiency of natural selection by eliminating LD between selected codons or combining them from different alleles. Our findings in grouse agree with this hypothesis as we found that higher rates of both balancing selection and gene conversion were associated with higher diversity at class II than class I.

Contrasting evolutionary histories of MHC class I and II genes in grouse were also revealed by their contrasting phylogenies. We found that class I alleles clustered strongly by genus, whereas *Centrocerucus* and *Tympanuchus* class II alleles did not form monophyletic clades. There are two non-exclusive mechanisms that may account for these differences. First, we found a strong signal of gene conversion between private *Centrocercus* and *Tympanuchus* class II alleles and the signature of inter-generic conversion events was less frequent for class I alleles. As increased recombination decreases phylogenetic signal, *Centrocercus* and *Tympanuchus* class II alleles generated by ancestral gene conversion were more likely to cluster together rather than by genus. Secondly, this pattern could be enhanced by the higher intensity of balancing selection acting on class II genes. Under strong balancing selection, MHC alleles are preserved and can persist beyond speciation events (Klein *et al*., 1993). Such trans-species polymorphism seems to be a general feature of MHC evolution and has been demonstrated in many vertebrate systems (Ottová *et al*., 2005; Cutrera and Lacey, 2007; Kamath and Getz, 2011), although less often in birds (Hess and Edwards, 2002).

It has to be kept in mind that sharing of identical MHC allelic variants between species may also imply recent speciation events or interspecific hybridisation (Nadachowska-Brzyska *et al*., 2012). Sharing of MHC alleles between species from the same genus has been found in several recently diverged taxonomic groups, such as *Xenopus* frogs (Bos and Waldman, 2006), *Spheniscus* penguins (Bollmer *et al*., 2007; Kikkawa *et al*., 2009), equids (Kamath and Getz, 2011) and primates (Doxiadis *et al*., 2006). The divergence between all three *Tympanuchus* species likely occurred within the last 0.5–2 million years, and in areas of geographic overlap, hybridisation has been observed between *T. cupido* and two other *Tympanuchus* grouse (Galla and Johnson, 2015). *Centrocercus minimus* is a recently described species and probably at an intermediate stage of speciation from *C. urophasianus*, driven mostly by reproductive isolation (Young *et al*., 2000; Oyler-McCance *et al*., 2010). Thus, recent divergence seems to be a reasonable explanation for the trans-species allele sharing within both grouse genera. On the other hand, we also recorded identical class I and class II allelic variants shared between *Centrocercus* and *Tympanuchus*. MHC allele sharing across genera has been observed rarely and is usually attributed to trans-species polymorphism resulting from retention of ancestral allelic variants (Go *et al*., 2002; Meyer-Lucht *et al*., 2008).

In conclusion, this is one of the first studies clearly demonstrating contrasting roles of selection and gene conversion in the evolution of MHC class I and class II in wild birds. Our results suggest that macroevolutionary mechanisms can act differently on genes involved in the immune response against intracellular and extracellular pathogens, but more data are needed to generalize this conclusion. Thus, we encourage comparative research on evolutionary histories of MHC class I and class II in other vertebrate systems, as well as studies

comparing the evolution of MHC and other immune genes involved in pathogen recognition.

DATA ARCHIVING

DNA sequences for the newly described MHC alleles will be deposited in GenBank (Accession nos: KU342536-KU342564). DNA sequences for the previously identified alleles are available in GenBank for MHC class I (Accession nos.: JX237361, JX237363–JX237364, JX237366, JX237368–JX237369. KF466475–KF466476, KF466478–KF466479 and KR779965-KR779979) and MHC class II (Accession nos.: FJ232512–FJ232514, FJ232516–FJ232518, GQ176848–GQ176851, HM011573–HM011577, HM011580 and KR779980-KR779993). MHC class I and class II genotypes used in the analyses are in Dryad: doi: 10.5061/dryad.35jt8.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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