Rapid loss of MHC class II variation in a bottlenecked population is explained by drift and loss of copy number variation

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Introduction

Changes in genetic variation following a population bottleneck depend on the interaction of drift, selection, recombination and gene flow (Crow & Kimura, 1970). The outcome of these interactions differs fundamentally between neutral genes and functional genes, which are under selection and often thought to be less affected by drift (Hedrick, 2001; Le Corre & Kremer, 2003). For example, in small populations experiencing genetic drift, variation at neutral loci may decline, whereas variation at expressed genes may be maintained by balancing selection (Hedrick, 2001). Several studies have tested this idea by comparing levels of genetic variation at putatively neutral markers (e.g. microsatellites) and those involved in immune function that are thought to be under relatively strong selection (Garrigan & Hedrick, 2003). One of the most common functional gene families used to examine balancing selection is the major histocompatibility complex (MHC), which plays a central role in vertebrate immunity and may influence population viability (Piertney & Oliver, 2006; Siddle et al., 2007; Radwan et al., 2010).

MHC genes are among the most polymorphic coding loci known in vertebrates (Klein, 1986), which is important for their role in the recognition and presentation of foreign antigens as part of the adaptive immune response. MHC molecules differ in their ability to recognize pathogens due to sequence variation at their peptide-binding region (PBR) (Potts & Wakeland, 1990; Apanius et al., 1997), and associations between particular MHC alleles or haplotypes and parasite resistance have been demonstrated across vertebrate taxa (Briles et al., 1983; Langelfors et al., 2001; Schad et al., 2005). Variation in the MHC is generated at multiple levels. First, the MHC is prone to gene duplication and deletion (Klein et al., 1993; Nei et al., 1997), which results in copy number variation (CNV) among individuals (Hosomichi et al., 2006; Anmarkrud et al., 2010; Doxiadis et al., 2010) as well as between species (Trowsdale, 1995; Kelley et al., 2005). Several models of multigene evolution have been proposed that may explain the evolution of CNV, including the accordion model (Klein et al., 1993), the...
birth-and-death model (Nei et al., 1997) and Associative Balancing Complex evolution (van Oosterhout, 2009). Second, allelic variation at individual loci is generated by recombination and mutation, and this variation is maintained by balancing selection acting on the PBR (Sommer, 2005; Piortney & Oliver, 2006; Spurgin & Richardson, 2010). Selection may also operate against deleterious mutations that have accumulated in the MHC (Shiina et al., 2006), and this can maintain a balanced polymorphism at the MHC as well (van Oosterhout, 2009).

In multigene families, such as the MHC, CNV could be lost due to drift in a similar fashion as allelic diversity is lost at single loci (Cutler et al., 2007; Schrider & Hahn, 2010). Different mechanisms have been demonstrated to generate CNV, including replication slippage, retrotransposition and recombination between duplicated sequences (Schrider & Hahn, 2010). Stochastic removal of particular haplotypes with a relatively large number of gene copies could eliminate several interacting (and co-evolving) genes at once, reducing genetic variation above and beyond that expected from the loss of heterozygosity at a single locus alone. Furthermore, duplicated alleles can occur at multiple loci (Chaves et al., 2010), which means that drift could cause the fixation of the same allele at different loci within the gene family. Such drift-across-loci could further exacerbate the loss of genetic variation during a population bottleneck in multigene families.

Despite the mechanisms generating variation at the MHC, many small populations exhibit low levels of variability, and some are even fixed for single MHC alleles (Hedrick et al., 2000; Miller & Lambert, 2004; Babik et al., 2009; Radwan et al., 2010). This suggests that drift can overwhelm balancing selection, although in some cases, MHC diversity appears to be maintained despite the loss of variation at neutral loci (Aguilar et al., 2004; van Oosterhout et al., 2006a; Mona et al., 2008). It has also been suggested that if genetic variation is depleted through a bottleneck, divergent alleles are more likely to be retained because they will confer resistance to a wider suite of potential pathogens (Hedrick et al., 2002; Hedrick, 2003). To date, no studies have directly compared the genetic diversity of the MHC in a wild population before and after a bottleneck using both historic and contemporary DNA samples. Furthermore, theory and computer simulation models have focused primarily on the effects of bottlenecks on single locus heterozygosity (Borghans et al., 2004; Ejsmond & Radwan, 2009) and do not consider CNV, which may be important in immune function and disease resistance (Siddle et al., 2010).

Here, we examined genetic variation in a population of greater prairie-chickens (Tympanuchus cupido pinnatus) before and after a bottleneck to test the relative effects of genetic drift and balancing selection on putative neutral (microsatellite) and functional (MHC class II B) genes. Over the past century, prairie-chickens have experienced one of the largest declines in range size of any bird species in North America (Anderson & Toepfer, 1999; Johnson et al., 2011), primarily because of habitat loss (Johnsgard, 2002; Johnson et al., 2011). For example, in Wisconsin, the species occurred in nearly every county in the state in the 1930s, but by the 1950s its range was reduced to seven (of 72) counties in the centre of the state. The population in central WI experienced a further decline of 50% during the 1950s to approximately 1200 individuals, and it has remained at that population size for the past 50 years (Anderson & Toepfer, 1999).

Our previous studies of greater prairie-chickens showed that genetic variation at neutral markers, such as microsatellites and the mtDNA control region, declined as a consequence of the bottleneck in Wisconsin (Bellinger et al., 2003; Johnson et al., 2004, 2007). In the present study, we used a subset of the same contemporary and historic samples used in the microsatellite study to directly measure the effect of the bottleneck on allelic variation in MHC class II B genes. We found a loss of MHC polymorphism (number of alleles in the population) similar to microsatellites (Johnson et al., 2004), a pattern that is consistent with the effect of drift. However, the loss of MHC variation within individuals (fewer alleles per individual) significantly exceeded the rate of loss expected from drift. Here, we show that this pattern of loss can be explained by drift acting on the number of loci (CNV) or the fixation of the same duplicated allele across multiple loci (drift-across-loci).

Materials and methods

Sampling and DNA extraction

To measure the change in MHC polymorphism through the population bottleneck, we examined sequences from 40 adults (20 contemporary and 20 historic) from Buena Vista Marsh, Portage County, WI, USA. Our sample consisted of a subset of the same historic (1951–1952) and contemporary (1996–1999) DNA samples that were previously analysed using microsatellites (Bellinger et al., 2003; Johnson et al., 2004) and mtDNA control region sequences (Johnson et al., 2004). The historic DNA was extracted from wings collected by F.N. and F. Hamerstrom in 1951–1952 during the last hunting season in Wisconsin (Bellinger et al., 2003). All historic DNA was extracted in a separate laboratory free of any other DNA or tissue samples from grouse (Johnson et al., 2004).

PCR, cloning and sequencing

We generated a 239-bp amplicon (excluding primers) of the PBR of MHC class II B (BLB) genes using the primers Blex2F (Eimes et al., 2010) and RNA R1 (Strand et al.,
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2007). This fragment begins at position 14 and ends at position 252 of the 270-bp exon 2.

PCRs contained approximately 100 ng of DNA. 0.2 μM of each primer (5’ phosphorylated), 25 μL of Phusion Flash High Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) and water to a total volume of 50 μL. Cycling conditions included an initial denaturation step at 94 °C for two min followed by 30 cycles of 94, 62 and 72 °C each for 30 s and a final extension of 72 °C for 10 min. Target PCR products were gel excised before purification in spin columns (Qiagen-QIAquick, Germantown, MD, USA). PCR amplicons were then cloned using Clonesmart blunt end high count kanamycin cloning kits with chemically competent cells (Lucigen, Middleton, WI, USA). Colonies were diluted in 20 μL of water, and gene products were amplified by whole colony PCR using the vector primers SL-1 and SR-2 in the Clonesmart kit. Whole colony PCR amplifications contained 3 μL of template, 0.625 μM of each primer, 0.5 mM of each dNTP, 1.5 mM MgCl2, 1× PCR buffer and 1.0 U of GoTaq Flexi polymerase in a total volume of 20 μL. The cycling conditions included an initial denaturation at 94 °C for 2 min followed by 30 cycles of 94, 55 and 72 °C each for 1 min and a final extension of 72 °C for 10 min. Sequencing was performed on Applied Biosystems (Carlsbad, CA, USA) 3730XL and 3130 automated DNA sequencers (University of Chicago Cancer Research Center). Sequences were deposited in Genbank (accession numbers FJ232512–FJ232514, FJ232516–FJ232520, GQ176848–GQ176851 and HM011573–HM011586).

RFLP and Southern blot

To estimate the number of MHC class II loci and to verify CNV, we performed a random fragment length polymorphism (RFLP) analysis using a probe that binds specifically to MHC class II of eight contemporary individuals. We digested seven micrograms of gDNA for 3 h with 20 units of PvuII-HF (New England BioLabs Inc., Ipswich, MA, USA), an enzyme that has been used successfully in other avian MHC studies (e.g. Miller & Lambert, 2004; Balakrishnan et al., 2010). The digested DNA was run on a 0.8% agarose gel for 19 h at 60 V in 0.5× Tris-Borate-EDTA buffer and then transferred by Southern blotting to a Hybond (GE Healthcare Life Sciences, Piscataway, NJ, USA) membrane following the manufacturer’s instructions. The blot was prehybridized at room temperature overnight in 50% formamide, 5× standard saline citrate (SSC), 10× Denhardt’s solution, and 250 ng/mL yeast RNA. The blot was then hybridized at 42 °C overnight with 32P-labelled 334 bp probe using random primer labelling. The probe was designed from an ampiclon of a contemporary individual using the primers Blex2F and E3R (Chaves et al., 2010) that extended from the 108th bp of exon 2 to the 85th bp of exon 3 using the first PCR conditions described previously. The homology of this PCR product to Galliform MHC Class II genes was verified by cloning and sequencing as described previously and by performing a BLAST (NCBI, Bethesda, MD, USA). The hybridization buffer was the same as the prehybridization, with the addition of dextran sulphate to a concentration of 10%. The blot was washed in 0.5× SSC, 0.1% sodium dodecyl sulfate (SDS) by rinsing briefly at room temperature, followed by two 30 min washes at 65 °C with a buffer change between each wash. Autoradiography was carried out for 24 h on Kodak (Rochester, NY, USA) BioMax film at −80 °C using an intensifying screen.

Sequence analysis

Ratios of nonsynonymous (d_S) and synonymous (d_K) substitution rates are often used as a test for (historic) selection at MHC loci, with d_S/d_K ratios greater than one suggesting balancing or positive selection (Garrigan & Hedrick, 2003). We tested for positive selection at peptide-binding codons (PBCs) and non-PBCs in MHC class II B sequences using the modified Nei-Gojobori/ Jukes-Cantor method (Nei & Gojobori, 1986) in MEGA v. 4 (Tamura et al., 2007). PBCs in our sequences were identified using the position of PBCs in HLA II (Brown et al., 1993).

Average pair-wise nucleotide diversity (d) and segregating sites (S) were calculated with DNASP 4.50.3 (Rozas et al., 2003). In addition, a phylogenetic network
was constructed using the program SplitsTree4 (Huson & Bryant, 2006) and employed the Neighbour-Net method (Bryant & Moulton, 2002) with Jukes-Cantor distances.

**Loss of genetic variation**

Genetic variation was expressed as the number of distinct alleles (or sequences) in the population sample (Ap) and the number of distinct alleles (sequences) per individual (Ai). These measures were computed by summing the alleles across loci, which allowed us to directly compare the temporal loss at the MHC with that at microsatellite loci. Mann–Whitney U tests were used to analyse whether the population bottleneck reduced Ai between temporal samples. The relative loss of Ai was calculated as the contemporary Ai divided by the mean historic Ai. The relative loss of Ai was compared between the MHC and microsatellite loci using a randomization test with 10,000 iterations to simulate the loss of MHC and microsatellite alleles.

**MHC simulations**

We simulated the loss of MHC diversity using an individual-based model and several demographic scenarios from molecular estimates of Ne for the Buena Vista population (Johnson et al., 2004). These values ranged from a maximum value of Ne = 1500 birds before the bottleneck down to an estimated Ne = 26 in the contemporary population for t = 30 generations (Johnson et al., 2004). Simulations focused on the loss of number of unique MHC sequences at the population level (Ap), as well as the loss of alleles per individual (Ai).

We first examined the hypothesis that drift within loci can explain our results by testing whether the loss of MHC variation was due to simulated population bottlenecks with Ne = 26, 50, 100, 500 and 1500 over 30 generations. We assumed there were five loci (based on the maximum of 10 alleles found in one individual; see Results later) that were completely linked and present in all individuals. The simulations took into account the probability of not observing all alleles per individual due to sequencing a finite number of clones (N = 23).

Drift within loci did not completely explain our results (see later), so we tested a second hypothesis that genetic drift accelerates the loss of genetic variation in multigene families due to the loss of CNV. For this hypothesis, we simulated the effects of genetic drift on: (i) the number of alleles within a single locus, (ii) fixation of the same allele across multiple loci and (iii) stochastic changes in CNV. If the same allele or sequence occurs at different loci, drift may not only reduce gene diversity (i.e. heterozygosity) within loci, but it can also fix the same allele at different loci. This can reduce the apparent copy number, and we refer to this as the ‘drift-across-loci’ hypothesis. For example, an individual may have five MHC loci, but if drift fixes all these loci for the same allele, its multilocus genotype is identical to an individual that is homozygous for a single locus. Haplotypes were constructed by randomly allocating 1 ≤ k ≤ 5 loci per individual, and drawing k from a uniform distribution. Alleles were then randomly allocated to these loci by sampling from the allele frequency distribution observed in the prebottleneck population. Hence, some alleles could occur at multiple loci, which introduced the possibility that the simulated loss of alleles per individual (Ai) could be affected by fixation or loss of alleles at more than one locus (drift-across-loci). Next, genotypes were generated by randomly combining the MHC haplotypes, and simulations were run with the same demographic scenarios and simulated sequencing effort (23 clones/individual), as described previously. The mean (95% CI) simulated Ap and Ai values were then compared with the values observed in the historic and contemporary population.

The drift-across-loci hypothesis provided a good fit to our results, but for completeness we also tested a more complex third hypothesis that a combination of genetic drift, balancing selection (s), gene conversion (c) and migration (m) could explain our results. Ultimately, we rejected this hypothesis because extremely high levels of gene conversion were required to explain our results (Fig. S1). Simulations were run 1000 times using a macro written for Minitab 12.1 (available from the authors upon request).

**Results**

**Loss of population variation (Ap)**

Genetic drift following the population bottleneck led to a reduction in MHC class II variation. The contemporary greater prairie-chicken population had only 14 distinct MHC class II B alleles, which was significantly fewer than the 22 alleles in the historic population (randomization test: P < 0.001). In total, we recovered 24 distinct sequences (alleles) from the combined populations: 10 were unique to the historic population, two were unique to the contemporary sample and 12 were present in both samples (Fig. 1). The loss of less common alleles in the population was consistent with that expected from genetic drift (Fig. 2).

The population-level loss of MHC variation, as measured by the loss of alleles across all class II B loci, was 33% (8 lost/24 total). We found previously that the number of haplotypes at the mtDNA control region declined by 50% from 10 in the historic period (haplotype diversity: 0.889 ± 0.013, N = 19) to five in the contemporary period [0.511 ± 0.128, N = 20; (Johnson et al., 2004)]. There was also a 26% reduction in number of alleles at microsatellite loci from the historic (mean of 8.2 ± 1.3 alleles/locus, N = 42) to the contemporary (mean of 6.1 ± 1.1 alleles/locus, N = 87) period when accounting for differences in sample size (Johnson et al., 2004). Thus, the loss of MHC genetic variation, at least
at the population level, was comparable to that found previously using putatively neutral markers in many of the same individuals (Johnson et al., 2004).

The historic and contemporary populations did not differ in average nucleotide divergence at either the PBCs (mean \( p \)-distance for both populations = 0.02 ± 0.03) or over the entire 239-bp fragment (mean \( p \)-distance for both populations = 0.09 ± 0.01). The contemporary and historic sequences intermixed in the network showing similar divergence among alleles in both samples (Fig. 3).

The ratio of nonsynonymous and synonymous substitution rates \( (d_{N}/d_{S}) \) at the PBCs (2.02) was significantly larger than one (codon-based \( Z \) test, \( Z = 2.01; P = 0.047 \)) for the contemporary population and approached significance for the historic sample (codon-based \( Z \) test, \( Z = 1.74; P = 0.062 \); Table S1).

**Loss of individual variation (Ai)**

Overall, the number of distinct MHC alleles per individual (Ai) declined by 44%, from a mean (±SD) of 6.20 (±2.14) in the historic population to 3.45 (±1.88) in the contemporary population (Mann–Whitney \( U \) test, \( Z = 3.48, P < 0.001 \); Fig. S2). For microsatellites, however, the number of alleles per individual (summed across six loci) declined by only 8% from 10.19 (±1.17) in the historic to 9.34 (±1.15) in the contemporary samples (Fig. S2).

**RFLP analysis**

Individuals had between two and four RFLP fragments, indicating at least four loci in some individuals. The banding pattern suggested MHC class II CNV as there were at least three distinct haplotypes among the eight individuals screened (Fig. S3).

**Computer simulations**

We first tested the hypothesis that genetic drift at five loci in combination with failed amplification of some alleles could explain the empirical results mentioned earlier (within-locus drift hypothesis). The simulation showed that even in the most extreme bottleneck scenario (\( N_e = 26 \)), the simulated number of alleles per individual (Ai = 5.61) remained considerably higher than the observed value (Ai = 3.45; Fig. 4). Therefore, we rejected the hypothesis that drift within loci in combination with the stochastic failure to amplify alleles could explain our results.

Next, we examined the drift-across loci hypothesis by simulating the effects of genetic drift on CNV and allowing for variation in the number of loci (between 1 and 5 per individual drawn from a uniform distribution). In this simulation, identical alleles can occur at multiple loci and drift can act across these loci. These simulations...
showed that the observed value of Ai (3.45) in the contemporary post-bottleneck population fell within the distribution of simulated values (mean = 4.69, 95% CI = 1.7–7.9; Fig. 5). The observed variance in Ai between individuals (3.53), which is a measure of CNV, also fell within the 95% CI (1.63–4.62) of variances in Ai of individuals from the simulations. Furthermore, the distribution of simulated Ap values (mean = 11.5, 95% CI = 5–16) included the observed Ap (14) of the contemporary population. Thus, our computer simulations support the hypothesis that genetic drift reduced variation both within and between loci to produce the large decline in number of MHC alleles per individual (Ai) and the relatively moderate decline in the number of alleles in the population (Ap).

**Discussion**

Greater prairie-chickens in Wisconsin lost a significant number of MHC class II alleles following a population bottleneck in the late 1950s. This finding is consistent with our previous study of the same population using microsatellites and mtDNA (Johnson et al., 2004), and it is consistent with random genetic drift. However, we also observed a dramatic loss of MHC variation at the individual level (the number of unique sequences per individual, Ai), and we showed that this loss was significantly greater for the MHC (44%) than for six (neutral) microsatellite loci (8%). We then used an individual-based model to test whether this loss of Ai could be explained by drift acting simply within loci, but rejected this hypothesis. We tested a more complex multilocus drift hypothesis, which proposes that drift reduced gene diversity (heterozygosity) within loci, as well as diversity between loci, by either fixing the same allele at different loci or by eliminating haplotypes with high gene copy number. Our simulations show this can result in a loss of CNV, and our empirical data appear to be best explained by this hypothesis.

The observed loss of MHC diversity at the population level is consistent with the results from other fragmented or bottlenecked populations (Radwan et al., 2010), as well as with simulations of bottlenecked populations (Ejsmond & Radwan, 2009). These studies show that selection at the MHC does not necessarily maintain variation over relatively short time scales. In contrast, other studies of bottlenecked populations have found evidence that selection may have maintained MHC variation (Aguilar et al., 2004; van Oosterhout et al., 2006a). In greater prairie-chickens, the $d_s/d_A$ ratios suggested selection at the MHC class II B region, but it is probably the result of selection over an evolutionary time scale (Eimes et al., 2010), rather than just the 50 years between our historic and contemporary samples. It has been suggested that if allelic variation is lost through a bottleneck, then alleles that are more

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**Fig. 3** Phylogenetic network of major histocompatibility complex class II B sequences (based on 239 bp) from four Galliform species: the greater prairie-chicken (Tycu; *Tympanuchus cupido*), red jungle fowl (Gaga; *Gallus gallus*), Japanese quail (Coja; *Coturnix japonica*) and domestic turkey (Mega; *Meleagris gallopavo*). Greater prairie-chicken sequences found only in the contemporary population are in boxes, sequences occurring only in the historic population are in bold and the remaining sequences are present in both time periods. All of the *Gallus* (AM489767–AM489775), *Meleagris* (DQ993255) and *Coturnix* (AB078884, AB265804 and AB282649–AB282651) sequences were downloaded from Genbank and originate from mapped loci.
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The MHC appears to follow a birth-and-death model of evolution, in which new genes arise via gene duplication and genes may be lost by deletion (Nei et al., 1997; Nei, 2005). This results in variation in MHC gene number between haplotypes both within (Hosomichi et al., 2006; Doxiadis et al., 2010) and across species (Trowsdale, 1995; Kelley et al., 2005). In the Japanese quail (Coturnix japonica), Hosomichi et al. (2006) identified between one and six transcribed class II B loci per haplotype. Our RFLP analysis indicates that MHC class II locus number is variable in greater prairie-chickens. Therefore, drift could have caused an increase in the relative frequency of haplotypes with fewer loci and a decrease in individual level MHC variability (Fig. 5). Additionally, identical alleles may have been present at multiple loci, as has been found in turkeys (Chaves et al., 2010) and guppies (McMullan, 2010), and drift could have caused multiple loci to become homozygous for the same alleles.

The roles of selection and drift in generating CNV are just beginning to be studied, particularly in natural populations (Nozawa et al., 2007; Schrider & Hahn, 2010). MHC copy number has been reduced and become fixed among inbred strains of laboratory mice (Cutler et al., 2007) and isolated populations of ornamental guppies (Poecilia reticulata; van Oosterhout et al., 2006b). In the Tasmanian devil (Sarcophilus harrisii), which is endangered as a consequence of a contagious cancer, MHC class I diversity is low and most of the variation is attributable to CNV, rather than sequence polymorphism (Siddle et al., 2010). Interestingly, Siddle et al. (2010) speculated that devils with a lower number of gene copies might be most resistant to the cancer, possibly as a consequence of a selective sweep. Our study suggests that a reduction in the number of gene copies may also occur simply through drift in the absence of any selection.

In conclusion, there was a significant loss of MHC class II B diversity following a population bottleneck in this Wisconsin population of greater prairie-chickens. The bottleneck decreased both the number of alleles in the population (Ap) and the average number of alleles present in individuals (Ai). Computer simulations suggest that these results can be explained by drift acting on the within- and between-locus gene diversity, as well as CNV between haplotypes. The loss of alleles could have been caused by fixation of the same allele across multiple loci or a reduction in the number of haplotypes with high divergent alleles.
gene copy numbers. Changes in CNV at the MHC are related to disease in humans and laboratory animals (Aldhous et al., 2010; Cahan & Graubert, 2010; Ye et al., 2010), but they are relatively unstudied in wild populations and could be an important contributor to population viability.

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References


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Additional Supporting Information may be found in the online version of this article:

**Figure S1** The mean (±95% CI) observed number of MHC alleles per population vs. the mean (±95% CI) observed number of MHC alleles per individual for the


historic sample (solid circle) and contemporary sample (open circle).

**Figure S2** The relative loss in number of alleles per individual ($A_i$) for MHC (solid circles) and microsatellites (open circles) expressed as $A_i$ divided by mean historic $A_i$ of the corresponding gene.

**Figure S3** RFLP/Southern blot of eight contemporary greater prairie chickens.

**Table S1** Variation in MHC class II B sequences of greater prairie-chickens before (Historic) and after (Contemporary) the population bottleneck.

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