Genetic variation of major histocompatibility complex (MHC) in wild Red Junglefowl (*Gallus gallus*)

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ABSTRACT The major histocompatibility complex (MHC) is a multi-family gene cluster that encodes proteins with immuno-responsive function. While studies of MHC in domesticated poultry are relatively common, very little is known about this highly polymorphic locus in wild Red Junglefowl (*Gallus gallus*), the natural progenitor of domestic chickens. We investigated the diversity of MHC within and among four wild Red Junglefowl populations across diversified natural habitats in South Central Vietnam. Based on a SNP panel of 84 sites spanning 210 Kb of the MHC-*B* locus, we identified 310 unique haplotypes in 398 chromosomes. None of these haplotypes have been described before and we did not observe any of the wild Red Junglefowl haplotypes in domesticated chickens. Analysis of molecular variance (AMOVA) revealed that 94.51% of observed haplotype variation was accounted for at the within individual level. Little genetic variance was apportioned within and among populations, the latter accounting only for 0.83%. We also found evidence of increased recombination, including numerous hotspots, and limited linkage disequilibrium among the 84 SNP sites. Compared to an average haplotype diversity of 3.55% among seventeen lines of domestic chickens, our results suggest extraordinarily high haplotype diversity remains in wild Red Junglefowl and is consistent with a pattern of balancing selection. Wild Red Junglefowl in Vietnam, therefore, represent a rich resource of natural genomic variation independent from artificial selection.

Key words: Red Junglefowl, Major Histocompatibility Complex, Adaptive variation, Balancing selection, Biodiversity

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INTRODUCTION

Our current livestock diversity originated from wild ancestors by altering the genome of these animals through a domestication process (artificial selection) over many thousands of years. Intensified contemporary selection programs, while very successful in improving agriculturally desirable traits, may have created a potential predicament: the extent of genetic variation within and among livestock breeds, strains, and lines—the cornerstones of agricultural diversity—have rapidly eroded in the past few decades (FAO, 2007; Groeneveld et al., 2010). Commercial poultry production relies on a few highly selected lines derived from a small number of breeds, replacing indigenous and non-commercial heritage breeds, and eliminating specialized research breeds in academia and industry (Fulton and Delany, 2003; Delany, 2006).

The current genetic composition of domesticated livestock contains only a small fraction of the genetic diversity present their wild ancestors, most of which are now unknown, endangered, or extinct. This situation is very different from species of crop plants whose ancestors often still remain in the wild, typically at the centers of their origin. Availability of the wild progenitors provides an invaluable source of genetic variation accessible for contemporary and future breeding initiatives (Tanksley and McCouch, 1997). If the wild progenitors of domesticated animals are known and accessible, maintenance of genetic diversity, for example, to combat disease and provide adaptive potential to changing environments, not only remains vital for the health of the natural populations but also presents a currently untapped source of genetic information to maintain and improve current and future agricultural diversity in livestock breeds and lines.

In this respect, Red Junglefowl (*Gallus gallus*) is an important species, representing one of the few remaining identifiable and direct ancestors to a domesticated animal line. Before spreading globally through human-mediated dispersal (Storey et al., 2012), chicken domestication is believed to have initiated in South and Southeast Asia from wild Red Junglefowl, perhaps with inclusion of another junglefowl lineage (Fumihito et al., 1994; Eriksson et al., 2008). Wild populations of Red Junglefowl still occur naturally in their native ranges

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(Johnsgard, 1999; Brisbin et al., 2002), a sizeable region extending from Southeast to Central Asia. The habitat preference of Red Junglefowl is broad but consists primarily of lowland tropical rainforests below an elevation of 600 meters. Such habitats throughout the Asian continent have been greatly modified in recent decades (Fuller and Garson, 2000).

In studies of Red Junglefowl, it is imperative to emphasize the importance of documenting their sampling origin, particularly in context with previous studies where samples from so-called "wild" Red Junglefowl were taken for DNA analysis. Japp and Hollander (1954) first proposed that the Red Junglefowl be the standard genetic wild type. However, in most, if not all, cases of previous genetic studies, wild junglefowl samples from Southeast Asia were obtained from captive (zoological park) birds captured from undocumented geographic localities (e.g., Berlin et al., 2008; Berthouly et al., 2010; Fumihito et al., 1994; Granevitze et al., 2007; Ngo et al., 2010; Worley et al., 2010; Mekchay et al., 2014). Even the Red Junglefowl female used to create the *Gallus gallus* reference genome sequence traces to the San Diego Zoo population, itself believed to be introgressed with White Leghorn alleles (M. E. Delany, University of California, Davis, CA; personal communication). The behavior of Red Junglefowl is extreme compared to many other species in the Phasianidae; it simply does not tolerate captivity (personal observation, Collias and Collias, 1996; Brisbin et al., 2002; Codon, 2012). Thus, any Red Junglefowl obtained from captive populations must have been crossed with domestic lines in order to maintain them. Indeed, only after 3 or 4 generations of crossing wild male Red Junglefowl to female domestic chickens (e.g., gà tre, "Bamboo chicken", a small Vietnamese heritage breed) will offspring survive and eventually tolerate a human presence (H. Nguyen-Phuc and M. E. Berres, unpublished data). Efforts to maintain wild-type Red Junglefowl have been attempted but apparently always end in failure, possibly due to genetic incompatibilities and/or specific nutrient deficiencies early after hatching (H. Nguyen-Phuc and M. E. Berres, unpublished data).

In chickens, the major histocompatibility (**MHC**) *B* locus occurs on chromosome 16 and possesses different gene content and organization than mammalian MHC. Nevertheless, many specific MHC genes exhibit functional equivalency (Guillemot et al., 1989; Kaufman et al., 1999a,b; Afanassieff et al., 2001; Rogers et al., 2003; Hunt et al., 2006). In contrast to domestic lines, the MHC complex in wild Red Junglefowl likely experiences intense balancing selection (negative frequency-dependent selection) (Hedrick, 1998), because it is subject to a large and possibly dynamic pathogen repertoire. Comparison of MHC structure and function in both wild and domestic individuals offers a model to study adaptive evolution at the molecular level (Edwards and Hedrick, 1998).

Historically, the most convincing associations between specific MHC haplotypes and pathogen response for any species are seen with domestic chickens. In chickens, specific MHC-*B* haplotypes have strong association with viral infection. Resistance to Marek's disease (Bacon, 1987) approaches 95% if an individual possesses the B^{21} haplotype yet is 0% with the B^{19} haplotype (Cole, 1968). The close relationship between MHC haplotype and role in disease resistance may also in part be due to its more compact synteny and absence of non-immune related genes (Kaufman et al., 1999a,b). But despite extensive study in commercial flocks (e.g., Fulton et al., 2006; Guangxin et al., 2014), as well as in non-commercial breeds (e.g., Izadi et al., 2011), MHC studies in wild Red Junglefowl remain rare, and those that do exist likely do not represent ancestral haplotypes.

We hypothesize that wild Red Junglefowl possess great diversity in their MHC-*B* haplotypes, in part due to pathogen inventories modulated by differences in geographical location and habitat types. Our hypothesis is further conditioned on the fact that commercial selection may reduce allelic diversity within domesticated chicken lines (e.g., Fulton et al., 2006; Muir et al., 2008, unpubl. data). Thus, extant populations of wild Red Junglefowl may still maintain a large variety of ancestral alleles. Calling attention to the genetic value of wild junglefowl populations by studying the population genetic structure and gene flow among natural junglefowl populations (H. Nguyen-Phuc and M. E. Berres, unpublished data) will inform landscape management efforts and help to conserve these wild populations.

Using an extensive single nucleotide polymorphism (**SNP**) panel spanning 210 Kb of the MHC-*B* locus (Fulton et al., 2016), this study examined levels of MHC diversity and variation in four natural populations of wild Red Junglefowl sampled from South Central Vietnam. Here, we present a study that specifically examines adaptive genetic diversity in the MHC-*B* region in wild Red Junglefowl obtained from four geographically and ecologically diverse habitats in South Central Vietnam.

MATERIALS AND METHODS

Field Sampling

During three dry seasons in 2012, 2013, and 2014, we live-captured Red Junglefowl with non-lethal walking snares (Bub, 1991) modified with local trapping customs. Domestic decoy roosters ("baiting cocks") trained to produce territorial vocalizations augmented our trapping efforts. A total of 199 birds were sampled from four unconnected sites. The sampling sites (average area = 50,000 ha) included Cát Tiên National Park and Đông Nai Cultural and Heritage Reserve (hereafter **CTN**, as the two sites are adjacent in the southwest) (CTN; n = 46), Hòn Bà Nature Reserve (**HBA**; n = 56), Lò Gò Sa Mát National Park (**LGO**; n = 39), and

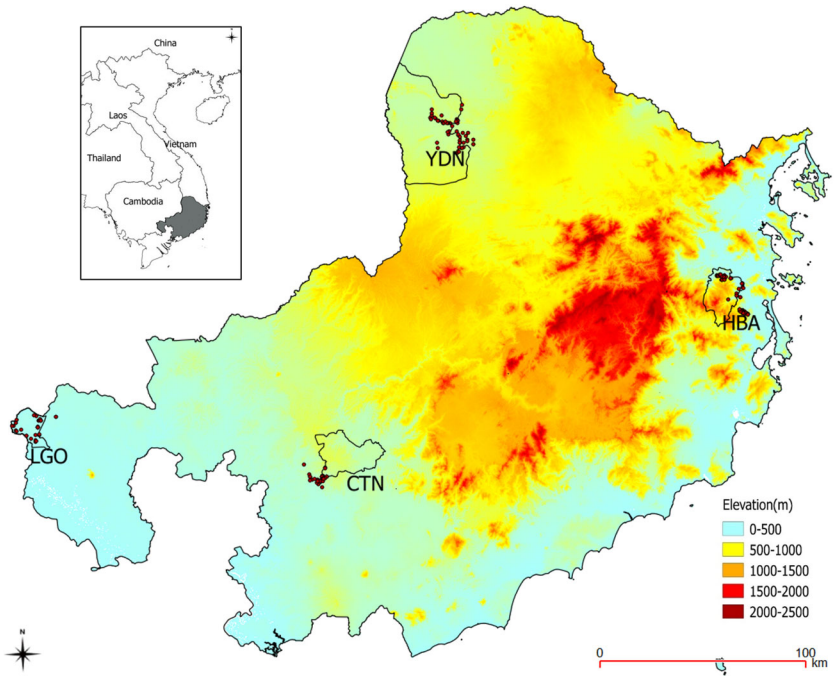

Figure 1. Location of Red Junglefowl sampling sites in South Central Vietnam. Cát Tiên National Park and Đồng Nai Cultural and Heritage Reserve (hereafter CTN, as the two sites are connected), Hòn Bà Nature Reserve (HBA), Lò Gò Sa Mát National Park (LGO), and Yok Đôn National Park (YDN).

Yok Đôn National Park (**YDN**; n = 58) (Figure 1). Site selection was based on the presence of suitable junglefowl habitat and a relatively symmetrical distance from the Annamite Mountain Range, a putative dispersal barrier. Two sites, CTN and LGO feature lowland tropical rainforest while HBA and YDN consisted of upland mixed deciduous forest and grassland. All sites were separated from each other by approximately 180 km and intervened with residential and agricultural habitats unsuitable for Red Junglefowl occupancy.

Like most other pheasant species (Phasianidae), Red Junglefowl occupy terrestrial habitats, often in forest understories containing bamboo (Poaceae). Morphological and demographic characters including elliptical wings, polygynous breeding system, and female promiscuity suggest Red Junglefowl have limited migration potential and natal dispersal is the primary mode of gene flow (Johnsgard, 1999). In a related study, Nguyen-Phuc and Berres (in preparation) established genetically a home range of approximately 5 km. For the current study, we sampled 163 roosters, 19 hens, and 17 juvenile chicks (< 3 months old, with rearing female). Average capture rates were estimated to be 0.92 bird per work day as the junglefowl were extraordinarily elusive, despite being strongly territorial during their mating season. We initially attempted to capture equal numbers of males and females. However, the use of snares alone had very low capture efficiency and required substantially more effort to check and maintain. With the assistance of decoy roosters, we had greater capture efficiency but mainly lured and captured the territorial male Red Junglefowl. The highest densities of Red Junglefowl occurred in bamboo-dominated forests where clear understory forest floors and seasonal natural fires provided sprouting food sources and predator-escape clearance for the ground-dwelling birds.

All birds sampled showed key phenotypic and behavioral characteristics consistent with pure (i.e., not introgressed with domesticated chickens) wild-type Red Junglefowl as previously described in their native range in Southeast Asia (Beebe, 1926; Delacour, 1977). They exhibited slender, blackish tarsi in both sexes, longer spur-lengths in male roosters, and complete absence of a comb in adult female hens. Morphologically, the males undergo a summer pre-basic molt of the neck hackles to an overall dark "eclipse" plumage following the breeding season, generally June–September, during the rainy season (Brisbin et al., 2002). The timing of capture efforts during the breeding season did not allow us to evaluate the presence of eclipse plumage for each male. In their natural habitats, Red Junglefowl are extremely timid and do not tolerate the presence of humans. However, we were able to visually record eclipse plumages from multiple males (without trapping) at each field site during late summer.

Collection of Genomic DNA and Genotyping

For each captured bird, 20 to 200 μL of blood was obtained from a brachial vein puncture and stored in a lysis buffer (0.1 M Tris-HCl pH 8.0, 0.01 M EDTA, 5% SDS) (Longmire et al., 2000). All individuals were marked with uniquely numbered aluminum leg bands. Genomic DNA was extracted from blood using the Promega Wizard DNA Isolation kit (Promega Corp.,

Madison, WI). The extraction quality was assessed visually with 1% agarose gel electrophoresis to confirm non-degraded, high molecular weight DNA.

DNA was genotyped at 84 sites distributed across a 210,744 base pairs (**bp**) of the MHC-*B* locus using a SNP panel originally described by Chazara et al. (2010) and subsequently modified (Fulton et al., 2016). This region ranges from Bzfp3 to CD1A1 and contains more than 40 described genes (GenBank AB268588). Originally sequenced by Shiina et al. (2007), this sequence established a framework for comparative MHC genomics in avian species (e.g., Hosomichi et al., 2006). These SNPs were adapted to the Kompetitive Allele-Specific PCR (**KASP**) high-throughput genotyping platform (Semagn et al., 2014), to create a panel of SNPs with high reliability for diverse sources including commercial lines of layers, broilers, and heritage chicken breeds (Fulton et al. this issue).

Haplotype Analyses

Since chickens are diploid organisms, the determination of a haplotype from a set of genotypic SNPs is not immediately possible. In the absence of extended pedigrees (which are rarely available for wild populations) construction of haplotypes from genotypic SNP information requires statistical inference (Browning and Browning, 2011). We used *PHASE* 2.1.1 (Stephens et al., 2001), a software that yields Bayesian estimates of haplotypes and their frequencies from genotypic data under the assumption of random mating from genotypic SNP data (simultaneously using all the 84 SNPs). During the haplotype reconstruction process, each allele in a SNP genotype is assigned to one or the other parental chromosome by estimating likelihoods of candidate haplotypes under a coalescent model (Stephens et al., 2001). The MHC haplotype reconstruction analyses were restricted to include only the four largest populations of Red Junglefowl sampled due to concerns that the haplotype phasing accuracy decreases with smaller sample sizes (Browning and Browning, 2009, 2011).

We also used *PHASE* to estimate the recombination rate ρ between SNPs across the study MHC region. Here ρ is the factor by which the recombination rate between any two loci exceeds the background population recombination parameter $\hat{\rho} = 4Ner$ (Posada, 2002; Rokas et al., 2003) itself estimated from the SNP panel data. Advantageously, *PHASE* generates a posterior probability distribution of the recombination parameter and can be checked for convergence of the sampling algorithm. When estimating recombination rates, we employed the MR model in *PHASE*, which makes explicit allowance for intragenic recombination. Runs consisted of 1,000 iterations as a burn-in, 1,000 secondary iterations, and a thinning interval of 1. The commonly cited value of $r = 0.0004$ per site (equivalent to 1 recombination event per million bp per generation) was used as the initial starting point. Each dataset was run 10 times with a different starting seed, and checked for convergence by checking consistency among haplotype frequency estimates and the goodness-of-fit measure for each of the 10 runs. The final haplotype assignments were taken from the replicate with the best average goodness-of-fit.

The presence and significance of recombination hotspots (sites exhibiting levels of recombination much larger than the background rate) was evaluated with *SequenceLDhot* (Fearnhead, 2006) using the recombination estimates computed by *PHASE*. This method used an approximate marginal likelihood method of Fearnhead and Donnelly (2002) to detect recombination hotspots from haplotype data. A likelihood ratio (LR) statistic for putative hotspots was calculated in regions spanning 2 Kb incrementing by 1 Kb. The nearest 7 SNPs were included for each LR test. If the LR exceeds a chosen recombination rate, the SNP or SNPs associated with that site are indicative of a recombination hotspot. A value of 10 corresponds to a false-positive rate of < 1 hotspot in 1.2 Mb. As our coverage region is much smaller (approximately 1/5), we chose a value of 5 because the test as originally formulated may be too conservative. A plot of the LR statistics can be visualized to assess differences in recombination at different positions along the MHC-*B* locus.

For measures of polymorphism and neutrality, we employed the software *DNAsp* 5.10.1 (Librado and Rozas, 2009) to calculate basic sequence statistics including haplotype diversity (H_d), nucleotide diversity (π) and Tajima's *D*. The *D* statistic (Tajima, 1989) is commonly used to distinguish between a neutrally evolving sequence from one evolving under a non-random process, e.g., directional or balancing selection. The number of pairwise differences between haplotypes (Rohlf, 1973) was also computed and based on a parsimony distance criterion, and used to create a minimum spanning tree to depict genetic distances among the haplotypes found in each population (Prim, 1957). The minimum spanning tree for the sampled MHC haplotypes was created using the online service "Interactive Tree of Life" (Letunic and Bork, 2011).

To capture the strength of linkage disequilibrium (LD) between pairs of SNPs in the MHC panel, we first computed for each SNP locus an exact test of Hardy Weinberg equilibrium (HWE) (Wigginton et al., 2005). Next, we estimated LD between each consecutive SNPs with the pairwise disequilibrium coefficient D' (Lewontin, 1964) using the software *Haploview* 4.2 (Barrett et al., 2005). Although LD is preferably estimated using high-frequency polymorphisms (Reich et al., 2001) we accepted the default parameters to include SNPs showing a minor allele frequency of at least 0.05. To define a set of consecutive sites between which there is little or no evidence of recombination—a haplotype block—we used the D'-based criteria of Gabriel et al. (2002) as implemented in *Haploview*, for each Red Junglefowl population separately.

Table 1. Haplotype diversity of Red Junglefowl sampled in four sites.

Site	CTN	HBA	LGO	YDN
Sample	46	56	39	58
Total chromosome number	92	112	78	116
Unique haplotypes	92	82	48	91
Segregating sites S	76	76	71	75
Haplotype diversity H_d	100%	98.97%	97.86%	99.39%
Nucleotide diversity π	0.2926	0.2846	0.2790	0.2878
Expected heterozygosity H_E	0.2894	0.2821	0.2753	0.2854
Tajima's D[1]	2.1236	2.1381	2.0641	2.2986

[1]All Tajima's D for neutrality are statistically significant ($P < 0.05$).

Finally, we estimated how genetic variance was partitioned within and among the MHC haplotypes in the four Red Junglefowl populations using an analysis of variance framework (Weir, 1996) for molecular data (**AMOVA**; Excoffier et al., 1992) implemented in the software *Arlequin* 3.5 (Excoffier and Lischer, 2010). This technique treats haplotype distances as deviations from an estimate of the group mean, and uses the squared deviations as an estimate of the variance. Significance of the covariance components associated with the three levels of genetic structure was tested in our data (haplotypes within individuals, haplotypes within populations, and haplotypes among populations). *Arlequin* also estimated covariance components to compute fixation indices, including F_{ST} among the four sampled populations. Statistical significance was tested by permuting individual genotypes among populations 10,000 times.

RESULTS

MHC Haplotype Variation

The KASP platform genotyped 84 loci distributed across 210,744 bp region of the MHC-*B* locus in 199 Red Junglefowl (398 chromosomes). Averaging 1 locus per 2.9 Kb, 79 sites (94%) were polymorphic. Haplotypes inferred by *PHASE* met or exceeded 85% for all SNPs. Stratified by each population, a total of 313 haplotypes were identified (Table 1). Three of these haplotypes (0.96%) were shared between populations: *haplotype 180* was shared between the sites HBA and LGO, *haplotype 178* between HBA and YDN, and *haplotype 241* between LGO and YDN. No sampling site shared any haplotypes with CTN. Thus, 310 unique haplotypes distributed among 398 chromosomes (78%) were identified, indicating extraordinarily high haplotype diversity in wild Red Junglefowl. All of the 310 unique haplotypes found in Red Junglefowl sampled in Vietnam, to the best of our knowledge, have not been previously reported in domestic chickens, either commercial lines or heritage breeds (see below).

Within each population, MHC haplotype diversity (H_d) was also considerable. All 92 haplotypes in CTN were unique to that locality. In the remaining three sites, H_d also approached 100% in HBA (99%; n = 112), LGO (98%; n = 78), and YDN (99%; n = 116) (Table 1). A few haplotypes occurred at higher frequencies in HBA, LGO, and YDN. In these samples, 8, 6, and 5 occurrences of the same haplotype were observed, respectively. The highest haplotype frequency recorded occurred in HBA where haplotype *147* was found in 8 of 112 chromosomes (7.14%).

Nucleotide diversity in the MHC-*B* locus was substantial and consistent in each population, averaging approximately 28%. The same was true for estimates of Tajima's D (average = 2.1, $P < 0.05$) showing strong evidence that the MHC-*B* locus in Red Junglefowl departed from expectations of neutrality (Table 1) and is consistent with a pattern of balancing selection.

Estimates of MHC Recombination

The overall background population recombination parameter $\hat{\rho}$ in the MHC-*B* locus in the four sampling populations was low, but consistent across each population: CTN: 0.0065; HBA: 0.0020; LGO: 0.0013; YDN: 0.0024 recombination events per bp per generation (Table 1). The recombination rates between SNP pairs was neither consistent across the MHC region nor was it similar between populations (Figure 2). In general, the region spanning approximately 180 to 235 Kb exhibited the lowest estimates of recombination in all four populations. The least amount of recombination occurred in the LGO population perhaps due to the fact that the number of haplotypes identified were nearly half that in the other three sampling sites. This occurrence was not a consequence of haplotype diversity since it was similar in magnitude. Rather, the sample size (specifically the number of chromosomes sampled)

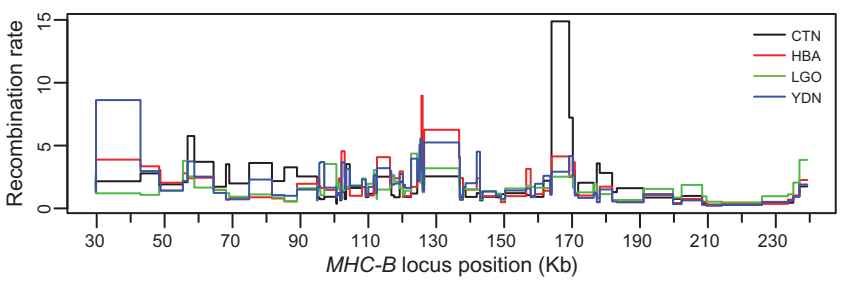

Figure 2. Recombination rates between 84 SNP sites spanning the MHC-*B* locus in four wild populations of Red Junglefowl.

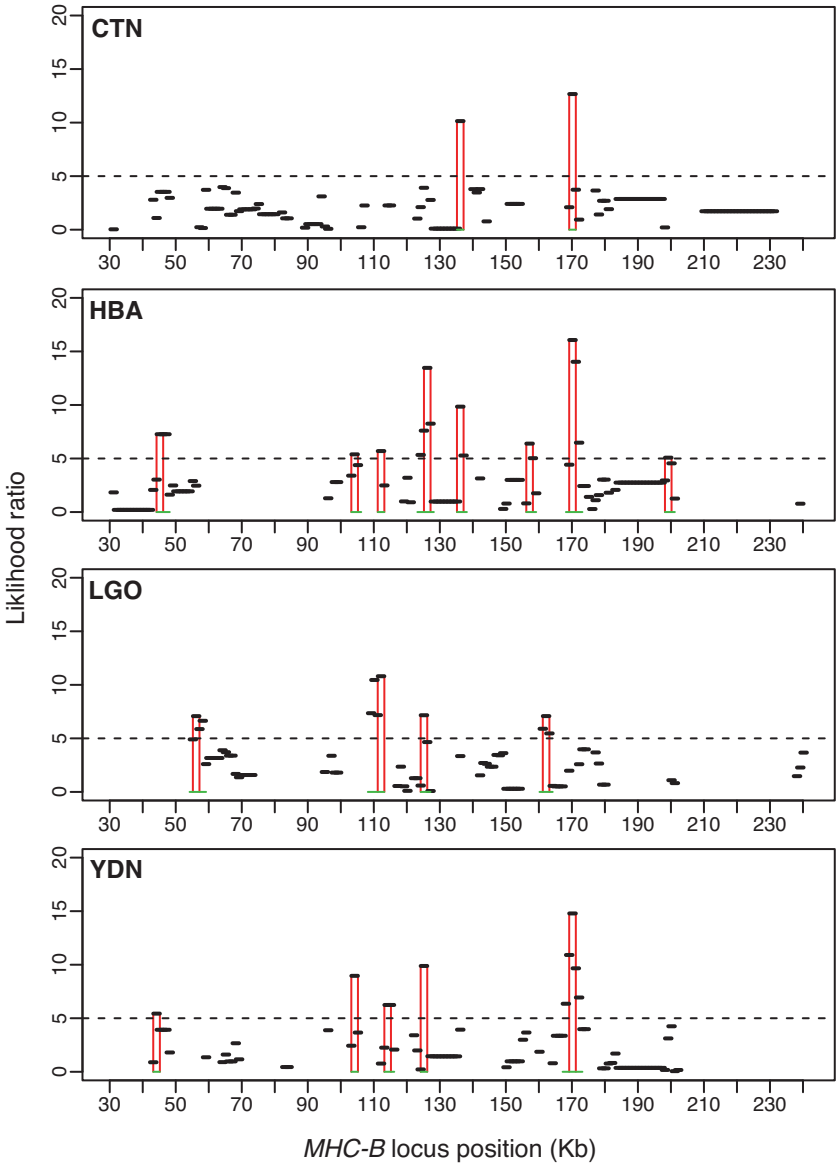

Figure 3. Recombination hotspots between 84 SNP sites spanning the MHC-*B* locus in four wild populations of Red Junglefowl. The likelihood ratio value chosen for significance (5) is depicted by the dashed line. Green lines show the extent (Kb) of the hotspot region.

was reduced compared to the other three sampling locations.

Several regions exhibited a ρ much higher than the average and background rates. Eleven recombination hotspots exceeding a likelihood ratio threshold of 5 were identified among the four sampling sites. Six hotspots occurred in more than one population (Figure 3). Individuals sampled from HBA exhibited more evidence of recombination hotspots than the other three sites. Two regions of increased recombination were shared between three populations at approximately 125 Kb (HBA, LGO, and YDN) and 170 Kb (CTN, HBA, and YDN). The former region spanned the MHC-*B* region from 122,472 to 136,539 bp and included genes BTN2, BG1, and BLEC4. The latter region spanned the MHC-*B* locus from 163,580 to 177,699 bp and included genes DMA (BMA1), DMB1, DMB2, BF1, and TAP1.

Estimates of MHC Linkage Disequilibrium

The population recombination rate affects the extent of LD (Hill and Robertson, 1968) and is an important facet in the evolutionary history of a population. Prior to measuring evidence for pairwise LD between each SNP site across the study MHC-*B* locus, SNP frequencies at each of the 84 sites were evaluated in terms of HWE expectations and minimum allele frequency (MAF). In both cases, failure to remove sites exhibiting these characteristics will bias the estimate of any LD. Three instances of deviation from HWE expectations were found ($P < 0.001$), two (SNPs 14 and 79) in HBA and one (SNP-65) in YDN. A limited but variable number of monomorphic loci also occurred in each population and along with the three sites exhibiting extreme allele frequencies, were removed.

Table 2. Analysis of Molecular Variance (AMOVA) in four Red Junglefowl populations.

Source of Variation	DF	Sum of Squares	Variance component	Percentage of variation
Among *K* populations	3	2.791	0.00417	0.83%
Among *N* individuals within *K* populations	195	101.187	0.02370	4.66%
Within *N* individuals	199	94.000	0.47236	94.51%

N = 199 Red Junglefowl, K = 4 populations.

Overall, very little evidence of LD across the MHC-*B* locus was present in any of the four sites and is consistent with the elevated inter-site recombination and hotspots we found by the *PHASE* and *SequenceLDHot* inferences. However, a few small LD blocks were identified. One present in all four sampling sites comprised SNPs 81 and 82, separated by 1,794 bp, exhibited a $D' = 1$ (complete linkage) and logarithm (base 10) of odds of linkage (LOD) > 2. This LD block contained two genes, a leukotriene B4 receptor (LTB4R1) and CD1A2 (CD1B) a cell-surface protein capable of presenting antigens to T cells (Miller et al., 2004). Another LD block common to the four sampling sites comprised SNPs 21, 22, and 23. Spanning 678 bp, this region contained the tripartite motif TRIM41 (Ruby et al., 2005).

Two additional LD blocks not common to all sampling sites were also observed. The first contained a putative NK receptor beta lectin gene, BLEC2 (Shiina et al., 2004, 2007; Chaves et al., 2009; Ye et al., 2012) and spanned MHC SNP sites 50 and 51 in LGO and YDN. The second spanned the MHC-*B* locus alpha chain DMA (BMA1; Chazara et al., 2011) at MHC SNP sites 61 and 62, but occurred only in individuals sampled from YDN. In HBA and to a lesser extent in CTN, high values of *D'* persisted in these areas but were not strong enough to meet significance requirements because of reduced D' and LOD scores.

MHC Population Structure

The most striking feature of the relationships among the 310 unique MHC haplotypes is a lack of spatial organization. The minimum spanning tree of MHC haplotypes for individual sampling sites displayed a dispersive pattern (Figure S1). Those that clustered were often consistent with close familial relationships (see Discussion). The overwhelming majority of genetic variation was partitioned within individuals (94.51%, $P < 0.001$). In contrast, substantially less genetic variation was attributable within (4.66%, $P < 0.001$) or among populations (0.83%, $P < 0.001$) (Table 2). Contrasting observations made with neutral loci (Nguyen-Phuc and Berres, in preparation), the overall F_{ST} among the four study sites was only 0.83% ($P < 0.001$), indicative of almost no geographical structure among MHC haplotypes in the four Red Junglefowl populations surveyed.

The mating system of Red Junglefowl is polygyny and males are highly territorial during the breeding season. Our field method of passive walk-in snares with decoy roosters mainly lured and promoted capture of dominant roosters in the flocks within our sampling localities (here, "flock" in a broad sense refers to an observed social unit). The average geographic distance between individual captures was 1.09 km depending on capture opportunities (a minimum of 0 m among birds in the same flock or family to a range of 14 to 26 km within a sampling area). As a consequence, we did not have complete knowledge about family structures that we could compare to our haplotype networks, except in the following three circumstances. In LGO, we knew the relationships of two families that were sampled less than 300 m apart. Each had a rearing hen and two young chicks. Of the six birds, we were able to match each family member to a specific haplotype. The first two families were defined by haplotypes 232 (hen), 233 to 235 (chicks) in one family and 255 (hen) and 254 (chick) in another family. Haplotypes of the remaining chick in the second family were not grouped together, likely due to inheritance from different males, as multiple sire mating does occur regularly in Red Junglefowl (Collias and Collias, 1996). Alternatively, a recombination event could also have taken place. In YDN, five MHC variants of haplotypes 289, 292, 277, 286, and 279 belong to five male Red Junglefowl sampled in the same vicinity. Average distance between these five birds was just more than 100 meters whereas haplotype 277 and 279 belong to two first-year non-territorial male roosters captured together.

DISCUSSION

Genes within the MHC are among the most polymorphic in vertebrate animals. As a result of their diversity, MHC molecules have received considerable attention in the fields of evolutionary and conservation biology, and especially in immunogenetics. The study of fitness effects of disease resistance and the costs of adaptive immune responses in avian species has been driven primarily by commercial enterprises seeking to create more disease resistant lines of poultry (e.g., Fulton et al., 2014). To date, no systematic evaluation of MHC in genuine wild Red Junglefowl has been conducted. We sampled 199 wild Red Junglefowl in four sites exhibiting geographically, ecologically, and environmentally diverse conditions in central Vietnam. Using a high-density SNP detection system, we assessed nucleotide diversity at 84 sites distributed across approximately 210 Kb of the MHC-*B* locus. The most striking result of this research is the extensive amount of nucleotide and haplotype variation characterized in

Table 3. Comparison of MHC haplotypes recorded in wild Red Junglefowl and 17 domestic lines.

Line type	No. Samples	No. Haplotypes	% Unique haplotypes
Red Junglefowl - CTN	46	92	100.00%
Red Junglefowl - HBA	56	82	73.21%
Red Junglefowl - LGO	39	48	61.53%
Red Junglefowl - YDN	58	91	78.44%
Red Junglefowl - Average			**78.30%**
[1]Broiler – Average (6 lines, n = 438)			5.54%
[1]Standard – Average (9 lines, n = 724)			1.97%
[1]Synthetic – Average (2 lines, n = 189)			3.14%
Domestic chickens - Average			**3.55%**

[1]Data obtained from Fulton et al., this issue.

the MHC-*B* locus: nearly 80% (310) of the 398 chromosomes analyzed exhibited a unique haplotype.

Originally, we hypothesized that Red Junglefowl were likely to demonstrate extensive MHC diversity. Because they were sampled in ecologically diverse sites, disconnected from inter-site migration, and perhaps exposed to differing pathogen repertoires, we also predicted a presence of strong population structure among MHC haplotypes. Although we observed a tremendous amount of MHC haplotype variation, there was a total lack of spatial organization among and within populations (Table 2, Figure S1). The genetic variation measured as expected heterozygosity (H_E) was always greater for MHC (between 0.2753 and 0.2894, Table 1) than for neutral markers (between 0.1243 and 0.1916; Nguyen-Phuc and Berres, in preparation).

Two other lines of evidence support the absence of MHC spatial pattern in wild Red Junglefowl. First, genetic differentiation, as measured by F_{ST} from the MHC data, was essentially zero (Table 2; 0.0083). The absence of among-population variation contrasted strongly with that observed in neutral markers (0.1400), even at the intraspecific population level (Nguyen-Phuc and Berres, in preparation). Second, the genetic variance attributable to within (4.66%) and among-population (0.83%) levels were only a small fraction of the total within level variance. Note that variance estimation in AMOVA has been derived under several different models and they may have different outcomes in their estimated covariance components. The fixed-population model employed here is considered limited to explaining evolutionary forces causing population differentiation (Weir, 1996). Nevertheless, the substantial amount of SNP variation observed at individual level (94.51%) strongly suggests diverse MHC-*B* locus variation occurs without regard to a geographical context in Red Junglefowl.

Agricultural Genetic Diversity

The best-studied MHC genes in birds come from those species of agricultural significance such as chicken, pheasant, quail, duck, and turkey (e.g., Kaufman et al., 1999b; Shiina et al., 2004; Shiina et al., 2007; Chaves et al., 2009; 2011). But unlike their mammalian counterparts, the genomic structure of MHC differs considerably within avian species (Hess and Edwards, 2002). This makes direct comparisons more challenging and confounded further by significant differences in genotyping technologies (e.g., Jacob et al., 2000; Miller et al., 2004). Our current study has a distinct advantage as we could compare it to haplotypes from 1,359 individuals sampled from 17 commercial lines (Table 3). Chickens from these domestic lines were genotyped with the same KASP technology at the identical MHC loci (Fulton et al., this issue). Without any doubt, the haplotype diversity of the wild-caught Red Junglefowl is increased substantially. No overlap was seen between the 310 unique Red Junglefowl MHC haplotypes and those found in commercial lines.

Fulton and Delany (2003) brought attention to the continuing rapid decline of both commercial and heritage poultry lines. Recent research evaluating influences of selection practices on commercial chickens suggests that it can reduce allelic diversity (Muir et al., 2008). Our current research also confirms less genetic diversity in commercial chicken lines: selective breeding practices for desirable agricultural traits have reduced significantly the level of genetic variation in MHC (Table 3). Importantly, the reduction in genetic diversity described by previous research is limited only to domesticated chickens, not their wild ancestors, Red Junglefowl. Given the introgressed and inbred nature of the *G. gallus* reference sequence, the loss of allelic diversity may even be greater. The unusually high polymorphism of Red Junglefowl MHC discovered in our study will further assist in understanding adaptive polymorphism and the genetic basis of pathogen resistance (Zuckerkandl and Pauling, 1965).

Adaptive Variation and Selection

The question as to why there is so much MHC-*B* locus diversity in Red Junglefowl remains unanswered. Extensive variation at MHC loci is generally thought to be maintained by balancing selection (Hess and Edwards, 2002), itself perhaps modulated by host-parasite coevolution. We estimated Tajima's *D*, a comparison of the average number of nucleotide pairwise differences (π) and the number of segregating sites (S) (Table 1).

In the absence of demographic changes (e.g., population expansion or contraction, high levels of inter-population migration), positive selection (selective sweeps) is indicated by negative values of D. Under the influence of balancing selection, alleles are kept at intermediate frequencies producing positive values of D because more pairwise differences exist relative to segregating sites. In all four populations, Tajima's D averaged 2.1 and was statistically significant at $P < 0.05$ (Table 1) indicating strong evidence that substitution patterns in Red Junglefowl MHC experience balancing selection.

The exact nature and major driving mechanisms of balancing selection are often debated (Hess and Edwards, 2002). In avian species, recent empirical studies support a predominant hypothesis of MHC-dependent mate choice where reproductive selection mechanisms maintain heterozygosity in natural populations (Von Schantz et al., 1989; Parker, 2002; Ekblom et al., 2007). However, a method to distinguish between loci within the MHC region and closely linked loci as the target of mate choice in these studies remains unclear (Tregenza and Wedell, 2000). Many other hypotheses have been proposed to account for disease-based selection to the extent of MHC diversity, particularly in model species, as previously mentioned.

Domestication of the chicken is thought to have occurred in Southeast Asia, probably present-day Vietnam or Southern China or both (Berthouly et al., 2010; Miao et al., 2013). Mitochondria-based sequence evidence suggests that the process of poultry domestication, like other livestock species, occurred in several places and probably deployed several divergent lineages of wild ancestors (Nishibori et al., 2005; Eriksson et al., 2008). Subsequently, wild alleles of genes in natural populations of the original progenitors may have been replaced through intensive crossbreeding with domestic stocks (Peterson and Brisbin, 1998; Berthouly et al., 2010). The ubiquity of human populations and widespread occurrence of free-ranging chickens in Southeast Asia are raising fears of introgression between wild Red Junglefowl and domestic chickens will irreversibly "contaminate" the ancestral pool of alleles. Our findings indicate that none of the analyzed Red Junglefowl haplotypes are shared with any haplotype in the commercial lines or even a limited number of individuals (n = 32) from domestic chickens we sampled in Vietnam (data not shown). Together, these findings suggest allelic introgression between wild and domesticized fowls has not occurred, at least in the four sites we sampled in Central Vietnam.

MHC Recombinants

In contrast to mammals, the close relationship between avian MHC haplotypes and role in disease resistance may in part be due to its more compact system and general absence of non-immune related genes (Kaufman et al., 1999b). From a population genetics point of view, recombination produces new haplotypes and increases the genetic variation in a population, by breaking up existing linkage between gene loci. But the relatively small size of the chicken MHC (at least compared to MHC in mammals) may restrict recombination between different loci. For example, recombinant individuals between the MHC-B to L and B to F loci are thought to be extremely rare in commercial chicken lines (Hala et al., 1989).

Our investigation of recombination in Red Junglefowl MHC suggests that while recombination within the MHC-B locus does occur, its magnitude is highly variable across both the entire MHC-B locus and between populations. At least two hotspots of recombination common to all four sampling sites were identified. However, despite the ostensible lack of evidence showing MHC recombination in domestic chickens, MHC-B locus recombination in natural populations of Red Junglefowl populations likely contributes to increased diversity. Recently, Fulton et al. (2016) provided evidence that MHC-B locus recombination may occur more frequently in domestic lines of chicken than previously thought.

The population recombination rate affects the extent of LD (Hill and Robertson, 1968). Unfortunately, studies of MHC LD in natural populations of birds remain rare. A recent study by Edwards and Dillon (2004) examined a 40-Kb region of MHC Class II locus in Red-winged Blackbirds (*Agelaius phoeniceus*). They found evidence of high LD only across a few hundred base pairs, a span nearly identical to what our current study discovered in Red Junglefowl. In contrast, Heifetz et al. (2005) found significant LD extending over several centimorgans in commercial populations of domestic chicken but these results may have limited relevance to comparisons with wild Red Junglefowl populations.

The presence of LD blocks depends on characteristics of the sample populations. While this study demonstrated very little LD exists in MHC in Red Junglefowl, all four populations had evidence of 2 to 4 LD blocks. Remarkably, two of these blocks were common across the four populations. All LD blocks were restricted primarily to regions less than 1 Kb although a roughly 2 Kb region was also identified. These findings mirror those found by Edwards and Dillon (2004) and also in MHC of the wild turkeys (*Meleagris gallopavo*) (Chaves et al., 2011). Generally, as effective population size (N_e) increases, the smaller the LD blocks will be. The similar magnitude and distribution of small LD blocks across four widely separated populations of Red Junglefowl suggests that not only is N_e large in these populations of wild Red Junglefowl, but also similar in size. Consistent with this interpretation are similar rates of recombination observed across the MHC-B locus.

However, blocks of LD can arise by chance even when recombination rates are uniform. If the recombination hotspots identified in the Red Junglefowl populations are genuine, then at least some aspect of the LD blocks is transferable between populations, an event that is

unlikely to occur at the large geographic scales between populations in our study. Importantly, this may suggest that some aspect of the environment (e.g., pathogens) is selecting for these non-random association of alleles. However, demographic transfer (dispersal) of LD blocks could certainly occur at smaller scales at least like that of our field sampling. But if recombination rates are in fact generally low, then LD blocks should be more reflective of historical recombination events since only very old recombination events can result in LD block boundaries that are shared between contemporarily isolated populations.

Sampling and Haplotype Diversity Analyses

Despite the obvious MHC-*B* locus diversity in wild Red Junglefowl, caution should be taken when interpreting the evolutionary origins of the haplotypes in the study. With balancing selection acting on MHC diversity, new MHC variants will arise and persist but need sufficient time for recombination (or mutation) to separate markers. These forces may act at very localized physical scales (even within a single exon). From our three topological 'family' clusters in LGO and YDN and from the observed diversity in CTN MHC-*B* locus haplotypes mentioned before, interpretation of Red Junglefowl MHC haplotype networks should be used only for a determination of haplotype similarity *per se*, rather than inferring any evolutionary relatedness among the birds and populations (Figure S1). Nevertheless, the shared linkage blocks are intriguing and may represent long-term selection for the presence of specific alleles of genes in these regions. We acknowledge that ascertainment bias arising from the MHC-*B* locus SNP panel may also distort population-specific allele frequencies and confound linkage analyses (Wakeley et al., 2001). While we have not explicitly addressed this issue here, we did not preferentially sample SNPs for intermediate or high frequencies. Thus, provided both rare and common SNPs are distributed at the same distance from any focal position, estimates of LD should not be overly biased.

Spatial Process of Adaptive Genetic Variation

In a geographical context, the diversity of MHC genes is also expected to be under the influences of spatial processes and local environmental factors. In a companion study using neutral markers (Nguyen-Phuc and Berres, in preparation), we identified considerable population structure in Red Junglefowl at both intra- and inter-population levels. Evidence of fine-scale genetic subdivision was detectable at distances as low as 5 km. Spatially explicit analyses also ruled out long-distance gene flow. Instead, the current population structure may have resulted from fragmentation of a formerly panmictic assemblage of populations. The spatial patterns of MHC variability are very different than neutrally based population structure. This has several implications in the evolution history of Red Junglefowl, as well as future management and breeding programs. Balancing selection, perhaps modulated in part by extensive recombination, has facilitated the generation and retention of MHC polymorphism in Red Junglefowl, perhaps to an extent that counters the effects of genetic drift and gene flow that shape patterns of neutral genetic variation. Adaptively derived diversity present in the wild Red Junglefowl MHC may be indicative of balancing selection and narrower tolerance to the underlying environments. However, our analysis so far has not been able to confirm this hypothesis, except to show that certain linkage blocks are common to some populations. Thus, neutral processes alone are insufficient to explain completely spatial genetic variation in wild Red Junglefowl. We conclude that MHC variation in domestic chickens is reduced considerably to that currently present in wild populations of Red Junglefowl in Vietnam.

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SUPPLEMENTARY DATA

Figure S1. Minimum spanning tree depicting relationships for 310 haplotypes identified in four wild populations of wild Red Junglefowl.

Supplementary data is available at PSA *Journal* online.

REFERENCES

Afanassieff, M., R. M. Goto, J. Ha, M. A. Sherman, L. W. Zhong, C. Auffray, F. Coudert, R. Zoorob, and M. M. Miller. 2001. At least one class I gene in restriction fragment pattern-Y (Rfp-Y), the second MHC gene cluster in the chicken, is transcribed, polymorphic, and shows divergent specialization in antigen binding region. J. Immunol. 166:3324–3333.

Bacon, L. D. 1987. Influence of the major histocompatability complex on disease resistance and productivity. Poult. Sci. 66:802–811.

Barrett, J. C., B. Fry, J. Maller, and M. J. Daly. 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21:263–265.

Beebe, W. 1926. A Monograph of the Pheasants. Dover Publications, New York.
Berlin, S., L. J. Qu, X. Y. Li, N. Yang, and H. Ellegren. 2008. Positive diversifying selection in avian *Mx* genes. Immunogenetics 60:689–697.
Berthouly, C., X. Rognon, T. N. Van, M. Gely, C. V. Chi, M. Tixier-Boichard, B. Bed'Hom, N. Bruneau, E. Verrier, J. C. Maillard, and J. R. Michaux. 2010. Vietnamese chickens: a gate towards Asian genetic diversity. BMC Genet. 11. doi:5310.1186/1471-2156-11-53
Brisbin, I. L., Jr., A. T. Peterson, R. Okimoto, and G. Amato. 2002. Characterization of the genetic status of populations of Red Junglefowl. J. Bombay Nat. Hist. Soc. 99:217–223.
Browning, B. L., and S. R. Browning. 2009. A unified approach to genotype imputation and haplotype-phase inference for large data sets of trios and unrelated individuals. Am. J. Hum. Genet. 84:210–223.
Browning, S. R., and B. L. Browning. 2011. Haplotype phasing: existing methods and new developments. Nat. Rev. Genet. 12:703–714.
Bub, H. 1991. Bird Trapping and Bird Banding: A Handbook for Trapping Methods All Over the World. Cornell University Press, Ithaca, NY.
Chaves, J. A., A. Ramis, R. Valle, A. Darji, and N. Majo. 2009. Avian influenza specific receptors expressed in the respiratory and gastrointestinal system from chickens, turkeys, ostriches, partridge, ducks and quail. J. Comp. Pathol. 141:277–277.
Chaves, L. D., G. M. Faile, J. A. Hendrickson, K. E. Mock, and K. M. Reed. 2011. A locus-wide approach to assessing variation in the avian MHC: the B-locus of the wild turkey. Heredity 107:40–49.
Chazara, O., F. Minvielle, D. Roux, B. Bed'hom, K. Feve, J. L. Coville, B. B. Kayang, S. Lumineau, A. Vignal, J. M. Boutin, and X. Rognon. 2010. Evidence for introgressive hybridization of wild common quail (Coturnix coturnix) by domesticated Japanese quail (*Coturnix japonica*) in France. Conserv. Genet. 11:1051–1062.
Chazara, O., M. Tixier-Boichard, V. Morin, R. Zoorob, and B. Bed'hom. 2011. Organization and diversity of the class II DM region of the chicken MHC. Mol. Immunol. 48:1263–1271.
Codon, T. 2012. Morphological detection of genetic introgression in Red Junglefowl (*Gallus gallus*). Master Thesis. Georgia Southern University, Statesboro, GA.
Cole, R. K. 1968. Studies on genetic resistance to Marek's disease. Avian diseases 12:9–28.
Collias, N. E., and E. C. Collias. 1996. Social organization of a Red Junglefowl, *Gallus gallus*, population related to evolution theory. Anim. Behav. 51:1337–1354.
Delacour, J. 1977. The Pheasants of the World. 2nd edn. Spur, Hindhead, UK.
Delany, M. E. 2006. Avian genetic stocks: The high and low points from an academia researcher. Poult. Sci. 85:223–226.
Edwards, S. V., and M. Dillon. 2004. Hitchhiking and recombination in birds: evidence from MHC-linked and unlinked loci in Red-winged Blackbirds (*Agelaius phoeniceus*). Genet. Res. 84:175–192.
Edwards, S. V., and P. W. Hedrick. 1998. Evolution and ecology of MHC molecules: from genomics to sexual selection. Trends Ecol. Evol. 13:305–311.
Ekblom, R., S. A. Saether, P. Jacobsson, P. Fiske, T. Sahlman, M. Grahn, J. A. Kalas, and J. Hoglund. 2007. Spatial pattern of MHC class II variation in the great snipe (*Gallinago media*). Mol. Ecol. 16:1439–1451.
Eriksson, J., G. Larson, U. Gunnarsson, B. Bed'hom, M. Tixier-Boichard, L. Stromstedt, D. Wright, A. Jungerius, A. Vereijken, E. Randi, P. Jensen, and L. Andersson. 2008. Identification of the Yellow skin gene reveals a hybrid origin of the domestic chicken. PLOS Genet. 4. doi:10.1371/journal.pgen.1000010.
Excoffier, L., and H. E. L. Lischer. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol. Ecol. Resourc. 10:564–567.
Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial-DNA restriction data. Genetics 131:479–491.
FAO. 2007. The State of the World's Animal Genetic Resources for Food and Agriculture. B. Richkowsky, and D. Pilling, eds. Food and Agriculture Organisation of the United Nations, Rome.
Fearnhead, P. 2006. SequenceLDhot: detecting recombination hotspots. Bioinformatics 22:3061–3066.
Fearnhead, P., and P. Donnelly. 2002. Approximate likelihood methods for estimating local recombination rates. J. Roy. Stat. Soc. B Stat. Methodol. 64:657–680.
Fuller, R., and P. Garson. 2000. Pheasants. Status Survey and Conservation Action Plan 2000–2004WPA/BirdLie/SSC Pheasant Specialist Group. IUCN, Gland. Switzerland and Cambrigde, UK and the World Pheasant Association, Reading, UK.
Fulton, J. E., A. M. McCarron, A. R. Lund, K. N. Pinegar, A. Wolc, O. Chazara, B. Bed'Hom, M. Berres, and M. M. Miller. 2016. A high-density SNP panel reveals extensive diversity, frequent recombination and multiple recombination hotspots within the chicken major histocompatibility complex B region between *BG2* and *CD1A1*. Genetics, Selection Evolution, in press.
Fulton, J. E., J. Arango, R. A. Ali, E. B. Bohorquez, A. R. Lund, C. M. Ashwell, P. Settar, N. P. O'Sullivan, and M. D. Koci. 2014. Genetic variation within the *Mx* gene of commercially selected chicken lines reveals multiple haplotypes, recombination and a protein under selection pressure. PLOS One 9:14. doi:10.1371/journal.pone.0108054.
Fulton, J. E., and M. E. Delany. 2003. Poultry genetic resources - Operation rescue needed. Science 300:1667–1668.
Fulton, J. E., H. R. Juul-Madsen, C. M. Ashwell, A. M. McCarron, J. A. Arthur, N. P. O'Sullivan, and R. L. Taylor. 2006. Molecular genotype identification of the *Gallus gallus* Major Histocompatibility Complex. Immunogenetics 58:407–421.
Fumihito, A., T. Miyake, S. I. Sumi, M. Takada, S. Ohno, and N. Kondo. 1994. One subspecies of the Red Junglefowl *Gallus gallus gallus* suffices as the matriarchic ancestor of all domestic breeds. Proc. Natl. Acad. Sci. U. S. A. 91:12505–12509.
Gabriel, S. B., S. F. Schaffner, H. Nguyen, J. M. Moore, J. Roy, B. Blumenstiel, J. Higgins, M. DeFelice, A. Lochner, M. Faggart, S. N. Liu-Cordero, C. Rotimi, A. Adeyemo, R. Cooper, R. Ward, E. S. Lander, M. J. Daly, and D. Altshuler. 2002. The structure of haplotype blocks in the human genome. Science 296:2225–2229.
Granevitze, Z., J. Hillel, G. H. Chen, N. T. K. Cuc, M. Feldman, H. Eding, and S. Weigend. 2007. Genetic diversity within chicken populations from different continents and management histories. Anim. Genet. 38:576–583.
Groeneveld, L. F., J. A. Lenstra, H. Eding, M. A. Toro, B. Scherf, D. Pilling, R. Negrini, E. K. Finlay, H. Jianlin, E. Groeneveld, and S. Weigend. 2010. Genetic diversity in farm animals - a review. Anim. Genet. 41:6–31.
Guangxin, E., R. N. Sha, S. C. Zeng, C. Wang, J. F. Pan, and J. L. Han. 2014. Genetic variability, evidence of potential recombinational event and selection of LEI0258 in chicken. Gene 537:126–131.
Guillemot, F., J. F. Kaufman, K. Skjoedt, and C. Auffray. 1989. The Major Histocompatibility Complex in the chicken. Trends Genet. 5:300–304.
Hala, K., R. Sgonc, C. Auffray, and G. Wick. 1989. Typing of MHC haplotypes in OS chicken by means of RLFP analysis. Pages 177–186 in B. S. Bhogal, and G. Koch eds. Recent Advances in Avian Immunology Research. Alan R. Liss, Inc., New York, NY.
Hedrick, P. W. 1998. Balancing selection and MHC. Genetica 104:207–214.
Heifetz, E. M., J. E. Fulton, N. O'Sullivan, H. Zhao, J. C. M. Dekkers, and M. Soller. 2005. Extent and consistency across generations of linkage disequilibrium in commercial layer chicken breeding populations. Genetics 171:1173–1181.
Hess, C. M., and S. V. Edwards. 2002. The evolution of the major histocompatibility complex in birds. BioScience 52:423–431.
Hill, W. G., and A. Robertson. 1968. Linkage disequilibrium in finite populations. Theoret. Applied Genet. 38:226–231.
Hosomichi, K., T. Shiina, S. Suzuki, M. Tanaka, S. Shimizu, S. Iwamoto, H. Hara, Y. Yoshida, J. Kulski, H. Inoko, and K. Hanzawa. 2006. The major histocompatibility complex (MHC) class IIB region has greater genomic structural flexibility and diversity in the quail than the chicken. BMC Genom. 7:322.

Hunt, H. D., R. M. Goto, D. N. Foster, L. D. Bacon, and M. M. Miller. 2006. At least one YMHCI molecule in the chicken is alloimmunogenic and dynamically expressed on spleen cells during development. Immunogenetics 58:297–307.

Izadi, F., C. Ritland, and K. M. Cheng. 2011. Genetic diversity of the major histocompatibility complex region in commercial and noncommercial chicken flocks using the LEI0258 microsatellite marker. Poult. Sci. 90:2711–2717.

Jaap, R. G., and W. F. Hollander. 1954. Wild type as standard in poultry genetics. Poult. Sci. 33:94–100.

Jacob, J. P., S. Milne, S. Beck, and J. Kaufman. 2000. The major and a minor class II beta-chain (B-LB) gene flank the Tapasin gene in the B-F/B-L region of the chicken major histocompatibility complex. Immunogenetics 51:138–147.

Johnsgard, P. A. 1999. The Pheasants of the World: Biology and Natural History. Smithsonian Institution Press, Washington, D.C.

Kaufman, J., J. Jacob, I. Shaw, B. Walker, S. Milne, S. Beck, and J. Salomonsen. 1999a. Gene organisation determines evolution of function in the chicken MHC. Immunol. Rev. 167:101–117.

Kaufman, J., S. Milne, T. W. F. Gobel, B. A. Walker, J. P. Jacob, C. Auffray, R. Zoorob, and S. Beck. 1999b. The chicken B locus is a minimal essential major histocompatibility complex. Nature 401:923–925.

Letunic, I., and P. Bork. 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res. 39:W475–W478.

Lewontin, R. C. 1964. The interaction of selection and linkage. I. General considerations; heterotic models. Genetics 49:49–67.

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Longmire, J. L., M. Maltbie, and R. Baker. 2000. Use of "Lysis Buffer" in DNA isolation and its implication for museum collections. Texas Tech University Museum, Lubbock, TX.

Mekchay, S., P. Supakankul, A. Assawamakin, A. Wilantho, W. Chareanchim, and S. Tongsima. 2014. Population structure of four Thai indigenous chicken breeds. BMC Genet. 15. doi:10.1186/1471-2156-15-40.

Miao, Y. W., M. S. Peng, G. S. Wu, Y. N. Ouyang, Z. Y. Yang, N. Yu, J. P. Liang, G. Pianchou, A. Beja-Pereira, B. Mitra, M. G. Palanichamy, M. Baig, T. K. Chaudhuri, Y. Y. Shen, Q. P. Kong, R. W. Murphy, Y. G. Yao, and Y. P. Zhang. 2013. Chicken domestication: an updated perspective based on mitochondrial genomes. Heredity 110:277–282.

Miller, M. M., L. D. Bacon, K. Hala, H. D. Hunt, S. J. Ewald, J. Kaufman, R. Zoorob, and W. E. Briles. 2004. 2004 Nomenclature for the chicken major histocompatibility (B and Y) complex. Immunogenetics 56:261–279.

Muir, W. M., G. K. S. Wong, Y. Zhang, J. Wang, M. A. M. Groenen, R. Crooijmans, H. J. Megens, H. Zhang, R. Okimoto, A. Vereijken, A. Jungerius, G. A. A. Albers, C. T. Lawley, M. E. Delany, S. MacEachern, and H. H. Cheng. 2008. Genome-wide assessment of worldwide chicken SNP genetic diversity indicates significant absence of rare alleles in commercial breeds. Proc. Natl. Acad. Sci. U. S. A. 105:17312–17317.

Ngo, T. K. C., H. Simianer, H. Eding, H. V. Tieu, V. C. Cuong, C. B. A. Wollny, L. F. Groeneveld, and S. Weigend. 2010. Assessing genetic diversity of Vietnamese local chicken breeds using microsatellites. Anim. Genet. 41:545–547.

Nishibori, M., T. Shimogiri, T. Hayashi, and H. Yasue. 2005. Molecular evidence for hybridization of species in the genus *Gallus* except for *Gallus varius*. Anim. Genet. 36:367–375.

Parker, T. H. 2002. Benefits of female mate choice in the red junglefowl. 3041978 Ph.D. Thesis. The University of New Mexico, Albuquerque, NM.

Peterson, A. T., and I. L. Brisbin. 1998. Genetic endangerment of wild Red Junglefowl *Gallus gallus*. Bird Conserv. Int. 8:387–394.

Posada, D. 2002. Evaluation of methods for detecting recombination from DNA sequences: Empirical data. Molec. Biol. Evol. 19:708–717.

Prim, R. C. 1957. Shortest connection networks and some generalizations. Bell System Techn. J. 36:1389–1401.

Reich, D. E., M. Cargill, S. Bolk, J. Ireland, P. C. Sabeti, D. J. Richter, T. Lavery, R. Kouyoumjian, S. F. Farhadian, R. Ward, and E. S. Lander. 2001. Linkage disequilibrium in the human genome. Nature 411:199–204.

Rogers, S., I. Shaw, N. Ross, V. Nair, L. Rothwell, J. Kaufman, and P. Kaiser. 2003. Analysis of part of the chicken Rfp-Y region reveals two novel lectin genes, the first complete genomic sequence of a class I alpha-chain gene, a truncated class II beta-chain gene, and a large CR1 repeat. Immunogenetics 55:100–108.

Rohlf, F. J. 1973. Hierarchical clustering using minimum spanning tree. Computer J. 16:93–95.

Rokas, A., E. Ladoukakis, and E. Zouros. 2003. Animal mitochondrial DNA recombination revisited. Trends Ecol. Evol. 18:411–417.

Ruby, T., B. Bed'Hom, H. Wittzell, V. Morin, A. Oudin, and R. Zoorob. 2005. Characterization of a cluster of TRIM-B30.2 genes in the chicken MHC B locus. Immunogenetics. 57:116–128.

Semagn, K., R. Babu, S. Hearne, and M. Olsen. 2014. Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Molec. Breed. 33:1–14.

Shiina, T., W. E. Briles, R. M. Goto, K. Hosomichi, K. Yanagiya, S. Shimizu, H. Inoko, and M. M. Miller. 2007. Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. J. Immunol. 178:7162–7172.

Shiina, T., S. Shimizu, K. Hosomichi, S. Kohara, S. Watanabe, K. Hanzawa, S. Beck, J. K. Kulski, and H. Inoko. 2004. Comparative genomic analysis of two avian (quail and chicken) MHC regions. J. Immunol. 172:6751–6763.

Stephens, M., N. J. Smith, and P. Donnelly. 2001. A new statistical method for haplotype reconstruction from population data. Am. J. Hum. Genet. 68:978–989.

Storey, A. A., J. S. Athens, D. Bryant, M. Carson, K. Emery, S. deFrance, C. Higham, L. Huynen, M. Intoh, S. Jones, P. V. Kirch, T. Ladefoged, P. McCoy, A. Morales-Muniz, D. Quiroz, E. Reitz, J. Robins, R. Walter, and E. Matisoo-Smith. 2012. Investigating the global dispersal of chickens in prehistory using ancient mitochondrial DNA signatures. PLOS One 7. doi:10.1371/journal.pone.0039171.

Tajima, F. 1989. Statistical-method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595.

Tanksley, S. D., and S. R. McCouch. 1997. Seed banks and molecular maps: unlocking genetic potential from the wild. Science 277:1063–1066.

Tregenza, T., and N. Wedell. 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. Molec. Ecol. 9:1013–1027.

Von Schantz, T., G. Goransson, G. Andersson, I. Froberg, M. Grahn, A. Helgee, and H. Wittzell. 1989. Female choice selects for a viability-based male trait in pheasants. Nature 337:166–169.

Wakeley, J., R. Nielsen, S. N. Liu-Cordero, and K. Ardlie. 2001. The discovery of single-nucleotide polymorphisms and inferences about human demographic history. Am. J. Hum. Genet. 69:1332–1347.

Weir, B. S. 1996. Genetic data analysis II: methods for discrete population genetic data. Sinauer Associates, Sunderland, MA.

Wigginton, J. E., D. J. Cutler, and G. R. Abecasis. 2005. A note on exact tests of Hardy-Weinberg equilibrium. Am. J. Hum. Genet. 76:887–893.

Worley, K., J. Collet, L. G. Spurgin, C. Cornwallis, T. Pizzari, and D. S. Richardson. 2010. MHC heterozygosity and survival in red junglefowl. Molec. Ecol. 19:3064–3075.

Ye, Q., K. He, S. Y. Wu, and Q. H. Wan. 2012. Isolation of a 97-kb Minimal Essential MHC B Locus from a New Reverse-4D BAC Library of the Golden Pheasant. PLoS One. 7:1–10.

Zuckerkandl, E., and L. Pauling. 1965. Molecules as documents of evolutionary history. J. Theoret. Biol. 8:357–366.