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Maintenance of MHC Class IIB diversity in a recently established songbird population

Danielle J. Whittaker, Amy L. Dapper, Mark P. Peterson, Jonathan W. Atwell and Ellen D. Ketterson

D. J. Whittaker (djwhitta@msu.edu), BEACON Center for the Study of Evolution in Action, Michigan State Univ., 1441 Biomedical and Physical Sciences, East Lansing, MI 48824, USA. DJW also at: Dept of Biology, Indiana Univ., 1001 East Third St., Bloomington, IN 47405, USA. – A. L. Dapper, M. P. Peterson, J. W. Atwell and E. D. Ketterson, Dept of Biology, Indiana Univ., 1001 East Third St., Bloomington, IN 47405, USA.

We examined variation at MHC Class IIB genes in a recently established population of dark-eyed juncos *Junco hyemalis* in a coastal urban environment in southern California, USA relative to an ancestral-range population from a nearby species-typical montane environment. The founding population is estimated to have been quite small, but we predicted that variation at the major histocompatibility complex (MHC) among the founders would nevertheless be preserved owing to the high functional significance of MHC. Previous studies of MHC in songbirds have had varying degrees of success in isolating loci, as passerines show extensive MHC gene duplication. In order to compare diversity in the two populations, we employed two published approaches to sequencing MHC Class II exon 2: direct sequencing with exon-based primers, and traditional cloning and sequencing with intron-based primers. Results from both methods show that the colonist population has maintained high levels of variation. Our results also indicate varying numbers of alleles across individuals, corroborating evidence for gene duplication in songbird MHC. While future studies in songbirds may need to take a genomic approach to fully understand the structure of MHC in this lineage, our results show that it is possible to use traditional methods to reveal functional variation across populations.

Reduction in population size, including founding events of new populations, often results in a loss of genetic diversity due to genetic drift. Measurements of such loss tend to be based on neutral markers such as microsatellites (Whitehouse and Harley 2001, Hauser et al. 2002, Larson et al. 2002). However, more relevant for assessing the viability or adaptive history of a population may be variation at functional loci, such as the major histocompatibility complex (MHC), a group of genes that plays an important role in mobilizing the adaptive immune response in vertebrates (Klein 1986) and may play a key role in survival of declining populations (Aguilar et al. 2004), as well as in mate choice (Penn and Potts 1999). MHC genes are highly variable, especially at the peptide-binding region (PBR), which is the portion of the protein that holds and presents peptides from pathogens to immune cells (Klein 1986). The shape of the PBR can affect which pathogens are detected by the immune system, and thus heterozygosity at MHC loci is thought to increase an individual's ability to fight a larger variety of pathogens (Penn et al. 2002), though some studies have found evidence that intermediate levels of heterozygosity, rather than maximum levels, may be optimal (Kalbe et al. 2009).

Many small isolated populations display reduced variation at MHC loci (Munguia-Vega et al. 2007, Radwan et al. 2007, 2010). However, variation at MHC loci may be maintained through natural selection despite reduction in population size, and several studies have found that some small populations retain considerable diversity at MHC loci (Richardson and Westerdahl 2003, Lukas et al. 2004). In the most extreme example, high levels of MHC diversity were observed in the San Nicolas Island fox, which is monomorphic at most other genetic markers, providing strong evidence for balancing selection at MHC loci in this population (Aguilar et al. 2004). Founding events, such as colonizing a novel environment with novel pathogens, can select for individuals with high levels of MHC heterozygosity or for individuals with different MHC alleles than those common in the ancestral population (Dionne et al. 2007, Spurgin and Richardson 2010).

We tested whether a recently isolated songbird population has lost variation at MHC loci, or whether the population's novel environment may have maintained variation or led to divergence in the MHC. Our study system is the *thurberi* race of the Oregon subspecies of dark-eyed juncos *Junco hyemalis thurberi* in San Diego County, California. Juncos are seasonally breeding, migratory emberizid sparrows that typically breed at higher elevations or northern latitudes. In San Diego County, populations of juncos breed in the

inland mountain ranges (i.e. >1500 m elevation; ≈ 70 km east of the Pacific Ocean) and migrate to warmer coastal regions for the winter, including to some urban habitats. Beginning in the early 1980s, a small number of juncos was observed to remain on the urban campus of the Univ. of California at San Diego (UCSD, Fig. 1) into the summer, and a breeding population became established on the campus that now lives there year-round (Yeh 2004). Despite being isolated for a relatively short period of time, the UCSD junco population has diverged in morphology and behavior (Yeh 2004, Yeh and Price 2004) from the Laguna Mountain population (Fig. 1). The colonist coastal population has also been observed to have significantly higher numbers of ectoparasites and pox lesion scars in birds in the UCSD population (J. W. Atwell, G. C. Cardoso, D. J. Whittaker, T. D. Price, E. D. Ketterson unpubl.), suggesting higher pathogen abundance at UCSD, possibly due to the milder climate and urban habitat. In a previous study of microsatellite diversity, the colonist population was estimated to have been founded by 10 or fewer breeding pairs, and it exhibits moderately reduced genetic diversity at neutral loci relative to several ancestral-range populations, including a likely candidate population near Laguna Mountain, California (Rasner et al. 2004). In this study, we directly compare diversity at MHC loci to neutral microsatellite diversity in these two populations.

The structure of mammalian MHC genes is fairly well established, especially in humans and mice (reviewed by Trowsdale 1995). In general, there are two classes of MHC genes involved in cellular recognition: Class I gene products target intracellular pathogens, while Class II gene products target extracellular pathogens (Klein 1986). Both classes of genes are highly variable at the peptide-binding region (PBR), which is encoded in the second and third exons of Class I MHC genes and the second exon of Class II MHC genes. Though the avian MHC also includes Class I and Class II genes, the structure and number of these genes are less understood. The first bird species in which MHC was investigated was the chicken *Gallus gallus*. It has a streamlined, 'minimal' MHC consisting of a total of 19 genes, with only two Class II loci (Kaufman et al. 1999). The hypothesis that all birds have a 'minimal essential MHC' (Kaufman et al. 1999) was dispelled when studies turned to the largest order of birds, the passerines (Westerdahl et al. 2000, Hess and Edwards 2002, Aguilar et al. 2006). The recently sequenced zebra finch genome appears to have

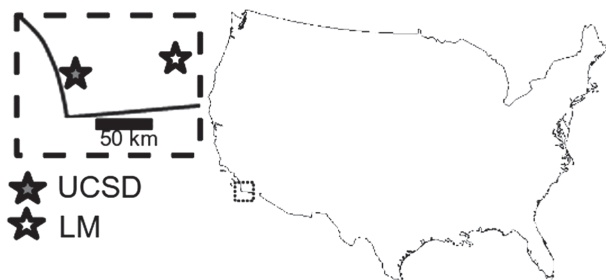

Figure 1. Map indicating location of field sites where blood samples were collected from free-ranging dark-eyed juncos in California, US. UCSD: Univ. of California, San Diego campus. LM: Laguna Mountain.

a MHC structure more complex than the mammalian MHC: at least four functional Class IIB loci, as well as five probable pseudogenes, have already been identified, with evidence for duplication of these loci (Balakrishnan et al. 2010). Extensive duplication has been reported in MHC Class II loci in another passerine, the common yellowthroat *Geothlypis trichas*, with at least 20 and possibly as many as 31 loci identified (Bollmer et al. 2010).

Evolution in avian MHC appears to involve frequent duplication and gene conversion events (Hess and Edwards 2002). Because of this history, exons may share sequence similarity across loci (Westerdahl 2007). Therefore, some researchers have advocated using introns to isolate loci, as these regions of the gene may escape conversion (Hess and Edwards 2002), and this approach has been successful in some avian species (Alcaide et al. 2008). However, studies taking this approach in passerine MHC have either failed to isolate a single locus (Miller and Lambert 2004) or have isolated a non-classical locus or apparent pseudogene (Hess et al. 2000, Aguilar et al. 2006). In one study, Canal and colleagues (2010) successfully targeted classical MHC Class IIB loci using intron-based primers in a passerine, but still co-amplified at least four loci (Canal et al. 2010). On the other hand, some studies that used primers within exon 2 (the peptide-binding region) appeared to isolate a single locus (Sato et al. 2001, Hess et al. 2006). Introns may be conserved across loci because of recent duplication events, and thus may not be useful for indicating the identity of loci (Hess and Edwards 2002).

Because of these difficulties, many studies on avian MHC have been unable to isolate individual MHC loci, and instead have co-amplified multiple alleles from different loci using polymerase chain reaction (PCR) and separated them using single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) methods (Griggio et al. 2011, Loiseau et al. 2011). Though these methods are relatively straightforward and capable of detecting allelic differences of even a single base pair (Myers et al. 1987, Sunnucks et al. 2000), isolating a single locus and knowing the exact sequence of alleles is preferable for many types of analyses, so many studies still rely on traditional cloning and sequencing methods (Eimes et al. 2011).

Thus, we had two secondary goals in this project: 1) to compare the effectiveness of intron-based primers vs exon-based primers, and 2) to attempt to find primers that isolate individual MHC loci in dark-eyed juncos. We used two previously published approaches, which differ considerably in cost and labor involved, to examine diversity at MHC Class IIB loci in these two populations of juncos: 1) amplifying MHC Class IIB exon 2 using primers located within the exon, and directly sequencing the amplified product, following methods described in Hess et al. (2006) and 2) designing primers for the flanking introns and cloning and sequencing the products, following Miller and Lambert (2004) and Aguilar et al. (2006). Here, we use the term amplicon to refer to the sequences amplified by a particular primer pair, even when more than two alleles are amplified in a single individual by that primer pair. Following Miller and Lambert (2004) and others, we also use the term alleles to refer to these sequences for simplicity, despite the fact that they may not belong to the same locus.

Methods

Sample collection

Study sites

The locations of our two study sites, the Univ. of California, San Diego campus (UCSD) and Laguna Mountain, are indicated in Fig. 1. The UCSD campus (the recently-established colonist population) is located at 32°40′N, 117°10′W, and is near sea level (100 m elevation). The habitat is a university campus made up of concrete pavement and grass lawns, plus several eucalyptus *Eucalyptus* spp. groves that cover about 10% of the total area of the campus, as well as plantings of ornamental exotic shrubbery (Yeh et al. 2007). The second study site (ancestral-range population) lies in the Laguna Mountain Recreation Area in the Cleveland National Forest (32°52′N, 116°25′W; elevation 1700 m). The vegetation is montane forest, dominated by trees including pines *Pinus* spp. and oaks *Quercus* spp. (Beauchamp 1986).

Samples

Using mist-nets and walk-in traps, we captured adult juncos at both sites during the breeding seasons of 2006 and 2007. We drew blood samples (~100 μl) from the wing vein and stored them in Longmire's solution (Longmire et al. 1992), a lysis buffer, at 4°C. Total genomic DNA was extracted using standard phenol-chloroform protocols (Sambrook et al. 1989).

Sequencing with exon-based primers

Initial amplification of MHC Class IIB exon 2 sequences in *J. hyemalis* was achieved using forward primer HOPE1 (5′-GAA AGC TCG AGT GTC ACT TCA CGA ACG GC-3′) and reverse primer HOPE2 (5′-GGG TGA CAA TCC GGT AGT TGT GCC GGC AG-3′) designed for Darwin's finches (Vincek et al. 1997). Loci were amplified in a final reaction volume of 50 μl with 1U Taq (GoTaq, Promega Corporation, Madison, WI), 1.5 mM $MgCl_2$ and 25 pmol of each primer with the following thermocycling conditions: 2 min at 94°C; 35 cycles of 94°C/30 s, 55°C/30 s, 72°C/30 s; and a final extension step at 72°C for 7 min. We cloned the resulting 260 bp products using Invitrogen TOPO TA Cloning kits, and sequenced 50 colonies for 2 individuals with ABI BigDye Cycle Sequencing Ready Reaction Kits and an ABI 3730 DNA Analyzer for a total of 100 sequences. We identified 10 unique sequences in one individual and four unique sequences in the second individual, suggesting the existence of at least 5 loci. In both individuals, we identified sequences with a divergent portion of the 5′ region and designed two forward primers on the basis of these sequences in an effort to isolate individual loci: Juhy-Ex1 (5′-TAC AAC CGG GTG CAG TTC GCG ATG-3′) and Juhy-Ex2 (5′-TCT ACA ACC GGC AGA TGT GGG CGA T-3′). These primers, paired with the conserved reverse primer HOPE2, amplify 118 and 117 bp regions, respectively (excluding the primer sequence itself), of the peptide-binding region (exon 2). These sequences begin at site 94 and 93, respectively, and end at site 218 in the 270 bp exon. The approximate location of the forward and reverse primers are indicated in Fig. 2. In both

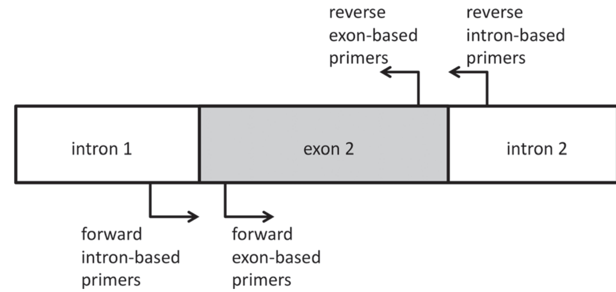

Figure 2. Schematic drawing of partial MHC Class IIB gene, showing the approximate locations of the forward and reverse intron- and exon-based primers.

amplicons this region encompasses 39 amino acids. We used the same mix as above with the following thermocycling conditions: 2 min at 94°C; 30 cycles of 94°C/30 s, 62°C/30 s, 72°C/30 s; and a final extension step at 72°C for 10 min. PCR products were purified using Qiagen PCR purification kits and directly sequenced with ABI BigDye Cycle Sequencing Ready Reaction Kits and an ABI 3730 DNA Analyzer. Sequences were assembled and aligned with the software CodonCode Aligner 3.0.

The resulting sequences contained a number of variable sites. No sequence showed more than two bases at any given site, suggesting that only two alleles were present and thus only a single locus. We assigned the ambiguous sites to alleles using the software PHASE 2.1 (Stephens and Donnelly 2003), which uses a Bayesian algorithm to estimate the most likely phase of each site. This method has previously been found to perform well with MHC class II sequences (Bos et al. 2007). Following Harrigan et al. (2008), who found that phases estimated with at least 70% probability matched the true alleles 100% of the time, we removed individuals with phase probabilities of $<$70%.

Sequencing using intron-based primers

We also amplified MHC Class IIB exon 2 along with the flanking introns in 10 individuals to assess how many loci might be found in juncos and to isolate a sub-set of them. We used primers designed to amplify the entire MHC Class IIB exon 2 (the peptide-binding region) with the primers themselves located in the conserved flanking regions: Int1f.7 (5′-ATG GGA CCC CAA AAG TGA TT-3′) and Int2r.1 (5′-CCG AGG GGA CAC GCT CT-3′) (Aguilar et al. 2006). We amplified these sequences in 50 μl PCR reactions containing 1U Taq (GoTaq, Promega Corporation), 1.5 mM $MgCl_2$ and 25 pmol of each primer with thermocycling conditions of: 2 min at 94°C; 30 cycles of 94°C/30 s, 55°C/30 s, 72°C/30 s; and a final extension step at 72°C for 7 min. We then cloned the PCR products using Invitrogen TOPO TA Cloning Kits and manufacturer-supplied protocols. We picked 5–30 colonies from each plate (most had 9–10 colonies), purified them using Invitrogen PureLink Plasmid Purification kits, and sequenced them using ABI BigDye Cycle Sequencing Ready Reaction Kits and the ABI 3730 DNA Analyzer. Sequences were assembled and aligned with the software CodonCode Aligner.

This process resulted in 96 unique sequences. We aligned these sequences using Clustal W (Larkin et al. 2007)

implemented in Geneious 4.8.5 (Drummond et al. 2010) along with published MHC sequences from seven passerine species that were most similar to the junco sequences based on a BLAST search (*Acrocephalus arundinaceus*: AAU24408; *Agelaius phoeniceus*: AF030995; *Carpodacus mexicanus*: AF241563S1; *Geospiza fortis*: AY064441.1; *Geothlypis trichas*: GQ247567.1; *Hemignathus virens*: AY583091; *Melanospiza richardsoni*: AY064454.1) plus one randomly selected human sequence (AF142457) as an outgroup, all downloaded from GenBank. We then constructed a bootstrapped neighbor-joining tree with 10 000 replications in PAUP 4.0 (Swofford 2002) implemented in Geneious 4.8.5 (Drummond et al. 2010) (Fig. 3). Using this tree and the sequence alignment, we identified two of the most divergent clusters of junco MHC sequences, which also had the most divergent intron sequences, and attempted to isolate individual MHC loci by designing primers that matched a portion of the intron on the 5′ end of these sequences. The two forward primers are Juhy-Int1 (5-′ACA ACG CCG GAT CTG TGT-3′) and Juhy-Int2 (F: 5′-GAG CTC TGG GGT GCT AAG G-3′). We used the same conserved reverse primer for both amplicons, located in the 3′ flanking intron: Juhy-IntR (5′-GGG GCA GCA AGC TCG TAG-3′). The locations of the forward and reverse primers within the gene are indicated in Fig. 2. Despite the placement of the reverse primer in the 3′ intron, we were unable to get high quality sequence at the 3′ end of exon 2. Using these primers we amplified sites 1 through 218 and 1 through 227, respectively, in exon 2.

Using these primers, we amplified and sequenced products using the same PCR, cloning and sequencing methods as described above. We attempted amplification and cloning in 45 individuals for Juhy-Int1 and 33 individuals for Juhy-Int2, and attempted to sequence about 10 colonies per locus per individual that was amplified and cloned. Not all amplifications were successful, not all cloning attempts

Figure 3. Neighbor-joining phylogram showing alignment of junco MHC alleles amplified using conserved intron primers; the two most divergent branches, used as a basis for designing new primers, are noted with dashed boxes.

resulted in 10 colonies, and not all colonies yielded clean or usable sequence. A subset of sequences (n = 9) displayed a 5 bp deletion in exon 2, likely rendering the allele non-functional. After deleting these putative pseudogenes and amplicons that were not confirmed in multiple PCRs, our dataset included Juhy-Int1 sequences for 26 individuals (10 from Laguna Mountain, 16 from UCSD), with an average of 8.7 sequenced colonies per individual (range 2–10), and Juhy-Int2 sequences for 10 individuals (4 from Laguna Mountain, 6 from UCSD), with an average of 6.1 colonies sequenced per individual (range 2–10). No chimeric sequences were detected.

Data analysis

To keep our estimates of MHC diversity conservative and to reduce the influence of errors in PCR and cloning, we collapsed similar sequences with two or fewer changes into one allele using the program Collapse 1.2 (Posada 2004). By default, when two sequences are detected by Collapse to be similar, the program arbitrarily assigns the first of the two in the dataset to represent both of them, rather than incorporating an ambiguous base in the sequence. We accepted the sequences assigned by the program, though we acknowledge that the true sequence cannot be known from this method.

We then aligned the sequences from all four amplicons (the two exon-based primer sets and two intron-based primer sets) with MHC sequences from the seven passerine species listed above and one human sequence using Clustal W (Larkin et al. 2007). For all analyses, we considered only the 140 bp stretch that was homologous across all sequences. This sequence begins at site 79 in the 270 bp exon and ends at site 218, and includes part of the forward primer site in the amplicons from exon-based primer sets. To test whether the sequences from the four amplicons formed four monophyletic clusters, we constructed a neighbor-joining phylogenetic tree in PAUP with 10 000 bootstrap replications. We also translated the sequences into amino acids and aligned them with a published human DRB allele (Brown et al. 1993) and known avian MHC Class II sequences using Clustal W in Geneious (Drummond et al. 2010).

For sequences generated using exon-based primers, we calculated gene diversity and allelic richness using the package FSTAT 2.9.3.2 (Goudet 1995), with statistical tests based on permutations of genotypes. The allelic richness measure corrects for unequal sample sizes in each population. We compared these diversity measures between the two populations using the Wilcoxon signed ranks test. For sequences generated using intron-based primers, we counted unique alleles per individual. Because we found more than two alleles using these primers in most individuals, we used the average percent difference (APD) method of estimating MHC diversity in a population, which is the average percentage of sequences that differ between individuals (Miller and Lambert 2004). Because the number of comparisons differed within each population and paired comparisons were not appropriate, we compared the APD values of the two populations for each intron-based primer pair using the Mann–Whitney U test.

For all primer sets, we calculated nucleotide diversity (π) (Nei 1987), the number of polymorphic sites, and the average number of differences between sequences (k) in each population in order to compare levels of diversity. We tested for population differentiation using nucleotide-sequence based statistics Ks* and Kst* and a permutation test in DnaSP ver. 5.10.00 (Hudson et al. 1992, Librado and Rozas 2009).

Microsatellites

In order to evaluate changes at neutral markers to directly compare with the MHC diversity measures, we genotyped the same birds at seven neutral dinucleotide repeat microsatellite loci – GF01b, Dpu01, Dpu16, JH_Ju05, JH_A03, JH_MM4.1, and JH_MM4.2 (Rasner et al. 2004, Price et al. 2008). We amplified these loci with fluorescently labeled primers (Operon; ABI) in multiplexed PCR reactions using Qiagen multiplex kits and manufacturer-supplied protocols in 10 μl reactions. The resulting product was then diluted (1:20) and mixed with a molecular size standard (GeneScan-500 LIZ, Applied Biosystems) and fragment size was analyzed using the ABI 3730 DNA Analyzer and GeneMapper 4.0 software. Each individual was genotyped a minimum of two times to confirm allele size and whether an individual was a heterozygote or homozygote. Only individuals with at least six genotyped loci were used in analyses of microsatellite variation (Laguna Mountain n = 61, UCSD n = 88). We calculated gene diversity and allelic richness using the package FSTAT 2.9.3.2 (Goudet 1995), with statistical tests based on permutations of genotypes.

Results

Table 1 summarizes the variability of sequences found using all four primer sets. For amplicon JUHY-Ex1, we amplified and sequenced 140 bp for 97 birds, yielding 67 different alleles that varied at 37 sites. For amplicon JUHY-Ex2, we sequenced 140 bp for 100 birds and found a total of 73 alleles and 48 variable sites.

Using primer set Juhy-Int1, we amplified and analyzed a 140 bp region and found 11 unique alleles across 26 individuals with 13 polymorphic sites, with 1–5 alleles per

Table 1. Population comparison of sequence-based statistics for 140 bp MHC Class II B exon 2 sequences amplified using the four primer sets: polymorphic sites (S), average number of differences between sequences (k), and sequence diversity (π). The populations are Laguna Mountain (LM) and Univ. of California San Diego college campus (UCSD). None of these measures differed between the Laguna Mountain and UCSD populations; see text for details.

Locus	Population	S	k	π
Juhy-Ex1	LM	37	12.02	0.086
	UCSD	36	12.36	0.088
Juhy-Ex2	LM	47	16.57	0.118
	UCSD	44	14.06	0.100
Juhy-Int1	LM	11	4.57	0.032
	UCSD	13	4.27	0.031
Juhy-Int2	LM	9	3.39	0.024
	UCSD	8	3.25	0.023

individual (average: 2.96; Table 1). For the 140 bp amplicon from Juhy-Int2, we found 9 unique sequences with 9 variable sites in 10 individuals, with 1–6 alleles per individual (average: 3.0; Table 1). Note that the number of alleles detected per individual should be considered a minimum estimate, as only 10 clones were sequenced per individual.

The full amplicons, including primer sequences and part of intron 1, for Juhy-Int1 (233 bp) and Juhy-Int2 (263 bp) have been deposited in GenBank (accession numbers JN861098–JN861108 and JN861089–JN861097). Amplicons Juhy-Ex1 and Juhy-Ex2 were less than 200 bp long and cannot be published on Genbank; these sequences are available from the authors by request. Translated exons from all four amplicons are shown aligned with passerine and human sequences in online Fig. 1.

Phylogenetic analysis

Amplicons Juhy-Int1 and Juhy-Int2 form reciprocally monophyletic clusters with 100% bootstrap support (Fig. 4). The Juhy-Ex2 amplicons form a clade with relatively low bootstrap support, while the Juhy-Ex1 sequences are unresolved (Fig. 4). The junco sequences do not form a monophyletic group relative to the other passerine sequences.

Population comparisons

UCSD and Laguna Mountain had similar measures of sequence diversity (number of polymorphic sites, average number of sequence differences, and sequence diversity) at all four MHC amplicons (Table 1, Wilcoxon signed ranks

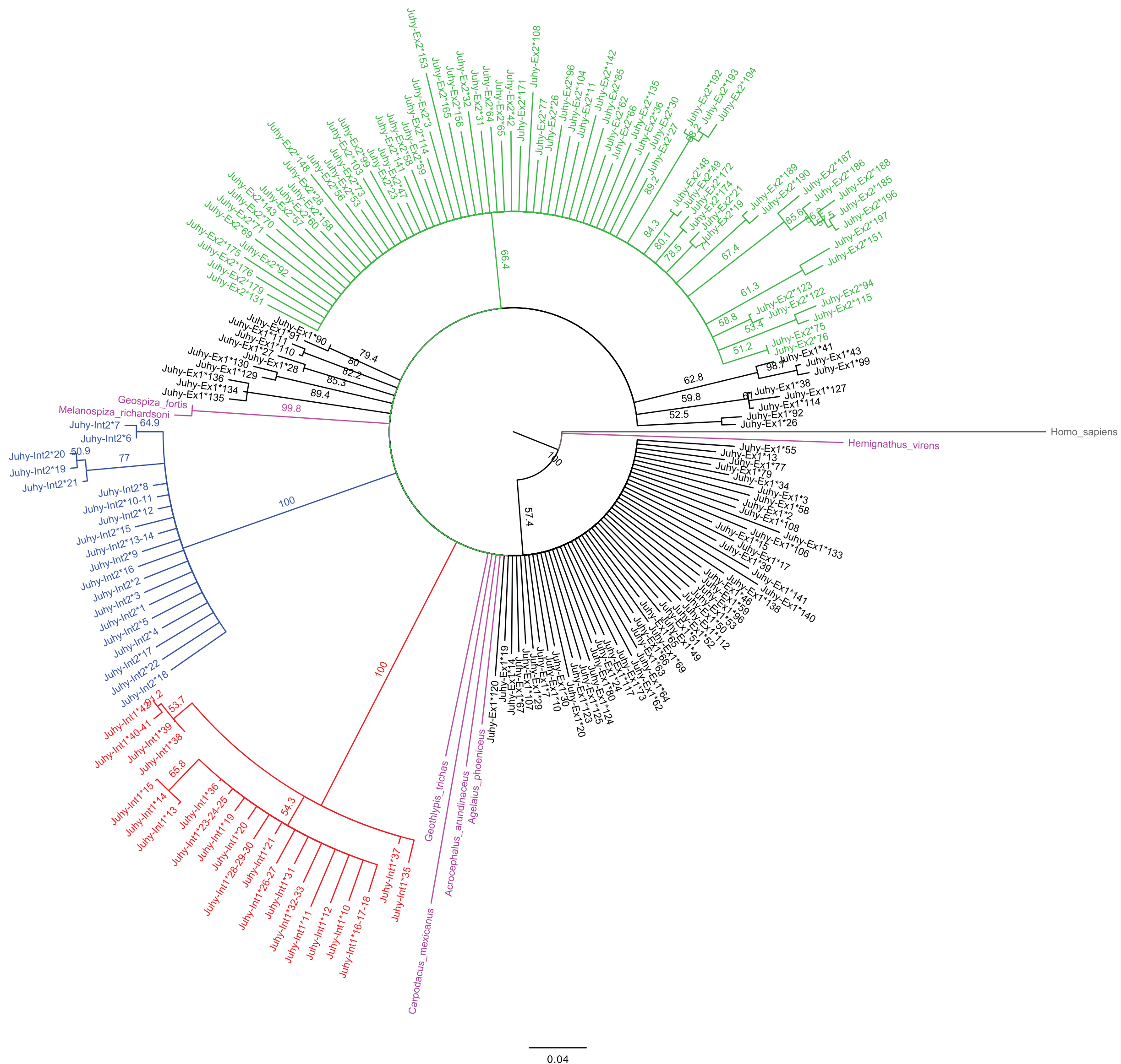

Figure 4. Neighbor-joining tree (10 000 bootstrap replications) showing relationships among 140 bp MHC Class II exon 2 sequences amplified with two exon-based primer sets (Juhy-Ex1: black; Juhy-Ex2: green) and two intron-based primer sets (Juhy-Int1: red; Juhy-Int2: blue). Avian outgroups are in purple; human sequence outgroup is in gray. Numbers on nodes indicate bootstrap support.

Table 2. Population comparison of number of individuals typed (N), number of alleles, observed heterozygosity, gene diversity, and allelic richness for microsatellite loci and MHC class II B exon 2 putative loci (140 bp) in the two populations surveyed, Laguna Mountain (LM) and Univ. of California San Diego campus (UC). For Juhy-Int1 and Juhy-Int2, average percent difference (APD) is given instead of gene diversity, and allelic richness was not calculated. Heterozygosity, gene diversity and allelic richness were compared between populations using the Wilcoxon signed ranks test, and APD was compared with the Mann–Whitney U test. *indicates comparisons that are significant at the 0.05 level.

	N		Number of alleles			Observed heterozygosity		Gene diversity/ APD		Allelic richness	
	LM	UC	LM	UC	Total	LM	UC	LM	UC	LM	UC
Microsatellite loci											
JH_A03	61	86	18	17	23	0.84	0.80	0.92	0.82	17.80	15.55
JH_MM4.1	61	88	17	12	19	0.69	0.77	0.86	0.79	16.94	10.86
JH_MM4.2	61	88	10	10	11	0.75	0.84	0.77	0.82	9.95	9.54
JH_Ju05	61	88	9	6	9	0.70	0.49	0.72	0.50	8.95	5.54
Dpu01	58	87	13	10	15	0.29	0.23	0.30	0.22	13.00	8.37
Dpu16	60	84	9	8	10	0.65	0.56	0.64	0.53	8.97	7.32
GF01b	59	87	18	14	19	0.86	0.90	0.90	0.87	17.93	13.18
Significance						ns		*		*	
MHC putative loci											
Juhy-Ex1	39	58	40	45	67	0.97	1.00	0.94	0.93	32.68	36.28
Juhy-Ex2	41	59	39	50	73	0.80	0.90	0.96	0.95	38.86	41.58
Juhy-Int1	10	16	8	10	11	1.00	0.83	0.53	0.59		
Juhy-Int2	4	6	8	8	9	1.00	0.94	0.79	0.74		
Significance						ns		ns		ns	

test, all comparisons $p > 0.1$). The two populations also showed no significant difference in heterozygosity or any allelic diversity measure (gene diversity, average percent difference (APD), or allelic richness) for any of the four MHC amplicons (Table 2, exon-based primers, Wilcoxon signed ranks test, all comparisons $p > 0.1$; intron-based primers: Mann–Whitney U test, $p > 0.1$). The populations also did not differ in the number of alleles detected in each individual at Juhy-Int1 (mean ± SD: Laguna Mountain 2.8 ± 1.14 alleles; UCSD 3.06 ± 1.39 alleles; $U = 88.5$, $p = 0.66$) or Juhy-Int2 (Laguna Mountain 3.5 ± 1.91; UCSD 2.67 ± 1.51; $U = 15.5$, $p = 0.48$).

At the sequence level, the populations did not show significant divergence at any of the four amplicons: Juhy-Ex1 – Ks*: 2.51, Kst*: −0.00165, $p = 0.749$; Juhy-Ex2 – Ks*: 2.72, Kst*: −0.00233, $p = 0.884$; Juhy-Int1 – Ks*: 1.54, Kst*: −0.04884, $p = 0.982$; Juhy-Int2 – Ks*: 1.37, Kst*: −0.0638, $p = 0.99$. We also tested divergence in allele frequency at the two exon-based MHC amplicons by estimating F_{ST} using Weir and Cockerham's (1984) theta in FSTAT. For Juhy-Ex1, $F_{ST} = -0.003$, indicating no differentiation. At Juhy-Ex2, $F_{ST} = 0.014$, suggesting little differentiation.

Microsatellite variation

At the microsatellite loci, the UCSD population had lower gene diversity and lower allelic richness than the Laguna Mountain population (Table 2). The difference between the two populations is statistically significant (Wilcoxon signed ranks test, $p = 0.043$ for gene diversity, $p = 0.018$ for allelic richness). Over the seven microsatellite loci, $F_{ST} = 0.036$ (95% confidence interval, 0.021–0.056), suggesting little differentiation at these loci. This estimate is somewhat lower than the pairwise mountain vs UCSD estimates in Rasner et al. 2004 ($0.060 < F_{ST} < 0.090$, moderate differentiation) (Rasner et al. 2004).

Discussion

Maintenance of MHC variation in colonist population

When compared to the ancestral-range population, the colonist juncos in the recently founded urban population at UCSD exhibited similar diversity at the major histocompatibility complex despite significantly reduced variation at neutral microsatellite loci. These results provide support for the hypothesis that selection has maintained MHC variation in the novel, smaller, and relatively isolated population. However, we did not find evidence for divergence at these loci between the two populations, despite differences in environment and possibly pathogen diversity and abundance.

Though geographically close, these two populations experience very different environments: the ancestral-range habitat at Laguna Mountain is highly seasonal (with a breeding season of ~3 months), while the colonists on the UCSD campus experience a mild Mediterranean climate with a longer, mild summer (and a breeding season lasting > 6 months) (Yeh and Price 2004). Pathogen species richness and diversity increases in areas with higher temperatures (Rohde and Heap 1998, Guernier et al. 2004), and in many pathogens, virulence and infectivity increase with temperature (Zheng et al. 2004). A positive relationship between temperature and MHC diversity has been noted in Atlantic salmon (Dionne et al. 2007), which may be mediated by this increase in pathogen diversity and virulence. A longer summer, as experienced by the UCSD juncos, likely means longer exposure to pathogens, which could also be a selective force for infectivity and virulence (André and Day 2005).

In addition to the climate difference, the UCSD junco population inhabits an urban environment, unlike the Laguna Mountain habitat, which is dominated by forest. Animals living in urban areas may experience higher rates

of infectious disease due to many anthropogenic factors, including environmental degradation that may facilitate the spread of disease, 'spill-over' of pathogens from domesticated animals, and the transportation or introduction of exotic species (Daszak et al. 2001, Dobson and Foufopoloulos 2001). These factors may select for high levels of MHC diversity in the UCSD junco population.

We have observed a much higher frequency of feather mites and evidence for previous pox virus infections in the UCSD population compared to Laguna Mountain (J. W. Atwell, G. C. Cardoso, D. J. Whittaker, T. D. Price, E. D. Ketterson unpubl.). Furthermore, in an unrelated common garden experiment, birds taken from the Laguna Mountain population showed a higher mortality rate in response to pox virus compared to the birds from UCSD, despite similar infection rates and vector exposure in captivity (J. W. Atwell, G. C. Cardoso, D. J. Whittaker, T. D. Price, E. D. Ketterson unpubl.). These data suggest that the UCSD population may be less susceptible to the pox virus, possibly because the virus is more prevalent in that environment and thus exerts a stronger selective pressure on the population.

Usefulness of these methods of amplifying MHC in passerines

We tried two previously published methods of amplifying and sequencing MHC Class II loci in a songbird to determine whether either or both was useful for comparing sequence-level MHC diversity in a colonist population relative to its ancestral range. Both of these methods, exon-based primers followed by direct sequencing (Juhy-Ex1 and Juhy-Ex2) and intron-based primers followed by cloning and sequencing (Juhy-Int1 and Juhy-Int2), revealed comparable levels of MHC diversity in the two populations.

When we used cloning and sequencing techniques with the two sets of intron-based primers, we found evidence for copy number variation in dark-eyed juncos: individuals had a minimum of 1–7 alleles amplified by these primer pairs, suggesting the possibility for wide variation in MHC copy number across all loci. The alleles amplified by these primers varied within the exon but not in the amplified portion of the introns, suggesting recent gene duplication followed by rapid divergence in the coding region. Recent data from common yellowthroats suggest that intraspecific variation in MHC Class II gene number is common in songbirds, with individuals in that study having up to 31 different Class II loci (Bollmer et al. 2010).

Other studies suggest that this pattern could be the result of ancient, not recent, duplication. Phylogenetic patterns based on exon 3 sequences from other species suggest that there are at least two separate lineages of avian Class IIB genes, with different functions, that predate modern bird lineages (Burri et al. 2010). The fact that the junco genes in this study did not form a monophyletic group relative to other passerines is consistent with the maintenance of multiple lineages across species.

The highly supported, reciprocally monophyletic clusters of the two amplicons from intron-based primers may indicate their identity as individual loci. However, avian exon 2 sequences from other species intermingle on a tree, more like the pattern seen with the Juhy-Ex1 sequences in this study, rather than forming clusters of individual loci (Burri et al. 2008). Targeting exon 2 may result in amplification of genes from different lineages that have similar sequences due to convergent evolution, confounding any interpretations of selection. Thus, the Juhy-Int1 and Juhy-Int2 sequences could be non-classical MHC loci with lower variability, a possibility made stronger by the fact that we chose the most divergent clusters of sequences from which to design our primers.

Our results from the exon-based direct sequencing method yielded similar conclusions about diversity in the two populations, and could offer a relatively inexpensive and fast way to sequence passerine MHC loci. However, the number of different alleles and varying sites found were much higher than those found using the intron-based primers. The higher diversity at these amplicons could indicate that these exon-based primers amplified multiple loci, or it could be that the way we chose the intron-based primers targeted sequences with lower levels of variation. Additionally, the fact that these amplicons cluster together in the phylogenetic analysis may be indicative of having designed primers that are motif-specific, rather than locus-specific. More traditional intron-based amplification and cloning and sequencing failed to isolate individual loci, most likely because of recent gene duplication and conversion, or ancient duplication and convergence.

Regardless of the limitations, both of the methods lead to the same conclusion about MHC diversity when comparing the two populations – that the colonist population does not have decreased MHC diversity relative to the ancestral-range population – suggesting that these methods may be sufficient for studies that aim to determine functional MHC diversity as an indicator of a population's genetic health or evolutionary history. That is, certain questions that call for a focus on the functional significance of MHC variability may override the need for locus specificity (Westerdahl 2007). However, for studies needing more detailed, locus-based information, such as those examining correlations between MHC variation and phenotypic characters, these methods are insufficient. Furthermore, previous studies have shown that avian MHC Class II loci can differ dramatically in expression levels, which can have major implications for survival (Jacob et al. 2000).

A new approach to passerine MHC is clearly needed. Recent advances in genomic technologies, especially next-generation sequencing techniques, yielding an increase in speed and a decrease in price, will lead to a better understanding of the structure of the passerine MHC (Lerner and Fleischer 2010), and may be better suited to detecting copy-number variation in MHC loci. Parallel 454 sequencing shows promise as a way to genotype MHC loci in the bank vole *Clethrionomys glareolus*, a non-model mammal with a complex MHC structure (Babik et al. 2009). This method has already been successfully implemented with one passerine, the collared flycatcher, revealing at least 9 loci and many pseudogenes (Zagalska-Neubauer et al. 2010).

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References

Aguilar, A., Roemer, G., Debenham, S., Binns, M., Garcelon, D. and Wayne, R. K. 2004. High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. – Proc. Natl Acad. Sci. USA 101: 3490–3494.

Aguilar, A., Edwards, S. V., Smith, T. B. and Wayne, R. K. 2006. Patterns of variation in MHC Class II B loci of the little greenbul (*Andropadus virens*) with comments on MHC evolution in birds. – J. Hered. 97: 133–142.

Alcaide, M., Edwards, S. V., Negro, J. J., Serrano, D. and Tella, J. L. 2008. Extensive polymorphism and geographical variation at a positively selected MHC class II B gene of the lesser kestrel (*Falco naumanni*). – Mol. Ecol. 17: 2652–2665.

André, J.-B. and Day, T. 2005. The effect of disease life history on the evolutionary emergence of novel pathogens. – Proc. R. Soc. Sci. B 272: 1949–1956.

Babik, W., Taberlet, P., Ejsmond, M. J. and Radwan, J. 2009. New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system. – Mol. Ecol. Resour. 9: 713–719.

Balakrishnan, C. N., Ekblom, R., Volker, M., Westerdahl, H., Godinez, R., Kotkiewicz, H., Burt, D. W., Graves, T., Griffin, D. K., Warren, W. C. and Edwards, S. V. 2010. Gene duplication and fragmentation in the zebra finch major histocompatibility complex. – BMC Biol. 8: 29.

Beauchamp, M. R. 1986. A flora of San Diego County. – Sweetwater River Press, National City, CA, USA.

Bollmer, J. L., Dunn, P. O., Whittingham, L. A. and Wimpee, C. 2010. Extensive MHC Class II B gene duplication in a passerine, the common yellowthroat (*Geothlypis trichas*). – J. Hered. 101: 448–460.

Bos, D. H., Turner, S. M. and Dewoody, J. A. 2007. Haplotype inference from diploid sequence data: evaluating performance using non-neutral MHC sequences. – Hereditas 144: 228–234.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. – Nature 364: 33–39.

Burri, R., Hirzel, H. N., Salamin, N., Roulin, A. and Fumagalli, L. 2008. Evolutionary patterns of MHC Class II B in owls and their implications for the understanding of avian MHC evolution. – Mol. Biol. Evol. 25: 1180–1191.

Burri, R., Salamin, N., Studer, R. A., Roulin, A. and Fumagalli, L. 2010. Adaptive divergence of ancient gene duplicates in the avian MHC Class II B. – Mol. Biol. Evol. 27: 2360–2374.

Canal, D., Alcaide, M., Anmarkrud, J. A. and Potti, J. 2010. Towards the simplification of MHC typing protocols: targeting classical MHC class II genes in a passerine, the pied flycatcher *Ficedula hypoleuca*. – BMC Res. Not. 3: 236.

Daszak, P., Cunningham, A. A. and Hyatt, A. D. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. – Acta Trop. 78: 103–116.

Dionne, M., Miller, K. M., Dodson, J. J., Caron, F. and Bernatchez, L. 2007. Clinal variation in MHC diversity with temperature: evidence for the role of host-pathogen interaction on local adaptation in Atlantic salmon. – Evolution 61: 2154–2164.

Dobson, A. and Foufopoloulos, J. 2001. Emerging infectious pathogens of wildlife. – Phil. Trans. R. Soc. B 356: 1001–1012.

Drummond, A. J., Ashton, B., Buxton, S., Cheung, M., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Thierer, T. and Wilson, A. 2010. Geneious ver. 4.8. – <www.geneious.com>.

Eimes, J. A., Bollmer, J. L., Whittingham, L. A., Johnson, J. A., Van Oosterhout, C. and Dunn, P. O. 2011. Rapid loss of MHC class II variation in a bottlenecked population is explained by drift and loss of copy number variation. – J. Evol. Biol. 24: 1847–1856.

Goudet, J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. – J. Hered. 86: 485–486.

Griggio, M., Biard, C., Penn, D. J. and Hoi, H. 2011. Female house sparrows ‘count on’ male genes: experimental evidence for MHC-dependent mate preference in birds. – BMC Evol. Biol. 11: 44.

Guernier, V., Hochberg, M. E. and Guégan, J.-F. 2004. Ecology drives the worldwide distribution of human diseases. – PLoS Biol. 2: 0740–0746.

Harrigan, R. J., Mazza, M. E. and Sorenson, M. D. 2008. Computation vs. cloning: evaluation of two methods for haplotype determination. – Mol. Ecol. Resour. 8: 1239–1248.

Hauser, L., Adcock, G. J., Smith, P. J., Ramirez, J. H. B. and Carvalho, G. R. 2002. Loss of microsatellite diversity and low effective population size in an overexploited population of New Zealand snapper (*Pagrus auratus*). – Proc. Natl Acad. Sci. USA 99: 11742–11747.

Hess, C. M. and Edwards, S. V. 2002. The evolution of the major histocompatibility complex in birds. – Bioscience 52: 423–431.

Hess, C. M., Gasper, J., Hoekstra, H. E., Hill, C. E. and Edwards, S. V. 2000. MHC Class II Pseudogene and genomic signature of a 32-kb cosmid in the house finch (*Carpodacus mexicanus*). – Genome Res. 10: 613–623.

Hess, C. M., Wang, Z. and Edwards, S. V. 2006. Evolutionary genetics of *Carpodacus mexicanus*, a recently colonized host of a bacterial pathogen, *Mycoplasma gallisepticum*. – Genetica 129: 217–225.

Hudson, R. R., Boos, D. D. and Kaplan, N. L. 1992. A statistical test for detecting geographic subdivision. – Mol. Biol. Evol. 9: 138–151.

Jacob, J. P., Milne, S., Beck, S. and Kaufman, J. 2000. The major and a minor class II B-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.

Kalbe, M., Eizaguirre, C., Dankert, I., Reusch, T. B. H., Sommerfeld, R. D., Wegner, K. M. and Milinski, M. 2009. Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. – Proc. R. Soc. B 276: 925–934.

Kaufman, J., Milne, S., Gobel, T. W. F., Walker, B. A., Jacob, J. P., Auffray, C., Zoorob, R. and Beck, S. 1999. The chicken B locus is a minimal essential major histocompatibility complex. – Nature 401: 23–925.

Klein, J. 1986. Natural history of the major histocompatibility complex. – Wiley.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., Mcgettigan, P. A., Mcwilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and

Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. – Bioinformatics 23: 2947–2948.
Larson, S., Jameson, R., Etnier, M., Fleming, M. and Bentzen, P. 2002. Loss of genetic diversity in sea otters (*Enhydra lutris*) associated with the fur trade of the 18th and 19th centuries. – Mol. Ecol. 11: 1899–1903.
Lerner, H. R. L. and Fleischer, R. C. 2010. Prospects for the use of next-generation sequencing methods in ornithology. – Auk 127: 4–15.
Librado, P. and Rozas, J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. – Bioinformatics 25: 1451–1452.
Loiseau, C., Zoorob, R., Robert, A., Chastel, O., Julliard, R. and Sorci, G. 2011. *Plasmodium relictum* infection and MHC diversity in the house sparrow (*Passer domesticus*). – Proc. R. Soc. B 278: 1264–1272.
Longmire, J. L., Gee, G. F., Handenkipf, O. L. and Mark, G. A. 1992. Establishing paternity in whooping cranes, *Grus americana*, by DNA analysis. – Auk 109: 522–529.
Lukas, D., Bradley, B. J., Nsubuga, A. M., Doran-Sheehy, D., Robbins, M. M. and Vigilant, L. 2004. Major histocompatibility complex and microsatellite variation in two populations of wild gorillas. – Mol. Ecol. 13: 3389–3402.
Miller, H. C. and Lambert, D. M. 2004. Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). – Mol. Ecol. 13: 3709–3721.
Munguia-Vega, A., Esquer-Garrigos, Y., Rojas-Bracho, L., Vazquez-Juarez, R., Castro-Prieto, A. and Flores-Ramirez, S. 2007. Genetic drift vs. natural selection in a long-term small isolated population: major histocompatibility complex class II variation in the Gulf of California endemic porpoise. – Mol. Ecol. 16: 4051–4065.
Myers, R. M., Maniatis, T. and Lerman, L. S. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. – Methods Enzymol. 155: 501–527.
Nei, M. 1987. Molecular evolutionary genetics. – Columbia Univ. Press.
Penn, D. J. and Potts, W. K. 1999. The evolution of mating preferences and major histocompatibility complex genes. – Am. Nat. 153: 145–164.
Penn, D. J., Damjanovich, K. and Potts, W. K. 2002. MHC heterozygosity confers a selective advantage against multiple-strain infections. – Proc. Natl Acad. Sci. USA 99: 11260–11264.
Posada, D. 2004. Collapse: describing haplotypes from sequence alignments. – <darwin.uvigo.es/software/collapse.html>.
Price, T. D., Yeh, P. J. and Harr, B. 2008. Phenotypic plasticity and the evolution of a socially selected trait following colonization of a novel environment. – Am. Nat. 172: S49–S62.
Radwan, J., Kawalko, A., Wojcik, M. and Babik, W. 2007. MHC-DRB3 variation in a free-living population of the European bison, *Bison bonasus*. – Mol. Ecol. 16: 531–540.
Radwan, J., Biedrzycka, A. and Babik, W. 2010. Does reduced MHC diversity decrease variability of vertebrate populations? – Biol. Conserv. 143: 537–544.
Rasner, C. A., Yeh, P., Eggert, L. S., Hunt, K. E., Woodruff, D. S. and Price, T. D. 2004. Genetic and morphological evolution following a founder event in the dark-eyed junco, *Junco hyemalis thurberi*. – Mol. Ecol. 13: 671–681.
Richardson, D. S. and Westerdahl, H. 2003. MHC diversity in two *Acrocephalus* species: the outbred great reed warbler and the inbred Seychelles warbler. – Mol. Ecol. 12: 3523–3529.
Rohde, K. and Heap, M. 1998. Latitudinal differences in species and community richness and in community structure of metazoan endo- and ectoparasites of marine teleost fish. – Int. J. Parasitol. 28: 461–474.
Sambrook, E., Fritsch, E. P. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. – Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
Sato, A., Mayer, W. E., Tichy, H., Grant, P. R., Grant, B. R. and Klein, J. 2001. Evolution of Mhc class II B genes in Darwin's finches and their closest relatives: birth of a new gene. – Immunogenetics 53: 792–801.
Spurgin, L. G. and Richardson, D. S. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. – Proc. R. Soc. B 277: 979–988.
Stephens, M. and Donnelly, P. 2003. A comparison of Bayesian methods for haplotype reconstruction from population genotype data. – Am. J. Hum. Genet. 73: 1162–1169.
Sunnucks, P., Wilson, A. C. C., Beheregaray, L. B., Zenger, K., French, J. and Taylor, A. C. 2000. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. – Mol. Ecol. 9: 1699–1710.
Swofford, D. L. 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. – Sinauer.
Trowsdale, J. 1995. 'Both man & bird & beast': comparative organization of MHC genes. – Immunogenetics 41: 1–17.
Vincek, V., O'huigin, C., Satta, Y., Takahata, N., Boag, P. T., Grant, P. R., Grant, B. R. and Klein, J. 1997. How large was the founding population of Darwin's finches? – Proc. R. Soc. B 264: 111–118.
Weir, B. S. and Cockerham, C. C. 1984. Estimating F-statistics for the analysis of population structure. – Evolution 38: 1358–1370.
Westerdahl, H. 2007. Passerine MHC: genetic variation and disease resistance in the wild. – J. Ornithol. 148: S469–S477.
Westerdahl, H., Wittzell, H. and von Schantz, T. 2000. Mhc diversity in two passerine birds: no evidence for a minimal essential Mhc. – Immunogenetics 52: 92–100.
Whitehouse, A. M. and Harley, E. H. 2001. Post-bottleneck genetic diversity of elephant populations in South Africa, revealed using microsatellite analysis. – Mol. Ecol. 10: 2139–2149.
Yeh, P. J. 2004. Rapid evolution of a sexually selected trait following population establishment in a novel habitat. – Evolution 58: 166–174.
Yeh, P. J., Hauber, M. E. and Price, T. D. 2007. Alternative nesting behaviours following colonisation of a novel environment by a passerine bird. – Oikos 116: 1473–1480.
Yeh, P. J. and Price, T. D. 2004. Adaptive phenotypic plasticity and the successful colonization of a novel environment. – Am. Nat. 164: 531–542.
Zagalska-Neubauer, M., Babik, W., Stuglik, M., Gustafsson, L., Cichoń, M. and Radwan, J. 2010. 454 sequencing reveals extreme complexity of the class II Major Histocompatibility Complex in the collared flycatcher. – BMC Evol. Biol. 10: 395.
Zheng, D., Mai, K., Liu, S., Cao, L., Liufu, Z., Xu, W., Tan, B. and Zhang, W. 2004. Effect of temperature and salinity on virulence of *Edwardsiella tarda* to Japanese flounder, *Paralichthys olivaceus* (Temminck et Schlegel). – Aquac. Res. 35: 494–500.

Supplementary material (Appendix J5504 at <www.oikosoffice.lu.se/appendix>). Appendix 1.