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Evolutionary genetics of *Carpodacus mexicanus*, a recently colonized host of a bacterial pathogen, *Mycoplasma gallisepticum*

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Abstract We present molecular data documenting how introduction to the eastern United States and an epizootic involving a bacterial pathogen has affected the genetic diversity of house finches, a cardueline songbird. Population bottlenecks during introduction can cause loss of genetic variation and may negatively affect a population's ability to adapt to novel stressors such as disease. Although a genome-wide survey using Amplified Fragment Length Polymorphism (AFLP) markers suggests little loss of genetic diversity in introduced populations, an epizootic of bacterial *Mycoplasma* has nonetheless caused dramatic declines in the eastern US population. Sequence analysis of a candidate gene for pathogen resistance in the Major Histocompatibity Complex (MHC) in pre- and post-epizootic population samples reveals allele frequency shifts since introduction of the pathogen, but similar shifts are also observed in control populations not exposed to the bacteria, and in a neutral non-coding locus. Expression studies using a novel subtractive hybridization approach indicate decreased expression of the class II MHC locus upon exposure to *Mycoplasma*, a pattern also seen in MHC class I loci in mice infected with cytomegalovirus and consistent with manipulation of the finch immune system by *Mycoplasma*. These results will be further expanded using experimental studies as well as examination of evolution of the pathogen genome itself.

Keywords Adaptive loci · Diachronic evolution · House finch · *Mhc* · Population bottleneck

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Introduction

With continued globalization, anthropogenically induced transport and introduction of non-native species to areas outside their native range is an ever increasing problem (Pimental et al. 2000). Most introductions negatively impact communities by displacing native species, in turn cascading into a greater reduction in biodiversity ultimately from the resulting loss of critical co-evolutionary partners (Case and Bulger 1991). We have been studying the evolutionary genetics of introduced and native house finches to understand the effect of human-induced introduction on their genetic diversity and consequently their potential to adapt to strong selective forces such as disease epidemics.

House finch distribution and introduced species status

House finches are cardueline finches native to the western United States (Hill 1994). Within the last

150 years, they have been introduced to the Hawaiian Islands and to the eastern United States, probably in the vicinity of Long Island in New York state (Grinnell 1911; Elliot and Arbib 1953; Hill 1993). Both introductions were facilitated by humans and probably involved a small number (< 100) of founding birds. Since the introduction to New York 60 years ago, the range of introduced birds has continued to expand westward and now the eastern and western populations intersect at several places in the Great Plains (Fig. 1, National Audubon Society, 2002). House finches are rare in this part of their range, however, possibly because of a low abundance of human-provided food in these areas compared to food abundance found in more densely human populated areas.

Population consequences of bottlenecks

One potential outcome of population introductions by a small number of founder individuals is a significant loss of genetic variation through random sampling of genes during the founding of the new population (Barton and Charlesworth 1987). The loss of alleles by drift can be especially profound at loci under overdominant selection, a consequence particularly important for highly polymorphic loci such as those found in the immune system. Theoretical modeling of population bottlenecks at loci under balancing selection suggests that populations must be founded by extremely small numbers of individuals (10 or fewer) and remain so for several generations to compromise their genetic diversity (Nei et al. 1975; Tajima 1989, Gilligan et al. 2005; Hoelzel et al. 2002; Leberg 2002). However, the consequences of this loss of diversity are unclear, because even mild bottlenecks can induce changes in the genetics of small populations in multiple ways, including loss of overall genetic diversity, allele number or overall heterozygosity. These population genetic measures are not all necessarily impacted to the same degree by a given bottleneck. In a typical population, many alleles will be roughly the same age, with the result that losing some alleles will reduce the overall number of alleles in the population even though total nucleotide diversity (average pairwise divergence of alleles) can remain the same. In particular, in the presence of disassortative mating, overall heterozygosity can also remain constant during a bottleneck (Wenick et al. 1998). For most populations, the pre-bottleneck level of genetic diversity will determine how much variation is left following drift of a given intensity and duration. Studies of silvereyes that undergo bottlenecks when they colonize new islands indicate that a single bottleneck is insufficient to negatively impact heterozygosity or nucleotide diversity at neutral loci (Clegg et al. 2002). In this paper, we examine the effects of population bottlenecks on overall genetic diversity, number of alleles and heterozygosity at a locus under balancing selection. Little empirical work has addressed the question of disease susceptibility due to loss of genetic diversity in vertebrates (Wenink et al. 1998; Slatkin 2004; Clegg et al. 2002); house finch introduction history provides a unique opportunity to do so.

History of house finch–*Mycoplasma* interaction

Just over a decade ago, house finches were exposed to a novel bacterial pathogen called *Mycoplasma gallisepticum* (MG). The outward clinical signs of mycoplasmosis are swelling around the eye and eventual eye

Fig. 1 Distribution of house finches in the continental United States. The species has also been introduced to the Hawaiian Islands. Stars indicate localities where we compare genetic variation before and after exposure to the *Mycoplasma*

closure if severe pathogenesis occurs. The spread and severity of MG in house finches over the course of the last decade has been tracked by the Cornell Lab of Ornithology through Project Feederwatch (Dhondt et al. 1998; Hosseiniet al. 2004). Within the first 5 years, the disease spread throughout the majority of the eastern United States. Almost as rapidly, the percentages of birds in each population that exhibited signs of infection decreased. Such a scenario leads to the prediction that allelic variation present in the eastern US. House Finches at the time of exposure to MG ultimately conferred resistance to MG and that alleles conferring resistance should have swept rapidly through the species to the extent that gene flow would allow.

AFLP study

Although the introduction of house finches to the eastern United States and Hawaii has been well documented, until recently only allozymes and mtDNA RFLPs have been used to address the question of the genetic consequences and source population of either introduced population (Benner 1991; Vasquez-Phillips 1992). More recently, by examining dominant AFLP markers, Wang et al. (2003) generated numerous nuclear markers and examined genetic variability and population subdivision across the range of this species. Wang et al. (2003) surveyed 172 individuals from 16 populations in the eastern United States, southeastern Canada, the Hawaiian Islands and Mexico, using Cassin's finch (*Carpodacus cassini*) and purple finch (*Carpodacus purpureus*) as outgroups. Of the 350 total markers, 71.2% were polymorphic among species and 60.2% were polymorphic within species. In general, heterozygosity and interpopulation divergence were low. In addition, tree analysis suggested that, of the two introduced populations (Hawaii and eastern United States), only the eastern population showed a signature, albeit a weak one, of having been derived from within the western US as would be predicted. In fact, assignment tests correctly assigned over 90% of individuals to their respective regions based on allele frequency differences among populations, implying that the multilocus AFLP profiles of each region were distinct, contrary to our prediction that introduced populations should be genetic subsets of the known population of origin, California. It does not appear that genetic variation has been compromised in the introduced populations at the AFLP loci, suggesting that the numbers of founders may have been large enough to have maintained variation throughout the genome despite the fact that allele frequencies in these populations have drifted enough to permit assignment of individuals to their correct population (Wang et al. 2003). A recent study, however, claimed to find significant reduction in microsatellite diversity as well as higher levels of isolation between populations in eastern US house finch populations (Hawley et al. 2006). Because this study sampled post-epizootic House finch populations, it is unclear whether the observed microsatellite diversities are due to demographic bottlenecks or to the MG epidemic itself. Examining genes potentially involved in MG resistance could differentiate between these alternatives.

House Finch behavioral ecology

House finches are a model system for studies of the evolution of plumage coloration and sexual selection, and their unique population history also makes them excellent models for studying the impact of introductions and exposure to pathogens. Geoff Hill and colleagues have been studying house finch mate choice and condition dependent plumage variation for the past 15 years and have made great strides in providing evidence for ''good genes'' hypotheses for female mate choice (Zahavi 1997). House finch male plumage varies from yellow to red and females generally prefer to mate with the reddest males. Hill has shown that making red feathers requires specific carotenoids that the birds cannot acquire directly from the environment—they must be synthesized from precursors, and such synthesis is known to be physiologically expensive (Hill 1991; Hill et al. 1999; Hill 2000). By experimentally limiting access to carotenoids, these researchers were able to make red birds more yellow; conversely, by giving yellow birds unlimited access to carotenoids yellow birds could be converted to red (Hill 1992; Hill and Montgomerie 1994; Hill et al. 1994). Additionally, redder birds that become infected with MG are more likely to clear the pathogen than more yellow birds, a result that lends support to the idea that good genes may be associated with genes of the immune system (Hill et al. 2004). The paradigm that ''good genes'' are thought to honestly indicate male's quality leads to the prediction that condition-dependent phenotypic traits utilized by females to assess male quality will be correlated with the frequency of advantageous immune system genes in post-epizootic populations of house finches.

MHC and house finches

The Major Histocompatibility Complex (MHC) contains the most polymorphic genes found in vertebrates

(Klein 1986). There are now numerous examples wherein MHC allelic variation has been linked to susceptibility to disease in humans (Hill 1991). A smaller number of studies have shown disease associations in other vertebrates, but the most striking examples of MHC disease associations are known from chickens (*Gallus gallus*). Several diseases including Marek's Disease and Rous Sarcoma virus show striking associations between MHC variability and resistance or susceptibility to these diseases (Wakenell et al. 1996). This taxonomic pattern could in part be due to the simple structure of the chicken MHC: only one of both class I and class II genes are highly expressed in chickens whereas in the mammalian MHC greater numbers are expressed at moderate levels (Kaufman et al. 1999).

We have been studying the MHC of songbirds (Passeriformes) to understand their genetic architecture, variability and contribution to disease susceptibility in natural populations (Edwards et al. 1995; Edwards and Hedrick 1998; Edwards et al. 1999; Hess and Edwards 2002). Our initial studies of MHC genes in passerines indicate that there are a variable number of MHC genes in different songbird species. When genomic blots are probed with MHC class II cDNAs, house finch genomic DNA in particular displays a number of fragments hybridizing at low levels (Edwards et al. 2000). A putative non-functional pseudogene isolated from a house finch cosmid library showed higher levels of variability than that found in the background house finch genome, most likely due to balancing selection prior to loss of function or to ongoing hitchhiking with a polymorphic MHC gene (Hess et al. 2000). This paper reviews our first attempts to measure variation in house finches at a functional, polymorphic MHC locus and to determine the effects of the MG epizootic on MHC variability in diachronic population samples.

Materials and methods

Analysis of MHC variation in diachronic population samples

We obtained pre- and post-exposure house finches from two localities: Ann Arbor, Michigan and Auburn, Alabama. Pre-exposure individuals from the period 1988–1990 were obtained from the collections at the Royal Ontario Museum and post-exposure samples were obtained from the collection of Geoff Hill. As a control for the effects of neutral drift on allele frequencies in evolving but unexposed populations, we analyzed birds from California unaffected by MG exposure but sampled over a similar time period separated from one another by a similar period of time (Fig. 1).

We amplified a portion of exon 2 of a class II B gene in birds from before and after exposure to MG. The second exon of class II B genes encodes all of the codons comprising the peptide binding region (PBR). The entire exon spans 270 base pairs and the region examined utilized primers sitting just inside the exon borders. The primers were designed by aligning previously published house finch cDNA (Edwards et al. 1995) with other bird species. The primers were designated HFMHCF (CAGGAGCTGTCGACCTCCGAG) and HFMHCR (GGGTAAAAATCGGGTAGTTGTGC). We amplified the MHC fragment with 35 PCR cycles (each 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s). The total amplified fragment is 212 bp. We sequenced these fragments directly from PCR products on either an ABI 377 or an ABI 3100. Sequences were then scored manually using the program Sequencher and haplotypes were inferred using HAPINFER (Clark 1990). We also examined an anonymous nuclear locus generated from a cosmid clone end sequence and designated anonymous locus 1 (ALHF1; 261 bp) to determine the contribution of demographic changes to fluctuations in genetic variability (Primers: ALHF1F: TGCTGAACGCTTTACTGCTT, ALHF1R: GAAGAGCAGCAGCCACACAG). We reasoned that any changes in frequencies at anonymous loci can be used as a measure of neutral drift against which changes at MHC allele frequencies can be compared.

We made two major predictions for expected change based on natural selection for resistance. In the event of a selective sweep, one would expect population size as measured by Θ (effective population size, Watterson 1975) to decrease drastically, consistent with a negative frequency dependent scenario. Conversely, if there has been strong selection for overdominance, we would expect a monotonic increase in heterozygosity over time. Given that we are examining selection over a short period of time, we can ignore the historical background selection that shaped diversity at this locus and focus on the specific predicted changes expected since the epidemic began. We measured variability and tested for selection using the DnaSP Version 3 package (Rozas and Rozas 1999).

Gene expression and adaptive evolution

A potential mechanism for combating pathogens in the absence of actual evolutionary change is through differential expression of mRNAs. Subtractive

hybridization is a technique used to identify loci whose expression profiles have changed between two cell types or physiological states by the isolation of loci that are expressed in one cell population but not the other. This is accomplished through hybridization of mRNAs of a test cell type with those of a control cell type and analyzing those mRNAs that are not expressed in the control type (or vice versa, for down regulated mRNAs). Although traditional subtractive hybridization methods have been successful in many applications, they require several rounds of hybridization and are not well suited to the identification of rare messages. We used a new PCR-based cDNA subtraction method, suppression subtractive hybridization (SSH) developed by Diatchenko et al. (1996). The process begins by converting both mRNA populations into cDNA. We refer to the cDNA that contains target (differentially expressed) transcripts as ''tester'', and the non-target cDNA as ''driver''. Tester and driver cDNAs are hybridized, and these hybridized sequences are then removed. The remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. SSH overcomes the problem of differences in mRNA abundance by incorporating a hybridization step that normalizes (equalizes) sequence abundance during the course of subtraction by standard hybridization kinetics. It eliminates any intermediate steps for physical separation of ss and ds cDNAs, requires only one subtractive hybridization round, and can achieve greater than 1,000-enrichment for differentially expressed cDNAs. To our knowledge, our method is the first application of this approach in wild birds.

Hatch-year finches were caught in cages at feeding areas in Auburn, Alabama during July and August 2000–2002 6 weeks after fledging and initially screened for MG by serum plate agglutination assay (SPA) (Luttrell et al. 1996, 1998) and PCR (Roberts et al. 2001). MG-negative birds were kept for 2 weeks in outdoor aviaries on ad libitum food and water and later inoculated with a culture of house finch MG via a bilateral ocular route (Farmer et al. 2002) and infection was assessed with SPA and PCR approaches 2 weeks later. The spleens of same sex infected and uninfected birds were collected and stored in RNA-later (Ambion Inc.) and total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA quantity and quality were determined spectrophotometrically by A260 and A260/280 ratio and quality was assessed using electrophoresis on a 1.2% agarose/formaldehyde gel. One microgram total RNA from each spleen of an uninfected and an infected house finch was used to prepare double stranded cDNA using the SMART PCR cDNA synthesis kit (CLONTECH Laboratories, Inc., Palo Alto, CA) and suppression subtractive hybridization (SSH) was performed using the CLONTECH PCR-Select^{TM} cDNA Subtraction Kit (Clontech, CA).

Results

Analysis of MHC variation in diachronic population samples

We found high variability as measured by Θ and π (nucleotide diversity, Nei 1987) in the eastern US population both before and after the epizootic (Fig. 2). In particular, much of this variability is concentrated at putative PBR codons. Our results indicate that there was neither a strong decrease in effective population size post selection (Θ: Pre-Michigan = 0.0593, Post-Michigan = 0.07313, Pre-Auburn = 0.05623, Post-Auburn = 0.05953, Mann Whitney U, $P > 0.05$) nor an increase in the number of heterozygous sites after exposure (Average number of heterozygous sites Pre-Michigan = 4.2, Post-Michigan = 6, Pre-Auburn = 3.667, Post-Auburn = 3.867, Mann Whitney U, $P > 0.05$). In addition, the average number of pairwise differences between alleles has not increased in response to selection (Pre-Michigan = 18.05, Post-Michigan = 20.00, Pre-Auburn = 14.09, Post-Auburn = 17.62, Pre-California = 20.45, Post-California = 21.12, $P > 0.05$). Other measures of selection across the entire MHC fragment were not significant compared to the neutral expectation (Tajima's D which measures how population variability deviates from the neutral expectation (Tajima 1989) Pre-Michigan = 0.85696,

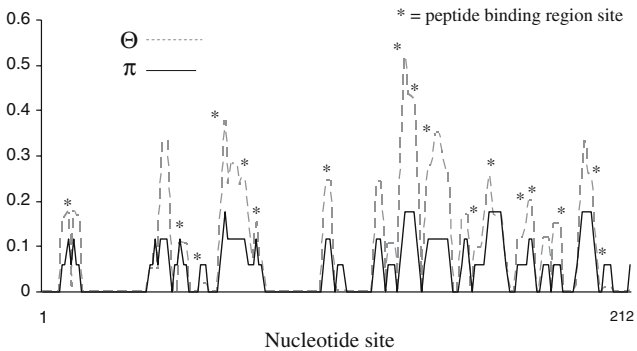

Fig. 2 Genetic diversity of house finches in Auburn, Alabama at the MHC locus. The dashed line represents Θ (Tajima 1993) and the solid line π (Nei 1987). The asterisks represent putative peptide-binding region codons inferred from X-ray crystallography studies in humans

Post-Michigan = 0.58462, Pre-Auburn = 0.54445, Post-Auburn = 0.56222, Pre-California = 0.63518, Post-California = 0.7991, all $P > 0.05$). For all of these values, the neutral anonymous sequence showed no deviation from the neutral expectation (Table 1).

Analogous to the prediction that selection should target specific genes rather than the entire genome, it may be that specific codons within the MHC class II exon 2 protein show a stronger signal of change over time than others (Hedrick et al. 1991). This would be particularly true if high levels of recombination dissociate the historical trajectories of different PBR sites, as recent measures in natural populations of birds and mammals have shown (Richman et al. 2003; Edwards and Dillon 2004). We therefore measured Tajima's *D* using a sliding window across the MHC exon (Fig. 3), including all samples from before and after the epizootic. The sliding window was 5 bp and shifted one base pair each iteration. If there has been no change in the selection profile before and after selection for MG resistance, we expect the values for Tajima's *D* to remain the same. Our data indicate that there are several locations that show marked shifts in *D* over time in both the Michigan and Auburn populations. In many cases, these sites are shared between different populations and time periods (sites 165, 171 and 192). All three of these sites are putative peptide binding region codons.

Gene expression and adaptive evolution

Genes are inferred to be up-regulated by MG when derived from experiments in which the infected bird cDNA served as the 'tester'. By contrast, genes are inferred to be down-regulated when derived from experiments in which the infected bird DNA is the 'driver'. To investigate whether MHC class II was regulated by mycoplasmal conjunctivitis in house finches, template cDNA that had been enriched for up- and down-regulated genes by SSH was amplified by PCR method with primers designed from MHC class II region (Fig. 4). Comparison of amplification patterns before and after the subtraction procedure demonstrated that the SSH approach increases MHC abundance, indicating that the SSH procedure was successful. Our results suggested that, contrary to a scenario in which MHC class II B is upregulated in response to pathogen infection, in fact MHC was down-regulated by exposure to MG. This conclusion stems from the observation that after subtraction we are left with considerably less cDNA in an infected bird compared to one with MG infection, emulating a quantitative approach by varying the number of cycles and comparing the intensity of product. Although we would predict that MG infection would result in increased expression of MHC class II, decreased expression does not necessarily indicate that the class II MHC was uninvolved in the immune response. Further studies of RNA production at different time points during the course of infection will be critical for understanding the detailed pattern of expression in MHC and other differentially expressed genes in house finches (Abendroth and Arvin 1999; Hengel et al. 1999), and also for testing in individuals with different MHC genotypes.

Discussion and future plans

We have compared levels of genetic variation of house finches introduced to the eastern United States to those found in their native Western US. Previous hypotheses

Table 1 Descriptive statistics for the new MHC locus and the anonymous locus

	Sampling-locality / Time					
	Pre-Michigan	Post-Michigan	Pre-Auburn	Post-Auburn	Pre-California	Post-California
Mhc						
No. of Individuals	19	10	10	25	9	8
No. of Var Sites	52	54	43	57	54	52
No. of Haplotypes	26	15	13	41	14	11
π	0.0852	0.0943	0.0665	0.0831	0.0965	0.0996
θ	0.0683	0.0824	0.0585	0.0716	0.0836	0.0838
Tajima's *D*	0.8569	0.5846	0.5444	0.5622	0.6351	0.7991
Anonymous locus-ALHFI						
No. of Individuals	11	13	5	36	7	9
No. of Var Sites	8	10	5	9	6	7
No. of Haplotypes	10	13	6	15	8	9
π	0.0063	0.0065	0.0062	0.0041	0.0054	0.0048
θ	0.0084	0.0111	0.0068	0.0079	0.0073	0.0078
Tajima's *D*	–0.82206	–1.37941	–0.37985	–1.27428	–0.93441	–1.30098

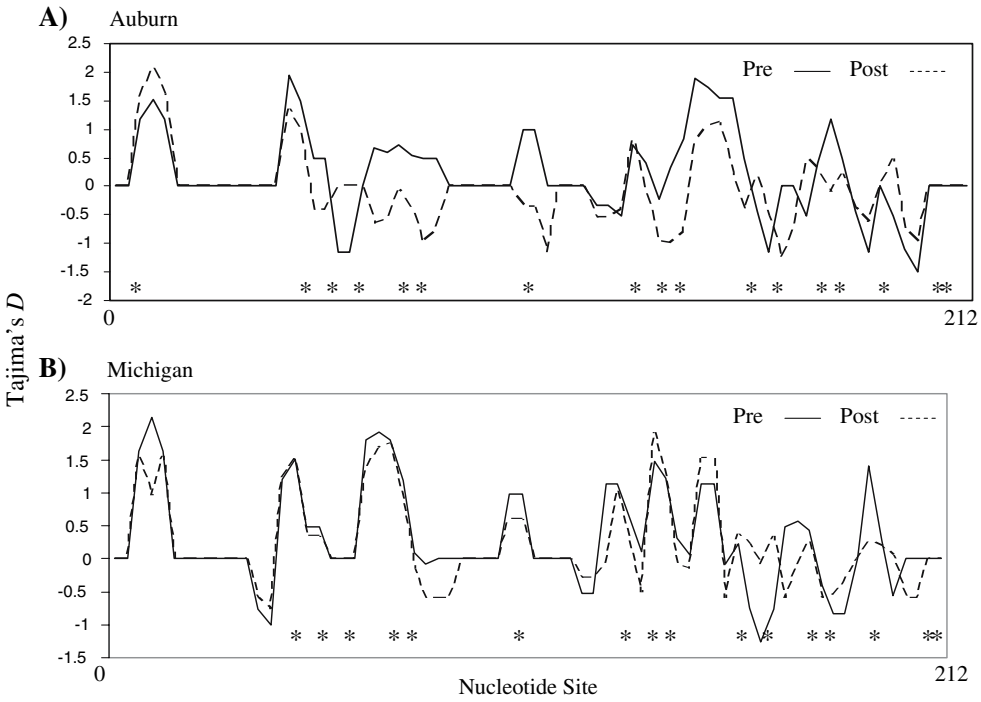

Fig. 3 Sliding window analysis of Tajima's *D* for the eastern US populations exposed to *Mycoplasma*. The solid lines are for the populations prior to exposure to the bacteria and the dashed lines after exposure. The asterisks represent putative peptide binding region codons as per Fig. 2

have postulated that the eastern population was founded by a relatively few number of founders and therefore should contain a subset of alleles from the founding population. Analysis comparing AFLP variation between these two populations shows that the eastern United States birds are distinct from, rather than a subset of, the Western birds, suggesting that, although little variation was lost through drift in the eastern US across the genome, drift nonetheless has shifted allele frequencies in the introduced birds to a detectable degree. However, we expect the effects of drift to be strongest in genes that have many variants expressed at low frequencies such as the genes of the MHC (Westerdahl et al. 2004). The combination of exposure to novel pathogens and loss of evolutionary potential through drift at immune system genes may

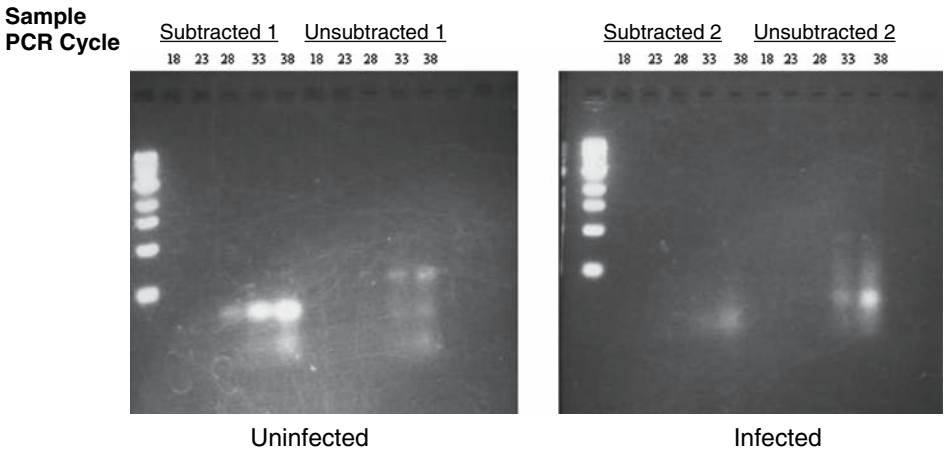

Fig. 4 Subtractive hybridization studies of class II MHC genes. (Left) The two gels show amplifications of cDNA from an individual infected with *Mycoplasma* (right) and an uninfected control (left). In each gel, the leftmost band is a DNA size marker. After subtractive hybridization we amplified conserved regions of the Class II region and found that there was considerably more product produced after subtraction in the uninfected (left) than in the infected (right) sample. This result indicates that the MHC was down-regulated in response to MG infection. See text for description of the subtractive hybridization procedure

lead to increased risk in populations that have gone through bottlenecks.

In this case, house finches do not seem to have suffered loss at genes of the MHC despite the observation of wild birds evolving resistance to a novel bacterial pathogen. We looked for evidence of selection for resistance to this disease at the MHC and found little support for selection acting at the level of the entire PBR exon. Nonetheless, we did find some examples of large shifts in selection patterns in analysis focused on specific PBR codons. These results are consistent with results from population comparisons of the human MHC which demonstrated that heterozygosities and other measures of polymorphism vary greatly across different amino acids and in general are much higher in peptide binding region codons compared to those that interact with the T-cell receptor (Hedrick et al. 1991; Reche and Reinherz 2003).

We have investigated the diversity of loci that exhibit up- or down-regulation in experimentally infected house finches to gain a broader view of the molecular basis of house finch response to MG infection (Wang et al. 2006). Our first attempts to examine expression levels of MHC using suppression subtractive hybridization showed there were 221 cDNA clones of 34 known genes and many novel transcripts were found to be differentially regulated in response to MG infection and confirmed with Northern blot analysis. Specifically, it appears that the MHC is down-regulated in the spleen by infection with MG. Admittedly, this first attempt only surveyed the change in expression levels in one individual finch. In loci that we identify to have altered expression patterns, we plan to assess population variation in mRNA levels. In addition, we plan on replicating these experiments in more individuals to determine whether there is variation in how expression levels change between individuals, especially those with differing genotypes at the MHC.

We have initiated a suite of genomic approaches to questions of the evolution of disease resistance in house finches, employing methodologies focused on the MHC as well as broad surveys of genome-wide variation. In addition to down regulation of the MHC in response to MG infection, we also found many other markers and genes with differing profiles of expression associated with MG infection. We will continue to explore these loci and their effect on MG resistance—these could be the loci, if they are genes of single effect, not multiple function like the MHC, that may show changes in Θ post-epizootic. Additional work on aspects of phenotypic evolution in house finches and molecular evolution of the bacterial pathogen itself will further our overall knowledge of avian host-parasite relationships and the evolution of disease resistance (Roberts et al. 2001; Hill et al. 2004).

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