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Toward an evolutionary genomics of the avian Mhc

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Summary: We review recent developments in the ongoing study of the evolution of the Mhc gene family in birds, with emphasis on class II B genes and results from songbirds obtained in our laboratory. Southern blots suggest a surprising diversity in Mhc class II gene number among various songbird species (Passeriformes). We have sequenced ~30 kb contigs from Mhc-bearing cosmid clones from two species, red-winged blackbirds (*Agelaius phoeniceus*) and house finches (*Carpodacus mexicanus*), whose demography, lifetime reproductive success, epizootics, parasitology and mate choice are among the best studied for natural populations of birds. Of three genes cloned from these species, only one appears strongly polymorphic, and one (from the house finch) is likely a pseudogene. All are similar in structure to those in chickens, albeit with introns intermediate in length between chickens and mammals. Phylogenetic analysis of available class II B peptide-binding region exons suggests that the overwhelming long-term force operating on avian genes sampled thus far has been post-speciation gene duplication and/or concerted evolution. These and other results suggest that the evolution of class II B genes in birds conforms to a mixture of several models of multigene family evolution proposed for the mammalian Mhc, incorporating ongoing homogenization, duplication and pseudogene formation. Large-scale sequencing studies in these and other species, though still in their infancy, will prove invaluable for studying the comparative structures of avian Mhcs, as well as patterns of selection, mutation and linkage disequilibrium at several scales.

Introduction

When it comes to science, birds are in many ways the poor sisters of mammals. In genetics and immunology, avian species almost inevitably receive the attention of the latest technologies after mammalian models do, and, in some instances, long after extensive work has been devoted to fish and amphibians. These trends certainly hold for genomic structure and evolution of the Mhc, despite the fact that the Mhc was first discovered in chickens (1). Although the entire chicken Mhc will likely be sequenced within the next few years, our knowledge of the short and long-term evolution and polymorphism of the Mhc in birds lags far behind that for mammals, and arguably for fish as well (2–4). Whereas the first surveys of variation in Mhc genes from natural populations of fish have already appeared (5, 6),

there is still no published study of variability at a single, well-characterized Mhc gene of birds in nature.

Recent studies of class I and II genes in birds reviewed here suggest that this dearth of comparative information on the avian Mhc has its origin at least in part in the unexpected challenges for characterization posed by the particular mode of Mhc gene family evolution when compared to mammals and as revealed primarily through comparative, interspecific studies (7–9). These same studies also herald the end of a decade-long era during which the chicken has, for lack of relevant data from other birds, played a phylogenetically disproportionate role in the quest to understand "the avian Mhc". This decade was marked at its beginning by the first genomic sequences for chicken Mhc genes (10, 11) and at its end by the publication of the first Mhc genomic sequences (albeit partial) from an avian species other than chicken (12). Understandably, the urge to generalize, to speculate on the evolution of the avian Mhc and to compare suggestive evolutionary trends to those in mammals has been strong during this period, but this urge has until recently proceeded primarily on knowledge from but a single species, with few comparative data. This is because insights of relevance to human health produced by studies of the avian Mhc, and of avian immunobiology generally, can accumulate faster by focusing in detail on a single species, and the research program of chicken geneticists has generally proceeded "vertically", revealing ever increasing detail on this single species. This history contrasts with the research programs of systematists and comparative biologists – for whom diversity and the comparative method is paramount and single-species detail less so – which typically proceed "laterally" across species once a new technology, focus of study or paradigm is introduced.

As a result, many interesting hypotheses raised on the basis of chicken data – such as Kaufman's provocative suggestion (13) that the avian Mhc is "minimal essential" (i.e. harbors fewer genes, junk DNA and intergenic regions than that of mammals, and hence is subject to stronger selection intensities per Mhc gene), or the idea that chicken Mhc introns are small because they occur on microchromosomes (14) – have still gone untested because the Mhcs of species relevant to these hypotheses, both birds and other vertebrates, have not yet been examined with the appropriate tools. Given what are by mammalian standards striking examples of Mhc-mediated infectious disease resistance and susceptibility in birds (reviewed in (15)), and the increasing realization by biologists that evolutionary thinking can fruitfully inform medical science, it will be interesting to monitor the future contributions to medicine of comparative avian Mhc studies now that the bottle of such studies has been uncorked. In reviewing our recent studies of avian Mhc evolution, we suggest several research protocols for this new comparative effort, which will productively combine the insights and reagents from chicken immunogenetics with attention to appropriate sampling of the avian phylogenetic tree.

Species sampling: phylogenetic criteria for inferring ancestral *Mhc* structures in birds

One obvious question in the evolutionary genetics of the avian Mhc is: what did the ancestral Mhc for birds look like? Was it small and compact like the chicken Mhc, or was it larger, perhaps dispersed on multiple chromosomes, as is suspected for frogs and some fish (16, 17)? It is worthwhile to ask how best to sample representatives from the avian tree so as to clarify the major features of Mhc evolution in birds most efficiently. Since parsimony inference of the ancestral Mhc structure for birds will depend primarily on structures found in representatives that branch deeply in the two basal lineages for birds, as well as on Mhc structure in crocodiles (the closest living relatives to extant birds), the answer, of course, depends on avian phylogenetic relationships (Fig. 1) (18). Some molecular and morphological data support the arrangement in which gamebirds, the focus of ongoing Mhc research by several groups, are thought to have branched off early among extant birds as the sister group to ducks and geese (Fig. 1A) (19, 20). By contrast, the relationships of groups such as perching birds (Passeriformes) are less certain. Traditionally, perching birds have been considered a relatively recent and rapid radiation, and what cladistic studies of morphology have been made, and to a certain extent DNA hybridization evidence, support this interpretation (reviewed in (19)). However, recent mitochondrial DNA analyses result in perching birds replacing ratites (flightless birds) as one of the two basal avian lineages (21, 22). If true, then understanding Mhc structure in perching birds will be critical, along with information from crocodiles, for defining the ancestral Mhc structure for birds. By contrast, studying Mhc structure of species embedded high up within either of the two basal clades of birds – as perching birds are traditionally – will shed less light on ancestral avian Mhc structure if parsimony is the criterion for inferring such structure on evolutionary trees (18). For example, if the mitochondrial tree (Fig. 1B) proves correct, the structure of the Mhc in ratites will influence reconstruction of the ancestral Mhc less than under the traditional tree (Fig. 1A).

Our studies have focused on songbirds (oscines), the lineage of perching birds with complex song-learning capacities and relatively large brain size. Of the ~5,300 species of perching birds, approximately 4,000 are songbirds. DNA–DNA

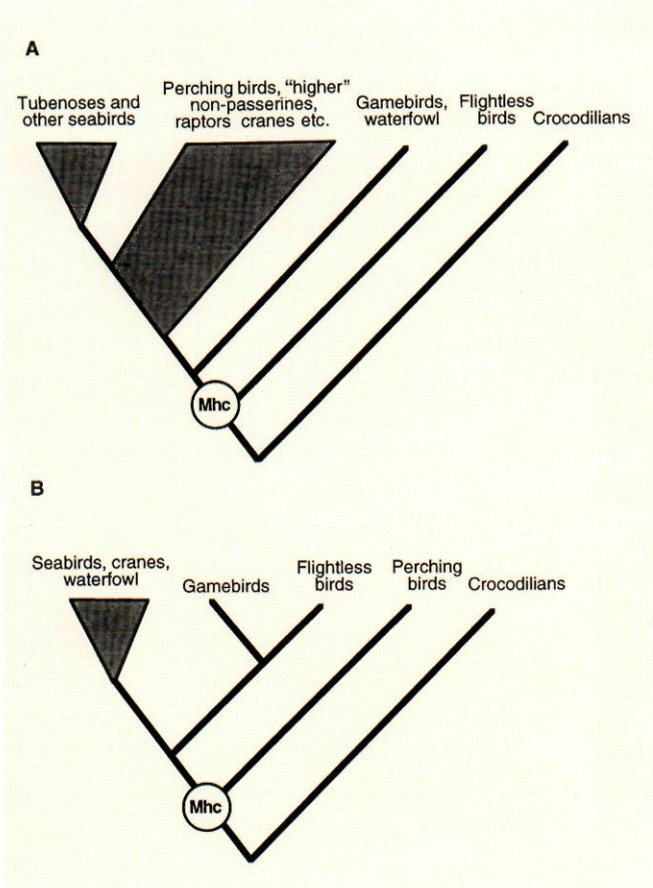


Fig. 1. Competing hypotheses for avian phylogenetic relationships with different consequences for the inferred ancestral structure of the *Mhc*. In each tree, the hypothetical ancestral *Mhc* is indicated.
A. Avian relationships as recovered by morphology and DNA–DNA hybridization studies. These relationships follow Mindell's summary on the Tree of Life Web Page (http://ag.arizona.edu/tree/eukaryotes/animals/chordata/dinosauria/aves/neornithes.html). The traditional and DNA hybridization trees conflict in many places, but agree in not placing perching birds as one of the two basal branches of the tree.
B. Relationships among birds as indicated by mitochondrial DNA data, following Härlid et al. (22).

hybridization studies suggest that songbirds are divided into two major clades, the Corvida, which includes crow-like birds and probably originated in Australia/New Guinea, and Passerida, which includes most other temperate and tropical oscine genera. The divergence time of Corvida and Passerida has been estimated at 55–60 million years ago (23) (although some molecular and fossil data favor more recent dates (24, 25)). Our most detailed investigations thus far have concentrated on two species of Passerida, red-winged blackbirds (*Agelaius phoeniceus*) and house finches (*Carpodacus mexicanus*) (7, 12), and there is also some information for a representative of the Corvida, scrub jays (*Aphelocoma coerulescens*) (7).

The above three species are of interest in the context of *Mhc* because each is common in North America and has been the focus of a wealth of ecological, evolutionary, genetic and endocrinological studies, making the payoff for cloning *Mhc* genes greater. The Florida scrub jay (*Aphelocoma coerulescens*) has been the subject of detailed long term studies of behavior, life history, demography and reproductive success (26), whereas other species in the genus *Aphelocoma* have long been model systems for the study of social behavior and phylogeny (27). House finches (*Carpodacus mexicanus*) and red-winged blackbirds (*Agelaius phoeniceus*) are important models in the study of sexual selection (28, 29), breeding systems (30), host–parasite interactions (31), phylogenetics (32) and population genetics (33, 34). House finches are currently undergoing a major mycoplasma epizootic in the eastern United States (35, 36), resistance to which could have an *Mhc* component. Thus, *Mhc* genes cloned from these species will be useful in both a phylogenetic and an ecological context.

One probe, many genes

Southern blots are a useful first step to gaining a genome-wide view of complexity of *Mhc* genes in birds. There are now a number of cloned class II B probes available for such use, including reverse transcriptase–PCR products spanning exons 1–4 in several songbird species (7). In addition to those from chickens, cloned class I sequences are also available from Japanese quail (*Coturnix japonica*) (37) and the great reed warbler (*Acrocephalus arundinaceus*) (38). Southern blots using same-species probes have revealed a surprising diversity in complexity among songbird species: whereas house finches appear to have a single strongly hybridizing band and 2–3 weakly hybridizing bands, blackbirds and jays have 8–11 weakly hybridizing bands (Fig. 2) (9).

By characterizing DNA fragments containing weakly hybridizing bands and detecting *Mhc*-like sequences on them, we have demonstrated that these weakly hybridizing bands do in fact contain *Mhc* genes or gene fragments, and are not simply the result of non-specific hybridization. Earlier suggestions that the complexity of *Mhc* class II genes in scrub jays was low, perhaps containing one or two genes (39), may require revision. These studies used a probe containing only highly variable peptide-binding region (PBR) sequences under fairly stringent hybridization conditions, a protocol that appears not to detect as many loci as when more conserved regions are used as probes (9). Further data from African greenbuls (*Andropadus virens*) supports the notion that number and complexity of class II B genes in songbirds may be substantial (A. Aguilar,

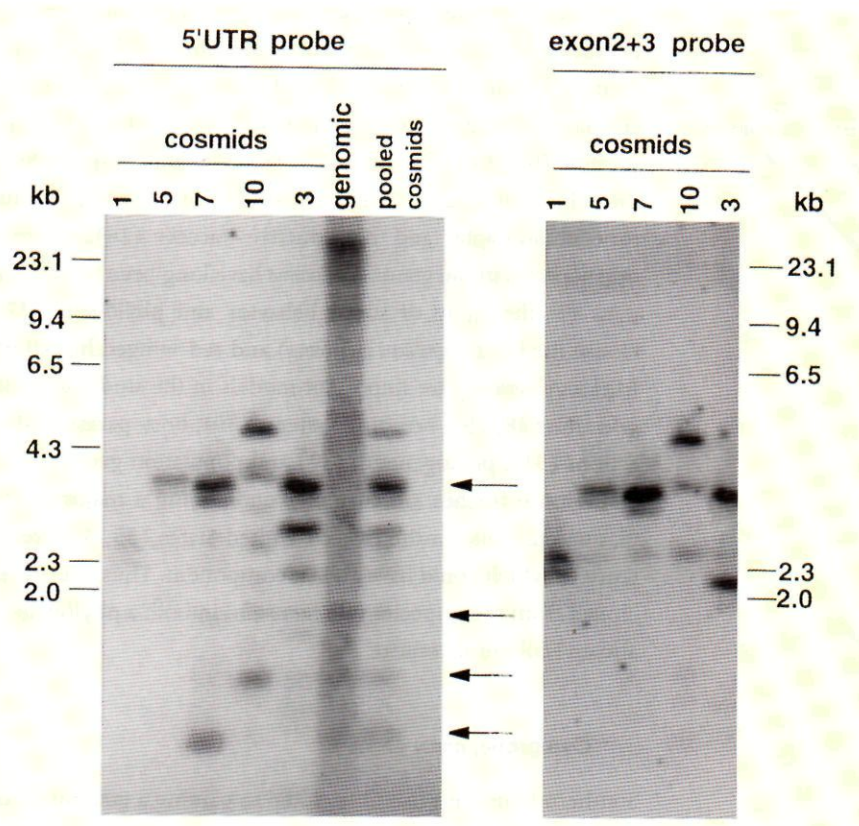


Fig. 2. Southern blots of red-winged blackbird probes on five Mhc-bearing blackbird cosmids and genomic DNA cut with Pst-I. Left, hybridization patterns recovered using a probe generated by PCR from genomic DNA and specific to the 5'-untranslated region of *Agph-DAB1*, an Mhc class II B gene in blackbirds (12). Arrows to the right of the panel indicate hybridizing bands shared in at least one cosmid and genomic DNA. The rightmost lane contains all five cosmid clones pooled. The primers used to generate the 5' probe were CGCCACTTTTGGGCAG (forward) and GGGCACCGCTGAGATC (reverse). Right, hybridization patterns from a probe generated from a cDNA clone (7) and spanning most of exons 2 and 3. Primers used to generate this probe are given in Edwards et al. (12).

unpublished data). New data from class I genes in songbirds is even more striking: Westerdahl and her colleagues' cDNA analysis of class I genes in reed warblers using a same-species probe suggested a highly redundant multigene family containing at least five genes (38).

Three of the four songbird species (all except house finch) for which Southern blot data are available exhibit an *Mhc* complexity exceeding that found for chickens. These differences are unlikely to be due solely to the increased detail with which the chicken *Mhc* is known. For example, a typical hybridization of a full length chicken class II B probe to chicken genomic DNA cut with Pst I yields 4–6 fragments per individual, some of which are weakly hybridizing (40). It is known that chicken class II probes will detect genes in both the canonical B-complex as well as the newly defined *Rfp-Y* system (41). This is an expected outcome because the sequence divergence between genes in these two clusters is less than 7% in conserved exons and the genes are much more closely related to one another than are typical mammalian class II genes (7). Thus in both chickens and songbirds, a single class II B probe containing conserved exons routinely detects multiple genes, and the number of bands detected on songbird blots appears greater than those for chickens (9). Nonetheless, in both ring-necked pheasants (*Phasianus colchicus*) and songbirds, the number of genes detected by systematic cDNA cloning is usually less than the number visualized on Southerns (8). Many of the "missing" genes may be unexpressed. If true, the Mhcs of songbirds may contain considerably more Mhc-like fragments and pseudogenes than does the chicken Mhc. Class I and II pseudogenes, the first to be reported for birds, have now been detected in reed warblers, house finches and possibly red-winged blackbirds (38) (C. Hess, J. Gasper, S. Edwards, in preparation). As suggested by Westerdahl et al. for class I genes (38), the complex hybridization patterns found at both class I and II loci in songbirds suggests that the Mhc of this lineage may not be "minimal-essential" as in chickens, perhaps containing higher numbers of pseudogenes and Mhc-gene fragments, a pattern more typical of the Mhc of mammals (42).

Sequence analysis of *Mhc*-bearing cosmid clones

We have reported on construction of cosmid libraries from two species, red-winged blackbirds and house finches, from which we have isolated Mhc-bearing clones and initiated complete characterization via shotgun sequencing (9, 12). The chicken Mhc was originally characterized with cosmids (10), and they provide inserts of a convenient size for analysis of new Mhcs. Whereas in simple Mhcs such as in house finches there is rea-

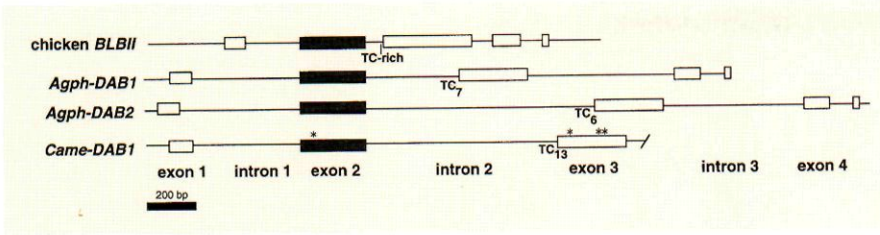


Fig. 3. Structure and relative sizes of four avian Mhc class II B genes, centered on exon 2. The genes are from red-winged blackbirds (*Agph-DAB1* and 2), house finches (*Came-DAB1*) and chickens. The β1 exon (exon 2) in each gene is shaded. Asterisks above exons in the house finch gene indicate putative frameshift mutations rendering it a pseudogene. The 5' upstream region is arbitrarily cut off at around 500 bp upstream of the start codon for each gene. Exons 1–5 and introns 1–4 are indicated for each gene, except for *Came-DAB1*, in which no structures downstream of exon 3 have been detected at the time of writing.

sonable possibility of cloning a large fraction of the class II genes in a few cosmids (9), more complex Mhcs such as found in blackbirds will require many more clones for adequate coverage. In our hybridization and sequencing studies so far, we have generally found a single class II B gene, with possibly one or more additional class II fragments, per cosmid clone. Analysis of five cosmids from blackbirds with probes generated from the 5' region upstream of a class II B gene, or from the second and third exons, supports this pattern (Fig. 2). Similar hybridization patterns are recovered with either the non-coding region or the coding region probes. This result also contrasts with what is known of the chicken map, which contains instances of class II genes within a few kilobases of one another, and suggests again that other birds may not conform to the minimal-essential rule as tightly as do chickens.

We have employed "large-scale" sequencing methods to characterize individual cosmids bearing Mhc genes in birds. Our protocol employs essentially the same techniques as those used in human genomics (43). Whole cosmids of interest are sequenced by the shotgun method, in which small (1.5–2.5 kb) sonicated fragments of the clones are subcloned into M13, followed by sequencing of one end of 800–900 randomly chosen subclones. New, more accurate chromatograms are generated from the raw sequence data using the base-calling program PHRED (44, 45), and assembled into longer contigs using both sequence overlap and sequence quality information by the program PHRAP (P. Green, unpublished, documentation available from: URL: http://bozeman.mbt.washington.edu/phrap.docs/phrap.html). The resulting contigs and chromatogram data are visualized using CONSED (46), which also aids in developing strategies to connect contigs via directed sequencing and PCR. We used the program SeqHelp (47) to identify coding regions, simple sequence repeats, transposable elements, and other features commonly encountered in vertebrate genomes.

Using these or similar methods, we have characterized three class II B genes at the genomic level (Figs 3 & 4) to date. Two of these genes are from blackbirds, and one, *Agph-DAB1*, we know is polymorphic. The third is from the house finch, *Came-DAB1*. All three genes have introns as large or larger than a typical chicken class II gene (although exon sizes are similar), making them the largest avian class II B genes characterized to date. The cosmid clone from which *Agph-DAB2* was derived corresponds to a weakly hybridizing fragment in blackbird genomic DNA, whereas the clone from which *Came-DAB1* derives corresponds to the single strongly hybridizing band in house finches. Despite our probing the finch library with a same-species cDNA and the correspondence of the clone to the single strong band in finches, *Came-DAB1* is apparently a pseudogene (C. Hess, J. Gasper, S. Edwards, manuscript in preparation). These results underscore the likelihood of redundancy even in songbird Mhcs that are relatively simple as judged by Southern blots, and the insufficiency of genomic hybridization signal for inferring functionality.

Features such as the presence of a (TC) microsatellite of varying length at the 3' end of intron 2 in each of the three songbird genes appear homologous to similar motifs in the same position in at least some chicken genes (48) and confirm ultimate derivation of songbird and chicken Mhc class II B genes from a single ancestral gene that likely possessed this motif (Fig. 3). However, in general, microsatellites are very uncommon in the songbird sequences. In addition, base compositional profiles of the three songbird genes compared to that of a functional chicken gene suggest maintenance of functional constraint via stabilizing selection, at least in the recent past (Fig. 4). Edwards et al. (12) noted that the base compositional bias of third positions of codons in *Agph-DAB1* were nearly as extreme as in functional chicken genes, and differed markedly between introns and exons. This result contrasts with the expectations of more even base compositions across functional

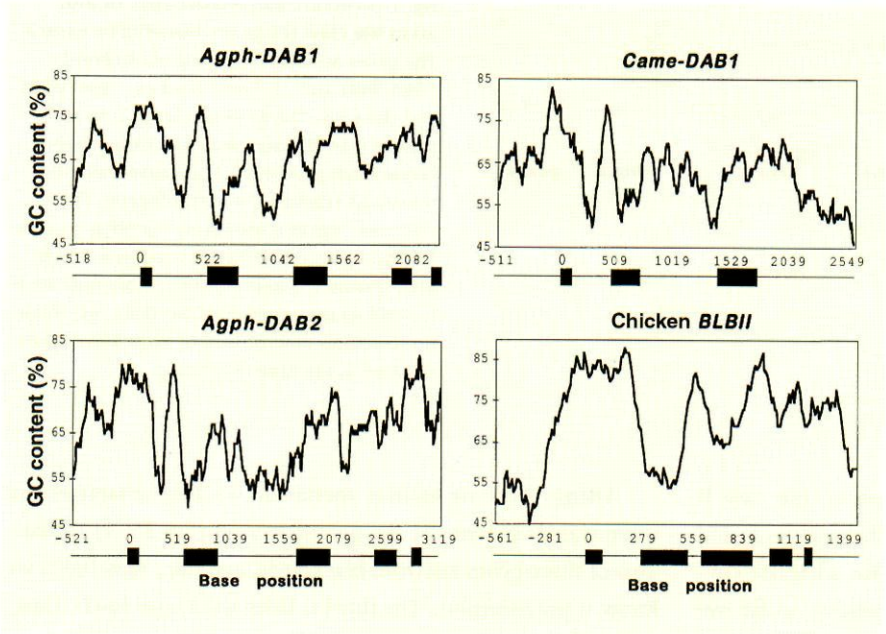


Fig. 4. Base compositional profiles of four class II B genes, three from songbirds and one from chicken. Gene names are as in Fig. 3. Below each plot is a schematic showing intron exon organization of each gene. The profiles were created using the PercentGC program written by T. Smith & A. Smit (unpublished), which calculates GC or AT content of a sequence using sliding windows of specified length and offset distance. We modified his program to generate profiles specific to each base and used a window length of 100 and an offset length of 10.

classes of sites for pseudogenes and, in addition to data on polymorphism, suggested that *Agph-DAB1* was functional. All four genes in Fig. 4 suggest possible CpG islands upstream of their respective start codons. In addition, whereas a deficit of Cs and Gs is evident in the second intron of both blackbird genes, in chickens this intron may be too short for AT mutation pressure to have any consequences, and the deficit does not appear strongly in the house finch gene (*Came-DAB1*). However, the similarity in base compositional profile between likely pseudogenes such as *Came-DAB1*, and functional genes (Fig. 4), suggests that base composition may be retained for a period after dysfunctionalization and alone is not a good guide to functionality.

In addition to revealing a variety of structural features and non-*Mhc* genes in the vicinity of *Mhc* genes (C. Hess, J. Gasper, S. Edwards, manuscript in preparation), shotgun sequence analysis of *Agph-DAB2* and *Came-DAB1* clones permits analysis of the base compositional context of individual *Mhc* genes (Fig. 5). Base composition in the nuclear genome of vertebrates has been most extensively studied at large scales, such as entire isochores (49), or at relatively small scales, such as in comparisons among codon positions within a gene. There are fewer studies of base compositional differences at the level of intron/exon organization of single genes, or at "mesoscales", such as individual cosmids. Base compositional variation on the scale of entire isochores is likely to affect primarily the mean base composition of an individual cosmid. The base compositional profile of these cosmids appears quite variable at the level of 1 kb windows, with a mean GC content for both the blackbird and finch contigs of 51.9% and 56.8% respectively. The large oscillations in base composition seen in Fig. 5 could in some cases to be attributable to CpG islands preceding, for example, the *Mhc* genes on each cosmid. However, CpG islands do not appear to explain all the oscillations. Since base composition reflects an equilibrium between mutation rates to different nucleotides, this variability in composition in the finch and blackbird cosmids might suggest spatial heterogeneity in mutational processes and/or selection. However, the heterogeneity of the base composition expected for a simple stochastic model of base substitution may be enough to explain the observed patterns.

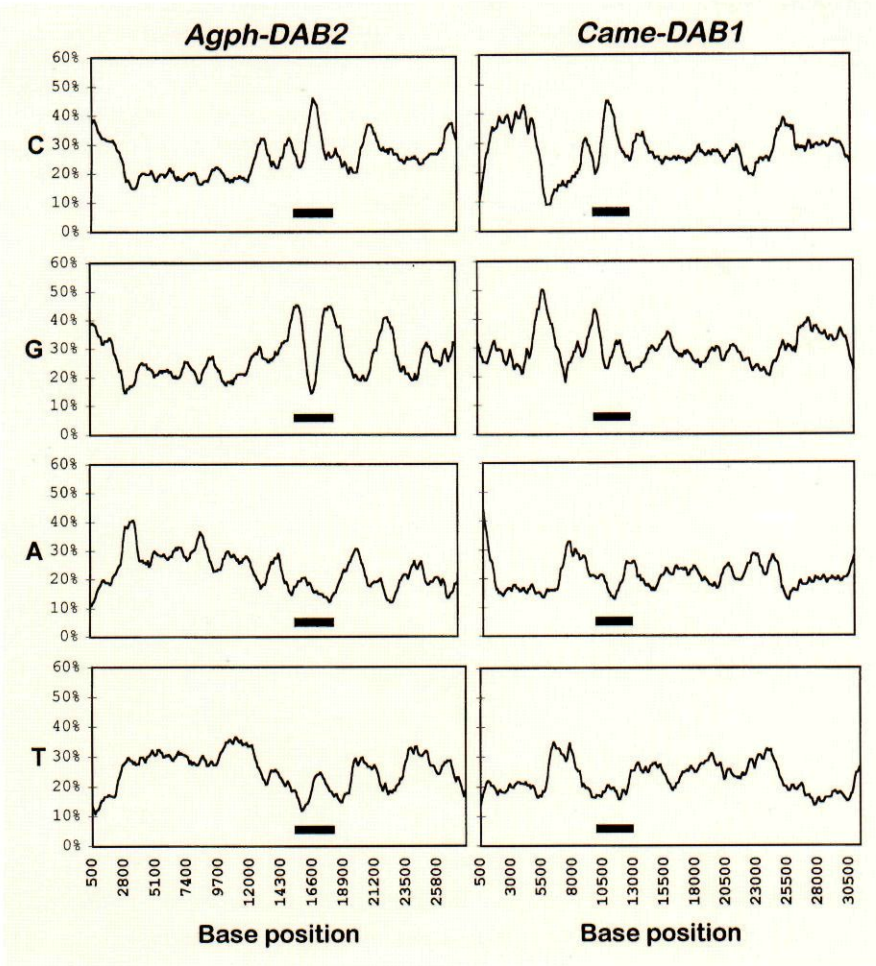


Fig. 5. Base compositional heterogeneity along two cosmid contigs for blackbird and finch. We used the program PercentGC as in Fig. 4, except the window size was 1,000 bases and the offset length 100. Black rectangles indicate the approximate size and location of the *Mhc* gene on each contig, beginning at the start codon and ending at the stop codon (*Agph-DAB2*) or at the limit of apparent sequence similarity to other bird *Mhc* genes (*Came-DAB1*).

An *Mhc* fragment consisting of an isolated class II B exon 4 sequence is present on the blackbird cosmid. In addition, genes unrelated to *Mhc* genes are detectable on the finch cosmid. The *Came-DAB1* cosmid also contains a serine–threonine kinase gene, whose products are involved in protein phosphorylation (50), and an open reading frame bearing high similarity to a zinc-finger Krüppel sequence, a widespread motif in eukaryotic transcription factors (51). These particular genes are not known to occur in the chicken *Mhc* (41, 52), and it is unlikely that these cosmids and those characterized thus far from chickens are alignable other than at the *Mhc* loci themselves. It is also apparent that the gene density on the songbird cosmids is much lower than in the chicken cosmids characterized thus far. The first large-scale sequences of the chicken cosmid clusters show that gene density in this part of the *Mhc* is extremely high: a Genbank entry (locus name GGBLOCUS) lists 19 genes or pseudogenes in ~92 kb, or a gene frequency of greater than 1 gene per 5 kb – a frequency certainly deserving of the label "minimal-essential"! At this frequency we would expect to see 12–13 genes in the ~60 kb we have characterized in two species thus far, as compared with the 2 or 3 we do in fact find (C. Hess, J. Gasper, S. Edwards, manuscript in preparation). Preliminary evidence from the blackbird suggests that several of our cosmids are linked together in larger clusters, but we have not yet determined the precise scale on which these cosmids are clustered nor whether they even map to the same chromosomal regions or parts of the genome. Linkage studies and analyses using fluorescent *in situ* hybridization will be useful in this regard.

Long-term multigene family evolution in the avian *Mhc*

The evidence for multiple class II B genes in the *Mhc* of songbirds suggests that gene duplication has been an important feature of *Mhc* class II evolution in this group; the question now remaining is: when did these duplications take place relative to speciation events, and are duplicate genes frequently retained over successive speciation events? The first comparative analyses of *Mhc* evolution between different avian lineages suggested that concerted evolution played an important role in the evolu-

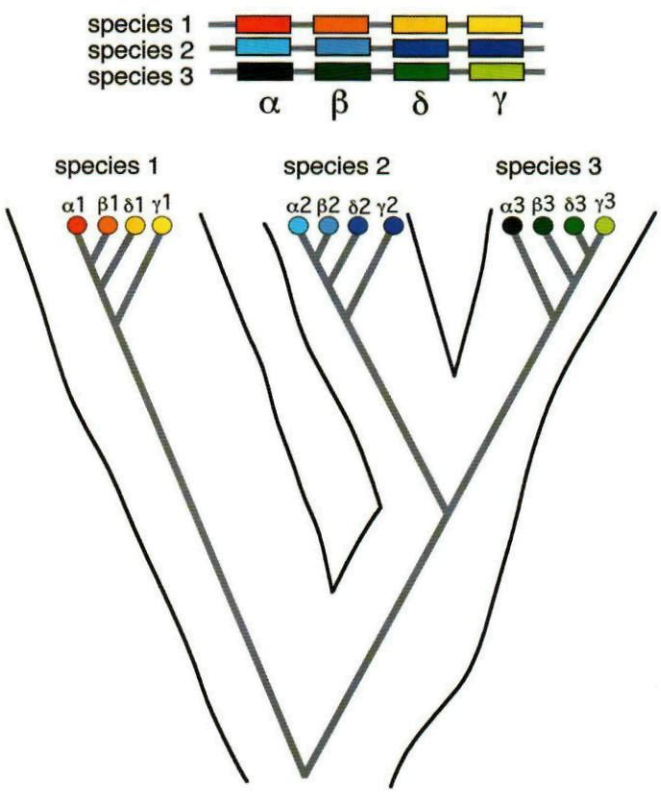


Fig. 6. The concerted evolution model and delayed duplication models of Mhc genes yield topologically identical gene trees. Top: schematic of four loci in a single chromosome in each species. Bottom: gene tree embedded in a species tree showing relationships in which all genes within a species are most closely related to one another. Such a gene tree can be explained either by a round of concerted evolution in each species after speciation or by duplication events within each species after speciation.

tion of avian Mhc genes (7). This conclusion was evident from the fact that different sequences within a given species, including those of chickens, not only clustered together to the exclusion of mammalian sequences, but clustered together to the exclusion of other bird sequences. This result suggested that the rates of homogenization observed in coding regions of class II B genes, particularly in exon 3, were higher than those observed in mammals. Indeed, in mammals, the class II genes appear to evolve largely in a divergent manner, because phylogenetic analysis suggests that paralogous genes that have duplicated in ancestral species have done so prior to most speciation events in mammals. However, the avian data are also consistent with a longer waiting time for gene duplications in a given lineage, or delayed duplication (Fig. 6). This latter model is similar to the "accordion" model proposed by Klein et al. for vertebrate class II B genes generally (53), in which a subset of ancestral Mhcs are founders for subsequent expansion via gene duplication in different vertebrate lineages. Given information only on coding regions, it is difficult to discriminate between the concerted evolution and the delayed duplication hypotheses. In order to test these ideas in greater detail, the cDNA sequences from which the concerted evolution hypothesis was first erected for avian Mhc genes needs to be augmented by sequence data at the genomic level. It is known that coding and non-coding regions of a multigene family can be subject to drastically different levels of sequence homogenization over time (54), and recent analyses of chicken and pheasant cDNAs suggest that untranslated regions (particularly 3') may escape homogenization more readily than coding regions (8). Thus comparison of coding and non-coding regions, ideally over blocks of sequence extending far away from the Mhc genes themselves, can be used to study in detail the long-term dynamics of avian Mhc genes.

Although interlocus gene conversion is known to occur in mammalian class II genes (55, 56), apparently the rates of such conversion are low enough and the size of converting tracts small enough to permit continued divergence of genes (57). Ohta (58) has suggested on the basis of non-synonymous/synonymous ratios that rates of interlocus gene conversion may be higher in non-human mammals. It will be an important goal of the future to quantify the differences in rates and patterns of interlocus gene conversion in birds and mammals and to determine whether ongoing genomic processes of the two systems differ only quantitatively or whether novel mechanisms are at work in one or the other system.

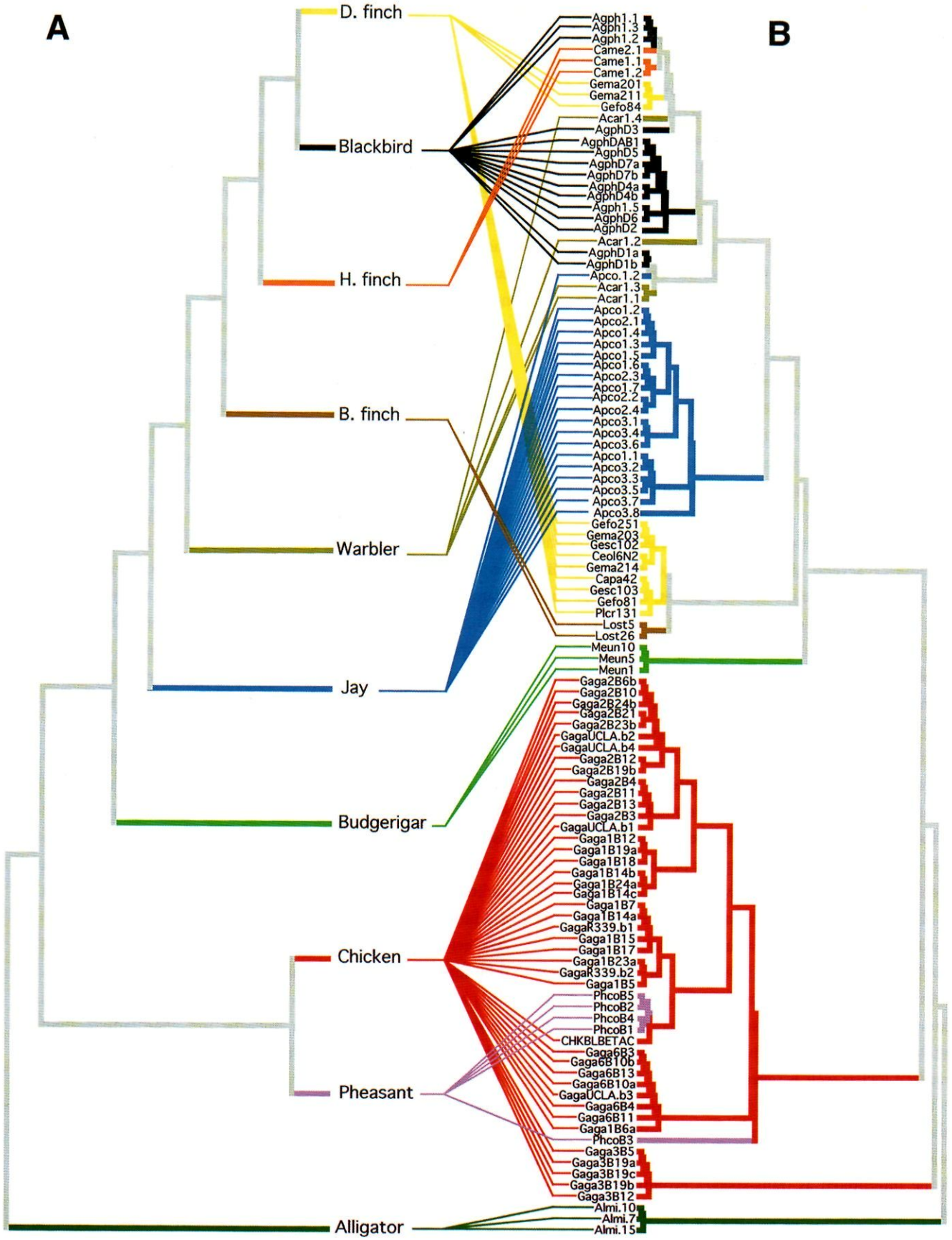
To evaluate the current data base for evidence of concerted evolution or delayed duplication in avian Mhc genes, we conducted a phylogenetic analysis of most of the available exon 2

sequences for avian class II B genes (Fig. 7). Generally the exon 2 sequences suggest strong clustering of genes within each species, with very little evidence for orthologous sequences clustering together to the exclusion of other sequences. Page (59, 60) has developed a method for estimating the minimum number of duplications in a gene family given phylogenetic relationships of the sequences and of the species from which those sequences were sampled. His method also allows one to estimate the number of gene deletions or unsampled gene copies that were expected to have occurred in a given species given the above information, and at what points in the tree these missing sequences occur.

We applied the above method to the tree in Fig. 7. This tree contains 106 sequences from ten species, including the American alligator (*Alligator mississippiensis*). Gene duplications and allelic divergences in this tree are visualized as "fans" spreading out from one tip of a species tree. Two or more smaller fans emanating from a single species indicate possible clusters of alleles corresponding to paralogous loci within those species; under an extreme scenario of recurrent concerted evolution, we would not expect to see any such broken fans. However, the analysis suggests several putative orthologous loci within which alleles diverge among different species, and every species except for Bengalese finch, house finch and alligator show such broken fans (these exceptions are likely due to small sample sizes of these species). Thus, there is some evidence for retention of orthologous loci in this data set. However, the number of inferred gene duplications/homogenization events is the predominant pattern; even different chicken loci cluster together to the exclusion of the songbird and pheasant sequences (7, 8). Were a similar analysis done for mammalian class II B loci, we would expect to see several small fans arising from each species and leading to all parts of the gene tree, not just particular subclades, consistent with largely divergent evolution of these loci. Thus this particular analysis supports the predominance of a concerted evolution/delayed duplication (accordion) model. Of course, for most of the songbird sequences the allele/locus relationships of a given sequence are poorly known; since in some cases more than two sequences were derived from a given individual, any given fan could include several loci as well as several alleles.

The analysis also recovered 29 "deep coalescences" i.e. instances in which ancestral polymorphism could have caused discrepancy between the gene and species trees. These could in principle be interpreted as trans-species evolution. Although trans-species evolution is suspected to occur in some avian lineages (61), current avian examples are not as clear cut as in other vertebrate groups (2, 62). In this light the degree of species-specific clustering of sequences in our updated tree (Fig. 7) suggests that the time scale over which retention of ancestral polymorphisms occur may be relatively short compared to mammals. However, the sequences we analyzed are very short, and it has been suggested that recombination and gene conversion occurs at PBR exons of class II genes of birds (7, 12), compromising traditional phylogenetic analyses such as ours. Furthermore, non-PBR exons and adjacent introns inevitably yield trees that differ from the PBR tree (7, 63–65). For these reasons and because the sampling of species is very limited, we treat the analysis in Fig. 7 as preliminary. We feel that non-PBR exons and non-coding regions are superior to PBR exons for recovering the true genealogy of *Mhc* genes, although phylogenetic analysis of the PBR exons can yield insight into other processes.

Despite the suggestion of high rates of interlocus gene conversion in avian class II genes, and hence a concerted evolution-type model, the presence of pseudogenes lends support to a different model of multigene family evolution, the birth-and-death model. In the birth-and-death model, gene deletion and dysfunctionalization (death) is balanced by duplication (birth) to maintain gene number in the long term. This model has also been suggested to characterize long-term evolution in the mammalian *Mhc* (57). However, it will be useful to characterize the long-term evolution of the *Mhc* family in more detail than that provided by a single model, especially since the divergent evolution and birth-and-death models are not really mutually exclusive. Several different gene families are now known to conform to the birth-and-death model, yet the rates of gene duplication and pseudogene formation appear to differ drastically in each. For example, of the three *Mhc* genomic sequences known for birds other than chicken, one and possibly two are pseudogenes, suggesting a ratio of functional class II genes to pseudogenes that is relatively high compared to mammals. In addition, the immunoglobulin V_H family appears to evolve via the birth-death process in both birds and mammals, yet available evidence suggests that the rates of gene duplication and pseudogene formation are much higher in birds than in mammals at these loci (66, 67). Quantitation of such differences between different vertebrate lineages and gene families will permit more robust comparisons within the framework provided by the birth-and-death model. Development of modern population theory specifying the shapes of phylogenetic trees of multiple genes and alleles in a gene family is sorely needed.


A
B
D. finch
Blackbird
H. finch
B. finch
Warbler
Jay
Budgerigar
Chicken
Pheasant
Alligator
Agph1.1
Agph1.3
Agph1.2
Came2.1
Came1.1
Came1.2
Gema201
Gema211
Gefo84
Acar1.4
AgphD3
AgphDAB1
AgphD5
AgphD7a
AgphD7b
AgphD4a
AgphD4b
Agph1.5
AgphD6
AgphD2
Acar1.2
AgphD1a
AgphD1b
Apco1.2
Acar1.3
Acar1.1
Apco1.2
Apco2.1
Apco1.4
Apco1.3
Apco1.5
Apco1.6
Apco2.3
Apco1.7
Apco2.2
Apco2.4
Apco3.1
Apco3.4
Apco3.6
Apco1.1
Apco3.2
Apco3.3
Apco3.5
Apco3.7
Apco3.8
Gefo251
Gema203
Gesc102
Ceol6N2
Gema214
Capa42
Gesc103
Gefo81
Plcr131
Lost5
Lost26
Meun10
Meun5
Meun1
Gaga2B6b
Gaga2B10
Gaga2B24b
Gaga2B21
Gaga2B23b
GagaUCLA.b2
GagaUCLA.b4
Gaga2B12
Gaga2B19b
Gaga2B4
Gaga2B11
Gaga2B13
Gaga2B3
GagaUCLA.b1
Gaga1B12
Gaga1B19a
Gaga1B18
Gaga1B14b
Gaga1B24a
Gaga1B14c
Gaga1B7
Gaga1B14a
GagaR339.b1
Gaga1B15
Gaga1B17
Gaga1B23a
GagaR339.b2
Gaga1B5
PhcoB5
PhcoB2
PhcoB4
PhcoB1
CHKBLBETAC
Gaga6B3
Gaga6B10b
Gaga6B13
Gaga6B10a
GagaUCLA.b3
Gaga6B4
Gaga6B11
Gaga1B6a
PhcoB3
Gaga3B5
Gaga3B19a
Gaga3B19c
Gaga3B19b
Gaga3B12
Almi.10
Almi.7
Almi.15

Polymorphism, concerted evolution and the quest for locus specificity in the avian *Mhc*

Kaufman & Salomonsen (15) have highlighted the relevance of polymorphism for both sequence diversity and expression levels for Mhc-linked disease resistance in birds. Focusing on class II B genes, Zoorob and colleagues (68) conducted the first systematic surveys of Mhc sequence diversity in birds. These patterns of nucleotide substitution in chicken class II B genes have been quantified and are consistent with the action of balancing selection at some loci, such as the BLB1/II family, but not loci such as the BLBIII group (39). Allelic diversity at BLBIII loci was characterized by a slight excess of synonymous substitutions in the PBR sites of exon 2, although at these sites and at non-PBR exon 2 sites the rates of synonymous and non-synonymous substitution were statistically indistinguishable. Therefore, the two chicken gene families BLBI/II and BLBIII show interesting contrasts in evolutionary dynamics, with a much more attenuated pattern of evolution, perhaps suggestive of stabilizing selection, at BLBIII.

To our knowledge there have been no other analyses of nucleotide variability at any Mhc loci in birds at what is known unambiguously to be a single polymorphic locus. Wittzell et al. (69) examined nucleotide diversity at pheasant class II B loci and found strong evidence for high diversity in the PBR, yet in some cases amplified more than two sequences from a single individual. After characterizing most of an Mhc class II B gene in red-winged blackbirds at the genomic level, including the entirety of the introns bounding the second (PBR) exon, Edwards et al. (12) examined nucleotide diversity using PCR primers placed in flanking introns yet still amplified more than two distinct sequences from several individuals! We pointed out that the sequences immediately flanking this second exon are conserved not only between long diverged species, but also between genes within the same species, and that divergence between genes and species was greater toward the middles of introns flanking exon 2. Even in some of Zoorob's studies, co-amplification of multiple chicken loci was not uncommon.

Although most of the sequence data produced in these studies suggest that avian Mhc genes are indeed subject to balancing selection as in mammals, it is clear that concerted evolution is homogenizing sequences immediately flanking the second exon of class II B genes to an extent that makes co-amplification of multiple loci a common occurrence (9). Analysis of similarities among genes will be a high priority for isolating sequences critical to single locus amplification in natural populations of birds. Such analyses will also permit evaluation of the joint effects of what appear to be diversity-reducing processes at avian Mhc loci, such as homogenization of duplicate genes, and diversity-enhancing processes, such as balancing selection – processes which can be in conflict with one another under some population genetic conditions (70, 71). The quest for locus specificity and the tools by which single locus polymorphism can be assayed in natural populations will be a critical bridge linking evolutionary genomics and applications of avian Mhc studies in molecular ecological contexts (72).

Still, genomic access to avian Mhc loci has much improved in the last few years. For example, primers flanking exon 2 of class II B genes of blackbirds amplify this exon in greenbuls (*Andropadus*), which are only distantly related to blackbirds (12) (A. Aguilar, unpublished data). The data base for avian class II B exon 2 is now large enough to permit rational design of PCR primers that amplify relevant regions in new clades of birds (Fig. 8) (61). Using such primers (Fig. 8), we amplified a segment of exon 2 in budgerigars (*Melopsittacus undulatus*), an avian model system for aging studies (73) and an exemplar of an avian clade (parrots) from which Mhc genes have not yet been examined. The sequences appear polymorphic in regions expected for functional Mhc loci (Fig. 8), and provide a critical inroad to determination of full length sequences. We can therefore expect more rapid accumulation of avian Mhc gene sequences in the near future.

Fig. 7. Reconciled tree analysis of avian class II B PBR sequences. A. Tree of species from which analyzed PBR sequences were obtained. The species relationships were extrapolated from Sibley & Ahlquist (20); of the species investigated here, Darwin's finches are likely most closely related to blackbirds (85) (K. Burns, personal communication). **B. Gene tree of 109 complete and partial PBR (exon 2) sequences extracted from Genbank.** The gene tree was generated by the neighbor-joining method (81) using a Kimura 2 parameter distance measure (82), and was rooted using three partial American alligator (*Almi*) sequences (39). H. finch = house finch (*Came*) (7, 39); D. finch = Darwin's finch (*Gema, Gesc, Gefo, Plcr, Ceol, Capa*) (61); Blackbird = red-winged blackbird (*Agph*) (7, 12, 39); B. finch = Bengalese finch (*Lost*) (83); Warbler = great reed warbler (*Acar*) (39); Jay = scrub jay (*Apco*) (7, 39); Budgerigar (*Meun*) (this work); Chicken (*Gaga*) (68) and Pheasant (*Phco*) (69). For the purposes of this analysis, all Darwin's finch sequences (61) were lumped into a single "species", albeit with an eye toward sampling all known major lineages within and between species. The budgerigar sequences were analyzed as presented in Fig. 8; each of the three sequences is thus a composite of two amplified alleles.

Conclusion

Avian genomes differ from mammalian genomes in a number of important respects, including patterns and origins of sex determination (74), presence of microchromosomes, smaller

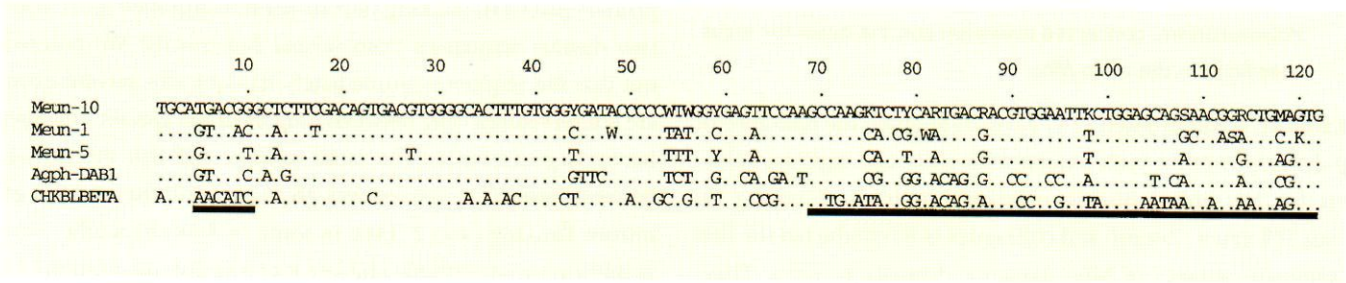


***Fig. 8.* Budgerigar (*Melopsittacus undulatus*) PBR sequences amplified with primers targeted to conserved regions in exon 2, sequenced directly, and analyzed with the PolyPhred program (84).** The standard IUPAC nucleotide code is used to designate nucleotide sites deemed heterozygous by PolyPhred. Bold lines at bottom indicate putative PBR domains. The primer sequences were (5'→3') AGGCAMRTCTACAACCGG (forward) and ACCCCGTAGTTGTKCCG (reverse). PCR was carried out under standard conditions, annealing temperature at 55°C for 35 cycles. These sequences have been deposited in Genbank (accession numbers AF114824–AF114826).

introns (75) and intergenic regions, lower frequency of simple sequence repeats (76) and mechanisms of somatic diversification of immunoglobulin genes (77), to name a few. Based as it is on the rigorous molecular genetics of chickens, the validity of the above statement at first seems compelling, yet potentially breaks down when we consider the taxonomic diversity of birds and the presumably equal diversity of their genomes (78). True, avian genomes are the most uniform in size for any vertebrate group (79), but within this uniformity is likely a high diversity in genome-wide processes. *Mhc* structure and complexity in birds undoubtedly reflects the complexity and diversity of their genomes – indeed, what is known of the chicken *Mhc* mirrors chicken genomic complexity surprisingly well (80). It will be crucial to monitor the particular ways in which these genomic reflections manifest themselves in future research. For the time being, however, we can claim only very fragmentary knowledge of the diversity of avian genomes as reflected in the *Mhc* of birds, and to conclude that the *Mhcs* of other birds will mirror the apparent simplicity and compactness of the chicken model is simply premature. Determining the timing and direction of evolutionary events – did the chicken *Mhc* arise from larger, perhaps more dispersed *Mhcs*, or is it representative of the common ancestral *Mhc* for birds? – will depend entirely on adequate sampling of *Mhc* diversity at a number of levels in the avian tree. We suspect that the powerful methods of modern genomics, informed with consideration of avian phylogenetic relationships and accompanied by rigorous statistical analyses, will reveal a number of surprises in the evolving story of *Mhc* evolution in birds.

References

1. Briles WE, McGibbon WH. Heterozygosity of inbred lines of chickens at two loci affecting cellular antigens. Genetics 1948;**33**:605.
2. Klein J, Satta Y, O'hUigin C, Takahata N. The molecular descent of the major histocompatibility complex. Annu Rev Immunol 1993;**11**:269–295.
3. Parham P, Ohta T. Population biology of antigen presentation by MHC class I molecules. Science 1996;**272**:67–74.
4. Edwards SV, Hedrick PW. Evolution and ecology of MHC molecules: from genomics to sexual selection. Trends Ecol Evol 1998;**13**:305–311.
5. Sato A, et al. Identification of major histocompatibility complex genes in the guppy, *Poecilia reticulata*. Immunogenetics 1996;**43**:38–39.
6. Miller KM, Withler RE, Beacham TD. Molecular evolution at *Mhc* genes in two populations of chinook salmon *Oncorhynchus tshawytscha*. Mol Ecol 1997;**6**:937–954.
7. Edwards SV, Wakeland EK, Potts WK. Contrasting histories of avian and mammalian *Mhc* genes revealed by class II B sequences from songbirds. Proc Natl Acad Sci USA 1995;**92**:12200–12204.
8. Wittzell H, Bernot A, Auffray C, Zoorob R. Concerted evolution of two *Mhc* class II B loci in pheasants and domestic chickens. Mol Biol Evol (In press).
9. Edwards SV, Nusser J, Gasper J. Characterization and evolution of *Mhc* genes from non-model organisms, with examples from birds. In: Baker AJ, ed. Molecular methods in ecology. Cambridge: Blackwell Scientific (In press).
10. Guillemot F, et al. A molecular map of the chicken major histocompatibility complex: the class II genes are closely linked to the class I genes and the nucleolar organizer. EMBO J 1988;**7**:2775–2785.
11. Xu Y, et al. Isolation and characterization of three class II major histocompatibility complex genomic clones from the chicken. J Immunol 1989;**142**:2122–2158.
12. Edwards S, Gasper J, Stone M. Genomics and polymorphism of *Agph-DAB1*, an *Mhc* class II B gene in red-winged blackbirds (*Agelaius phoeniceus*). Mol Biol Evol 1998;**15**:236–250.

13. Kaufman J, Völk H, Wallny H-J. A "minimal essential Mhc" and an "unrecognized" Mhc: two extremes in selection for polymorphism. Immunol Rev 1995;**143**:63–88.
14. Kaufman J, Skjoedt K, Salomonsen J. The MHC molecules of nonmammalian vertebrates. Immunol Rev 1990;**113**:83–117.
15. Kaufman J, Salomonsen J. The "minimal essential MHC" revisited: both peptide-binding and cell surface expression level of MHC molecules are polymorphisms selected by pathogens in chickens. Hereditas 1997;**127**:67–73.
16. Flajnik MF, et al. A novel type of class I gene organization in vertebrates: a large family of non-MHC-linked class I genes is expressed at the RNA level in the amphibian *Xenopus*. EMBO J 1993;**12**:4385–4396.
17. Bingulac-Popovic J, et al. Mapping of *Mhc* class I and class II regions to different linkage groups in the zebrafish, *Danio rerio*. Immunogenetics 1997;**46**:129–134.
18. Maddison WP, Donoghue MJ, Maddison DR. Outgroup analysis and parsimony. Syst Zool 1984;**33**:83–103.
19. Cracraft J. The major clades of birds. In: Benton MJ, ed. The phylogeny and classification of the tetrapods, volume 1: amphibians, reptiles, birds. Vol 35A. Oxford: Clarendon Press; 1988. p. 339–361.
20. Sibley CG, Ahlquist JE. The phylogeny and classification of birds: a study in molecular evolution. New Haven: Yale University Press; 1990.
21. Mindell DP, et al. Phylogenetic relationships among and within select avian orders based on mitochondrial DNA. In: Mindel DP, ed. Avian molecular systematics and evolution San Diego: Academic Press; 1997. p. 213–247.
22. Härlid A, Janke A, Arnason U. The complete mitochondrial genome of *Rhea americana* and early avian divergences. J Mol Evol 1998;**46**:669–679.
23. Sibley CG, Ahlquist JE. The phylogeny and classification of the Australo-Papuan passerine birds. Emu 1985;**85**:1–14.
24. Wilson AC. Time scale for bird evolution. In: Ouellet H, ed. Proceedings of the XIX International Ornithological Congress; 1986 Jun 22–29; Ottawa, Canada. Ottawa: National Museum of Natural Sciences; 1988. p. 1912–1917.
25. Feduccia A. Explosive radiation in Tertiary birds and mammals. Science 1995;**267**:637–638.
26. Woolfenden GE, Fitzpatrick JW. The Florida scrub jay: demography of a cooperative-breeding bird. Princeton (NJ): Princeton University Press; 1984.
27. Peterson AT, Burt DB. Phylogenetic history of social evolution and habitat use in the *Aphelocoma* jays. Anim Behav 1992;**44**:859–866.
28. Hill GE. Plumage color is a sexually selected indicator of male quality. Nature 1991;**350**:337–339.
29. Weatherhead PJ, Boag PT. Genetic estimates of annual and lifetime reproductive success in male red-winged blackbirds. Ecology 1997;**78**:884–896.
30. Gibbs HL, et al. Realized reproductive success of polygynous red-winged blackbirds revealed by DNA markers. Science 1990;**250**:1394–1397.
31. Weatherhead PJ, Metz KJ, Bennett GF, Irwin RE. Parasite faunas, testosterone and secondary sexual traits in male red-winged blackbirds. Behavioral Ecology and Sociobiology 1993;**33**:13–23.
32. Lanyon SM. Polyphyly of the blackbird genus *Agelaius* and the importance of monophyly in comparative studies. Evolution 1994;**48**:679–693.
33. Ball RM Jr, et al. Phylogeographic population structure of red-winged blackbirds assessed by mitochondrial DNA. Proc Natl Acad Sci USA 1988;**85**:1558–1562.
34. Gibbs H, et al. Detection of a hypervariable DNA locus in birds by hybridization with a mouse MHC probe. Mol Biol Evol 1991;**8**:433–446.
35. Luttrell MP, Fischer JR, Stallknecht DE, Kleven SH. Field investigation of *Mycoplasma gallisepticum* infections in house finches (*Carpodacus mexicanus*) from Maryland and Georgia. Avian Dis 1996;**40**:335–341.
36. Dhondt AA, Tessaglia DL, Slothower RL. Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. J Wildl Dis 1998;**34**:265–280.
37. Shiina T, et al. Isolation and characterization of cDNA clones for Japanese quail (*Coturnix japonica*) major histocompatibility complex (*MhcCoja*) class I molecules. Immunogenetics 1995;**42**:213–216.
38. Westerdahl H, Wittzell H, von Schantz T. Polymorphism and transcription of *Mhc* class I genes in a passerine bird, the great reed warbler. Immunogenetics 1999;**49**:149–157.
39. Edwards SV, Grahn M, Potts WK. Dynamics of *Mhc* evolution in birds and crocodilians: amplification of class II genes with degenerate primers. Mol Ecol 1995;**4**:719–729.
40. Briles WE, Goto RM, Auffray C, Miller MM. A polymorphic system related to but genetically independent of the chicken major histocompatibility complex. Immunogenetics 1993;**37**:408–414.
41. Miller MM, et al. Two *Mhc* class I and two *Mhc* class II genes map to the chicken *Rfp-Y* system outside the *B* complex. Proc Natl Acad Sci USA 1994;**91**:4397–4401.
42. Gongora R. Presence of solitary exon 1 sequences in the *HLA-DR* region. Hereditas 1997;**127**:47–50.
43. Rowen L, Koop B, Hood L. The complete 685-kilobase DNA sequence of the human β T cell receptor locus. Science 1996;**272**:1755–1762.
44. Ewing B, Green P. Base-calling of automated sequencer traces using PHRED. II. Error probabilities. Genome Res 1998;**8**:186–194.
45. Ewing B, Hillier LD, Wendl MC, Green P. Base-calling of automated sequencer traces using PHRED. II. Accuracy assessment. Genome Res 1998;**8**:175–185.
46. Gordon D, Abajian C, Green P. *Consed*: a graphical tool for sequence finishing. Genome Res 1998;**8**:195–202.
47. Lee M, Lynch ED, King M-C. SeqHelp: a program to analyze molecular sequences utilizing common computational resources. Genome Res 1998;**8**:306–312.
48. Zoorob R, Behar G, Kroemer G, Auffray C. Organization of a functional chicken class II B gene. Immunogenetics 1990;**31**:179–187.
49. Bernardi G. The human genome: organization and evolutionary history. Annu Rev Genet 1995;**29**:445–476.
50. Kruse M, Muller IM, Muller WEG. Early evolution of metazoan serine/threonine and tyrosine kinases: identification of selected kinases in marine sponges. Mol Biol Evol 1997;**14**:1326–1334.
51. Struhl K. Helix-turn-helix, zinc-finger, and leucine-zipper motifs for eukaryotic transcriptional regulatory proteins. Trends Biochem Sci 1989;**14**:137–140.
52. Bernot A, Zoorob R, Auffray C. Linkage of a new member of the lectin supergene family to chicken *Mhc* genes. Immunogenetics 1994;**39**:221–229.
53. Klein J, Ono H, Klein D, O'hUigin C. The accordion model of *Mhc* evolution. In: Gergely J, Petraryi G, eds. Progress in immunology. Vol VIII. Heidelberg: Springer-Verlag; 1994. p. 137–143.
54. Irwin DM, Wilson AC. Concerted evolution of ruminant stomach lysozymes. J Biol Chem 1990;**265**:4944–4952.
55. Zangenberg G, Huang M-M, Arnheim N, Erlich HA. New *HLA-DPB1* alleles generated by interallelic gene conversion detected by analysis of sperm. Nat Genet 1995;**10**:407–414.
56. Böhme J, Högstrand K. Timing and effects of template number for gene conversion of Major Histocompatibility Complex genes in the mouse. Hereditas 1997;**127**:11–18.
57. Nei M, Gu X, Sitnikova T. Evolution by the birth-and-death process in multigene families of the vertebrate immune system. Proc Natl Acad Sci USA 1997;**94**:7799–7806.

58. Ohta T. On the pattern of polymorphism at major histocompatibility complex loci. J Mol Evol 1998;**46**:633–638.
59. Page RDM. Maps between trees and cladistic analysis of historical associations among genes, organisms, and areas. Syst Biol 1994;**43**:58–77.
60. Page R, Charleston MA. From gene to organismal phylogeny: reconciled trees and the gene tree/species tree problem. Mol Phylogenet Evol 1997;**7**:231–240.
61. Vincek V, et al. How large was the founding population of Darwin's finches? Proc R Soc Lond B Biol Sci 1997;**264**:111–118.
62. Graser R, et al. Trans-species polymorphism of class II Mhc loci in danio fishes. Immunogenetics 1996;**44**:36–48.
63. Satta Y, Mayer WE, Klein J. Evolutionary relationship of *HLA-DRB* genes inferred from intron sequences. J Mol Evol 1996;**42**:648–657.
64. Cereb N, Hughes AL, Yang SY. Locus-specific conservation of the *HLA* class I introns by intra-locus recombination. Immunogenetics 1997;**47**:30–36.
65. Bergström TP, Josefsson A, Erlich HA, Gyllensten U. Recent origin of *HLA-DRB1* alleles and implications for human evolution. Nat Genet 1998;**18**:237–242.
66. Ota T, Nei M. Divergent evolution and evolution by the birth-and-death process in the immunoglobulin VH gene family. Mol Biol Evol 1994;**11**:469–482.
67. Ota T, Nei M. Evolution of immunoglobulin VH pseudogenes in chickens. Mol Biol Evol 1995;**12**:94–102.
68. Zoorob R, et al. Chicken major histocompatibility complex class II B genes: analysis of interallelic and interlocus sequence variance. Eur J Immunol 1993;**23**:1139–1145.
69. Wittzell H, von Shantz T, Zoorob R, Auffray C. Molecular characterization of three Mhc class II B haplotypes in the ring-necked pheasant. Immunogenetics 1994;**39**:395–403.
70. Ohta T. Role of diversifying selection and gene conversion in the evolution of major histocompatibility complex loci. Proc Natl Acad Sci USA 1991;**88**:6716–6720.
71. Walsh JB. Sequence-dependent gene conversion: can duplicated genes diverge fast enough to escape conversion? Genetics 1987;**117**:543–557.
72. Von Schantz T, et al. MHC genotype and male ornamentation: genetic evidence for the Hamilton-Zuk model. Proc R Soc Lond B Biol Sci 1996;**263**:265–271.
73. Ogburn CE, et al. Cultured renal epithelial cells from birds and mice: enhanced resistance of avian cells to oxidative stress and DNA damage. J Gerontol 1998;**53A**:B287–B292.
74. Fridolfsson AK, et al. Evolution of the avian sex chromosomes from an ancestral pair of autosomes. Proc Natl Acad Sci USA 1998;**95**:8147–8152.
75. Hughes AL. Small genomes for better flyers. Nature 1995;**377**:391.
76. Primmer CR, et al. Low frequency of microsatellites in the avian genome. Genome Res 1997;**7**:471–482.
77. McCormack WT, Tjoelker LW, Thompson CB. Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion. Annu Rev Immunol 1991;**9**:219–241.
78. Edwards SV. Diversity of birds. In: Knobil E, Neill JD, eds. Encyclopedia of reproduction. New York: Academic Press; 1999. p. 358–369.
79. Tiersch TR, Wachtel SS. On the evolution of genome size of birds. J Heredity 1991;**82**:363–368.
80. McQueen HA, et al. CpG islands of chicken are concentrated on microchromosomes. Nat Genet 1996;**12**:321–324.
81. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;**4**:406–425.
82. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 1980;**16**:111–120.
83. Vincek V, et al. Molecular cloning of major histocompatibility complex class II B gene cDNA from the Bengalese finch, *Lonchura striata*. Immunogenetics 1995;**42**:262–267.
84. Nickerson DA, Tobe VO, Taylor SL. PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic Acids Res 1997;**25**:2745–2751.
85. Baptista L, Trail P. On the origin of Darwin's finches. Auk 1988;**105**;663–671.