CSIRO PUBLISHING
www.publish.csiro.au/journals/emu

Review

Emu, 2010, **110**, 233–241

The Zebra Finch genome and avian genomics in the wild

Christopher N. Balakrishnan[A,C], *Scott V. Edwards*[B] *and David F. Clayton*[A]

[A]Department of Cell and Developmental Biology and Institute for Genomic Biology, University of Illinois, Urbana-Champaign, IL 61820, USA.
[B]Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA.
[C]Corresponding author. Email: cbala@igb.uiuc.edu

Abstract. The Zebra Finch (*Taeniopygia guttata*) is the first species of passerine bird with a complete genome sequence, making it an exciting time for avian evolutionary biology. Native to Australia and the Lesser Sunda Islands, this species has long played an important role in the study of ecology, behaviour and neuroscience. With the sequencing of its genome, the Zebra Finch now also represents an important model system for evolutionary and population genomics. The production of a genome sequence for the Zebra Finch will have far-reaching impacts on the study of avian biology. Here we discuss the genomic resources available for the Zebra Finch, including the genome sequence itself, and some of the ways in which they will facilitate the study of avian diversity. We also highlight recent examples from the literature that have already begun to leverage Zebra Finch genomic tools towards the study of birds in nature.

Introduction

The Zebra Finch genome is the first passerine genome to be sequenced and only the second avian genome to be sequenced after that of the Chicken (Red Junglefowl, *Gallus gallus*). Zebra Finches are estrildid finches (family Estrildidae), a group of 140 species found in Africa and Australasia (Goodwin 1982). They are also a passerine, or perching bird, belonging to a clade (order Passeriformes) comprising over 5000 species and representing over half of all bird diversity. With the second avian genome in hand, it will now be possible to draw conclusions about the general features of the avian genome as well as the unique features of the genomes of both Zebra Finches and Chickens.

As a passerine, the Zebra Finch represents a lineage with the rare trait of producing learned vocalisations, a trait they share with humans and only a few other vertebrates. They are also highly social, living in often large colonies, and display a complex system of vocal communication in which individual males can be identified on the basis of song. The choice of the Zebra Finch as a target species for whole genome sequencing was motivated in large part by its critical and growing role as the best experimental model of vocal learning and communication (Doupe and Kuhl 1999; Brainard and Doupe 2002; Jarvis 2004). The Zebra Finch is also notable in its adaptation to the arid zone of Australia (e.g. Zann 1996; Perfito *et al*. 2007) and Richard Zann championed the development of the Zebra Finch, and birds in general, as ecological and evolutionary model systems (Zann 1996). As a result, the Zebra Finch and some its close relatives are now important model systems for the study of adaptation and speciation (Smith 1993; Sorenson *et al*. 2003; Balakrishnan and Edwards 2008; Pryke and Griffith 2009*a*, 2009*b*). The Zebra Finch genome will bolster efforts in these fields as well. Our primary aim here is to place the Zebra Finch and its genome in a comparative context, highlighting some of the ways in which the Zebra Finch genome can facilitate evolutionary studies across the passerine radiation.

Nuts and bolts of Zebra Finch genomics

A genome represents the totality of deoxyribonucleic acid (DNA) in an individual's cells. The effort to sequence the Zebra Finch genome emerged through a series of commitments by the National Institutes of Health in the USA, beginning in 2002 with the construction of a Zebra Finch Bacterial Artificial Chromosome (BAC) library (Arnold and Clayton 2002; see below), followed by production of a physical genetic map (Clayton *et al*. 2005*a*), and finally by a draft assembly of the whole genome sequence itself (Clayton *et al*. 2005*b*; Warren *et al*. 2010). The Zebra Finch genome was generated using DNA derived from a single Zebra Finch – a captive male bird from the colony of Dr Art Arnold of the University of California, Los Angeles.

The construction of a genome involves two key steps: sequencing and assembling. Owing to limitations of current sequencing chemistry, the genome is first sheared into short fragments (~1000–2000 base pairs (bp)) and these fragments are then sequenced (so-called 'shotgun sequencing'). This of course leads to the issue of assembly, in which the sheared fragments must be reconnected to one another and linked to their respective chromosomes. Most genomes, including that of the Zebra Finch, are sequenced to a coverage of 6×, meaning that six times as many bases are sequenced as exist in the genome. This redundant sequencing is done to ensure adequate overlap between the small sequence-fragments, allowing them to be assembled. Although, on average, each base pair is sequenced six times, the stochastic nature of the sequencing process means

© Royal Australasian Ornithologists Union 2010
10.1071/MU09087 0158-4197/10/030233

that some areas are not sequenced at all, and some areas are sequenced many more than six times.

The first draft assembly of the Zebra Finch genome has now been completed (Warren *et al*. 2010) and is publicly accessible (http://www.songbirdgenome.org, accessed 17 July 2010). Altogether, the sequenced genome spans 1.2 gigabases (Gb, or billion base pairs) of which 1 Gb has been assigned to 33 chromosomes and three linkage groups. A total of 17 475 genes have been predicted, representing roughly 90% of the expected gene content of the genome. The missing 10% of genes is expected, even given 6× coverage. As indicated above, either by chance, or by the specific properties of the region in question (some regions just do not sequence easily), portions of the genome simply have not yet been sequenced.

Zebra Finch genomic resources

Before the actual genome sequencing had even begun, three key genomic resources had already been developed. First, a BAC library was produced at the Arizona Genomics Institute (Luo *et al.* 2006). BAC libraries are a type of large-insert genomic library and represent the foundation of genome-sequencing efforts (e.g. Couzin 2002; Hillier *et al*. 2004; Warren *et al*. 2008). Long genomic DNA fragments, of between 100 and 200 kilobases (kb) are cloned into a bacterial vector and these constructs can then be propagated in bacteria. These long inserts are useful for accessing large genomic regions surrounding genes of interest (e.g. Luo *et al*. 2006) and are also the basis for physical mapping of the genome (linking of genome sequence data to physical chromosomes). Another Australian bird for which a BAC library exists is the Emu (*Dromaius novaehollandiae*; Shedlock *et al*. 2008).

Expressed sequence tag (EST) databases are another key set of resources. ESTs are sequences of complementary DNA (cDNA) derived from transcribed ribonucleic acid (RNA) or messenger RNA (mRNA); they are a large collection of sequences, often from a specific tissue, that include many different genes. Genes that are transcribed into RNA are a major source of functional information for the Zebra Finch. Over the last 5 years, cDNA sequences derived from brain-expressed genes have been gathered by a number of research groups and organised into online databases (e.g. Replogle *et al*. 2008). The efforts of the different research groups have now been unified into a searchable database 'ESTIMA songbird' (Table 1). Sequences of expressed transcripts are essential to the genome annotation process as they provide direct evidence of gene structure to complement gene-prediction algorithms.

A third important resource, developed before the completion of the Zebra Finch genome, was a linkage map for the Zebra Finch (Stapley *et al*. 2008). By genotyping a pedigreed population using large sets of genetic markers dispersed across the genome (i.e. determining the type of variation in the genome at many different places across the chromosomes), linkage mapping allows the estimation of the physical position of markers in the genome (a marker simply being any type of DNA feature that can be easily amplified and characterised by tools such as the polymerase chain reaction (PCR)). These data have provided a valuable validation of the Zebra Finch assembly because of the strong correlation between the physical location of the markers achieved by linkage mapping and the locations of those same markers by shotgun sequencing. Linkage mapping also sets the stage for making associations between genotype and phenotype (e.g. Forstmeier 2005). Linkage mapping studies have now been carried out in a diversity of passerine species (e.g. Hansson *et al.* 2005; Åkesson *et al*. 2007; Backström *et al*. 2008*a*; Jaari *et al.* 2009).

Two online databases are essential tools for examining the complete genome: the University of California – Santa Cruz (UCSC) genome browser (Kent *et al*. 2002; Karolchik *et al.* 2003), and the Ensembl genome browser (Birney *et al*. 2001; Table 1). Both of these provide an interface with which one can map sequences of interest to genomes, search for genes of interest, and extract sequence data. Both of these databases also provide access to comparative sequence data from other completed genome projects. Below we describe several recently published case studies illustrating the utility of the Zebra Finch genome and the associated resources for ecological and evolutionary studies. We also highlight some of the first findings from the analysis of the Zebra Finch genome itself.

Multilocus phylogeography and population genetics

Advances in population genetic theory, in particular the development and now common usage of models rooted in the coalescent theory (reviewed in Wakeley 2008), have made it clear that single-locus gene trees can vary from gene to gene; the tree for a single gene need not resemble the species history. The failure of a single locus such as mitochondrial DNA (mtDNA) to

Table 1. Web resources for avian genomics
All websites were last accessed on 29 July 2010

	URLs	Summary
Zebra Finch genome	http://www.songbirdgenome.org	Central website for basic information regarding the Zebra Finch genome
Comparative genomics		
UCSC genome browser	http://genome.ucsc.edu	All-purpose genome browser, particularly useful for exporting data tables
Ensembl	http://www.ensembl.org	All-purpose genome browser, particularly useful for comparing gene sequences from published complete genomes
Aviangenomes.org	http://aviangenomes.org	A developing resource for comparative genomics of birds
Gene expression		
ESTIMA	http://www.illinois.edu/goto/songbird	Database of Zebra Finch brain cDNA sequences
ZeBRA	http://ignrhnet.ohsu.edu/finch/songbird/index.php	Online brain atlas showing gene expression patterns in the brain

accurately reflect population history could occur due to deterministic factors (e.g. sex biased gene flow or natural selection) or stochastic factors such as incomplete lineage sorting (Avise 1994). Mitochondrial DNA data, which has long been the workhorse of phylogeography, are now routinely supplemented with data from the nuclear genome and indeed discordance among loci is commonplace (e.g. Canestrelli and Nascetti 2008; Gompert *et al*. 2008; Brito and Edwards 2009; Edwards and Bensch 2009). Discordance among loci, however, should not be thought of as hindering multilocus approaches, but rather as providing added resolution into the central tendency of genes in the genome and demographic history at the population and species levels (Balakrishnan *et al*. 2010*a*). The Zebra Finch genome has already increased the accessibility of sequence-based nuclear markers for population genetic studies of songbirds. Having a genome, however, not only aids in the development of the markers themselves, but also provides important supplementary information, such as their placement on the chromosome, their distance from known coding genes and their genomic copy number. These details can contribute greatly to our understanding of patterns of molecular evolution and phylogeography.

Zebra Finch population genetics

In a recent study, Balakrishnan and Edwards (2008) used sequence data from the developing Zebra Finch genome project to characterise patterns of polymorphism and divergence in wild Zebra Finches. Markers were designed based on published BAC sequences that, as mentioned above, are long tracts of DNA. By sequencing sets of markers within such tracts, Balakrishnan and Edwards were able to characterise both patterns of divergence among Zebra Finch populations, and also patterns of linkage disequilibrium (LD) in the genome. LD is a measure of the strength of correlation among the allelic states at different sites in the genome. The extent of LD is an indicator of genomic properties, such as the recombination rate, as well as of demographic parameters. Balakrishnan and Edwards found higher LD in the Lesser Sundas subspecies of Zebra Finch (*Taeniopygia guttata guttata*) than in Australian populations (*T. g. castanotis*), supporting the hypothesis that Zebra Finches had passed through a population bottleneck, or founder event, during the colonisation of the islands. The ability to map genetic distances among loci in combination with coalescent analyses therefore contributed to the ability to answer questions about historical demography (Fig. 1).

The extent of LD is also a key determinant of the feasibility of associating genomic variation with phenotypic variation. For example if LD is high, one can find associations between a genomic polymorphism and at trait by genotyping a locus in the same genetic region as the causal mutation. In contrast, if LD is very low, one essentially has to genotype the causal polymorphism itself to find an association between the genotype and phenotype. Two recent studies of LD in captive Zebra Finches (Backström *et al*. 2010; Stapley *et al*. 2010) suggest that LD is fairly high in these populations, a property that will facilitate mapping traits (e.g. Forstmeier 2005). This contrasts with wild, out-bred populations of Zebra Finch that indicated lower levels of LD (Balakrishnan and Edwards 2008). This difference in LD

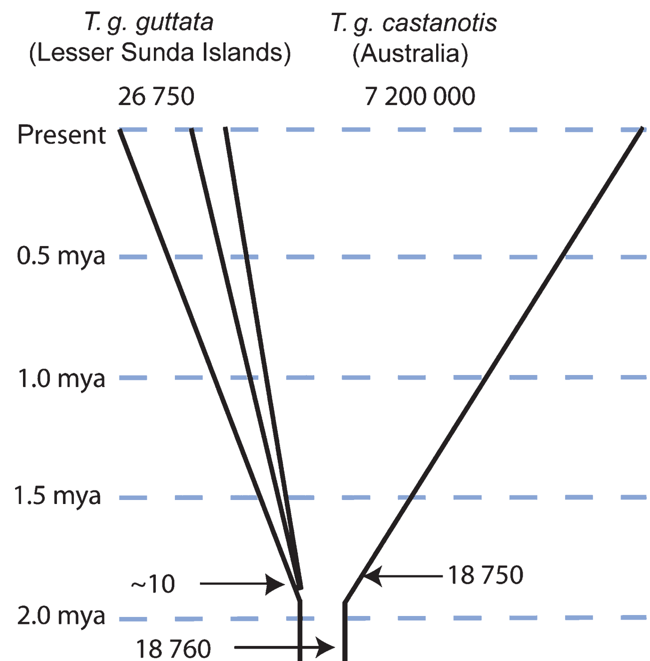

Fig. 1. Divergence history of Zebra Finches based on multilocus analysis of 30 nuclear loci (Balakrishnan and Edwards 2009). The two Zebra Finch subspecies are estimated to have diverged between 1 and 2 million years ago (mya) when a small number of founders (estimated at ~10, but see Balakrishnan and Edwards 2009) colonised the Lesser Sunda Islands. Numbers shown represent effective, not actual, population sizes of the ancestral, descendent, and founding populations; the population in Australia is characterised by a history of expansion.

between wild and captive populations is expected based on population genetics theory.

Facilitating the genomics of other non-model avian species

The Zebra Finch genome, however, is not only useful for studies of Zebra Finches. A recent study of White-throated Sparrow (*Zonotrichia albicollis*) provides an exceptional example of how Zebra Finch genomic resources can be used to characterise the genetics of a relatively divergent avian lineage (Thomas *et al*. 2008). The White-throated Sparrow represents perhaps the best avian example linking genetic and behavioural variation. Two colour-morphs exist – tan and white-striped (Lowther 1961) – and these phenotypes correlate with a chromosomal inversion (Thorneycroft 1966, 1975). The colour-morphs are further correlated with striking behavioural variation: white-striped males sing more, are more territorial and are more promiscuous than tan-striped birds, whereas tan-striped birds of both sexes invest more in parental care than white-striped birds (e.g. Tuttle 2003). The polymorphism is maintained because these birds tend to mate disassortatively, each preferring to mate with the other morph (Lowther 1961; Houtman and Falls 1994). Cytogenetic mapping studies previously mapped the inversion to Sparrow chromosome two (Thorneycroft 1966; 1975), but the use of Zebra Finch genomic tools has provided added resolution to the history of the inversion. Thomas *et al*. (2008) used probes

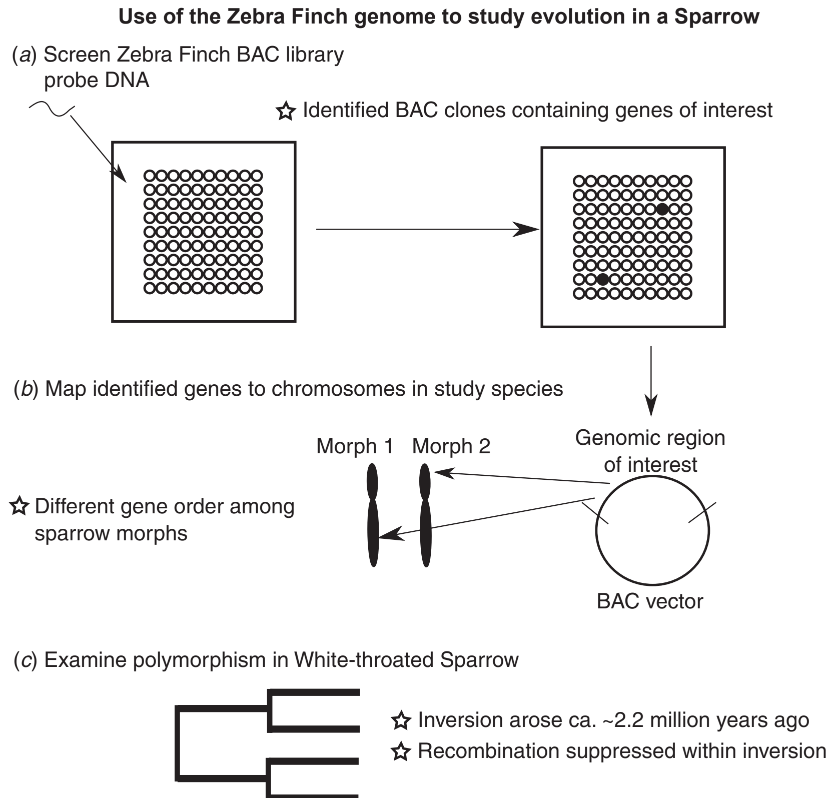

Fig. 2. Strategy used by Thomas *et al.* (2008) to characterise a chromosomal inversion in the White-throated Sparrow (*Zonotrichia albicollis*). (*a*) Zebra Finch BAC clones that map to the genomic region of interest were identified; (*b*) BAC clones were FISH mapped to alternative versions of the Sparrow chromosomes clarifying structural differences among populations; and (*c*) primers were designed using Zebra Finch genomic data to amplify with PCR and sequence portions of the Sparrow chromosome. Polymorphism data revealed the divergence time of the two chromosomal morphs and suppressed recombination within the inversion.

targeted to the known region of the inversion to screen the Zebra Finch BAC library (Fig. 2). Probes were either 'universal', designed from Chicken–human sequence comparisons (Kellner *et al.* 2005), or avian, designed from comparisons of sequencing reads from the Zebra Finch and the Chicken genomes (Thomas *et al.* 2008). Once BAC clones were identified, they were mapped onto the Sparrow chromosomes using fluorescence *in situ* hybridisation (FISH). In FISH mapping, BAC sequences are labelled with a fluorescent dye and then bound to chromosome spreads such that the physical placement of the BACs can be visualised by microscopy. By placing a series of Zebra Finch BACs on alternative forms of the Sparrow chromosomes, Thomas *et al.* found that Sparrow chromosome 2 was actually orthologous to Chicken chromosome 3, and were able to characterise the details of the inversion that had taken place. As expected, in the alternative forms of the chromosomes, the BAC markers were arranged in different orders. The FISH mapping data, however, also revealed that the inversion was complex and in fact was the result of multiple inversions (Thomas *et al.* 2008). These researchers further used alignments of Chicken and Zebra Finch sequences to design PCR primers targeted to sequences within and around the inversion. By characterising polymorphism in the inversion, they were then able to estimate that the inversion arose ca. 2.2 million years ago. Polymorphism data also supported the prediction that recombination was suppressed within the inversion (Thomas *et al.* 2008).

Genomic markers for population genetics

In the study described above, alignments of Chicken and Zebra Finch sequences were used to design primers that would likely amplify in many bird groups. This highlights one of the benefits of having a second avian genome: by identifying conserved regions of the genome; it is possible to design primers that will be useful across bird groups. Backström *et al.* (2008*b*) used the same approach at a genome-wide scale, designing primers spanning the genome based on conserved regions in the Chicken and Zebra Finch genomes. Backström *et al.* (2008*b*) tested the utility of these primers in five bird species: Chicken, Peregrine Falcon (*Falco peregrinus*), Collared Flycatcher (*Ficedula albicollis*), Blue Tit (*Cyanistes caeruleus*), and Great Reed-Warbler (*Acrocephalus arundinaceus*). Karaiskou *et al.* (2008) similarly identified microsatellite sequences in Zebra Finch EST databases and tested these loci in seven species of passerine and one species of owl. Both of these studies reported roughly 80% success in the

amplification of the designed loci across species. These primer sets should have broad utility across bird species and will facilitate the use of multilocus data in avian population genetics.

Whereas universal primers are now easily accessible, one possible drawback of such approaches is ascertainment bias (e.g. Wakeley *et al*. 2001). By amplifying regions of the genome that are particularly conserved, or linked to ESTs, one might not accurately represent patterns of genomic polymorphism. A particularly powerful class of genomic markers that are less subject to such biases are anonymous nuclear loci (e.g. Jennings and Edwards 2005; Balakrishnan and Edwards 2008; Lee and Edwards 2008). Anonymous loci are loci selected at random from genomic libraries generated from the species of interest. Anonymous loci are sequence-based markers, which offer several advantages over microsatellites (Brito and Edwards 2009). Because these markers are anonymous, until now we have had no information on where they fall within the passerine genome (but see Dawson *et al*. (2006) for an example mapping microsatellites to the Chicken genome). Ideally, anonymous markers would represent a sampling of chromosomal locations. The Zebra Finch genome allows us to map anonymous loci, at least from passerines, to the genome. We can test whether they are, in fact, intergenic or whether they are closely linked to a gene. We can also ensure they are single-copy markers, and not duplicated, which would complicate the genotyping process. Of course such tests are really tests of the markers from the test species on the Zebra Finch genome, and do not guarantee the chromosomal location of a marker, for example, in the test species. But they can provide insight, particularly if the genome arrangements of the test species and Zebra Finches are conserved.

A recent study on Red-backed Fairy-wrens (*Malurus melanocephalus*) developed 29 anonymous loci and used them to reconstruct the divergence history for the group (Lee and Edwards 2008). To examine the chromosomal distribution of the markers used in that study, we used BLASTn in the Ensembl browser to place markers on Zebra Finch chromosomes. Given the evolutionary distance between fairy-wrens and Zebra Finches, and that the chosen markers are non-coding, we were somewhat surprised to find strong BLAST hits for all of the loci examined (Table 2). As expected from anonymous markers, we found hits across at least 14 chromosomes (1 locus mapped to an unmapped region), and most markers only had strong hits in one place (Table 2). In contrast, the Zebra Finch study described above (Balakrishnan and Edwards 2008) used primers based on existing BAC sequence data. Six of the seven marker sets used in that study are now found to map to chromosome 1a (the other

Table 2. Mapping of 29 anonymous nuclear loci from the Red-backed Fairy-wren to the Zebra Finch genome

The variation in percent identity between Red-backed Fairy-wren (*Malurus melanocephalus* (MAME)) loci and the Zebra Finch genome highlight the fact that anonymous loci represent a genomic sampling of polymorphism, and will reflect the variability among loci in evolutionary rate. Nonetheless, most Fairy-wren loci produced only one strong match to the genome, increasing confidence that the marker in the Fairy-wren is homologous to its closest match in the Zebra Finch genome. Exceptions to this are indicated with an asterisk. % ID, % sequence identity

Locus	Locus length (bp)	Hit length (bp)	% ID	Chromosome: position
MAME_AL01	337	331	85.50	Chr1A: 6480559–6480885
MAME_AL02	451	252	86.51	Chr9: 26032035–26032283
MAME_AL03	453	453	95.14	Chr2: 70618937–70619387
MAME_AL04	579	230	81.91	Chr23: 2241012–2241239
MAME_AL05	553	554	85.38	Chr3: 47997479–47998030
MAME_AL06	445	269	92.94	Chr2: 147513094–147513359
MAME_AL07	494	489	86.50	Chr7: 8968530–8969016
MAME_AL08	467	353	83.29	Chr5: 27224773–27225122
MAME_AL09	495	320	81.88	ChrUn: 36507638–36507957
MAME_AL10*	229	171	68.42	ChrZ: 49371592–49371756
MAME_AL11	396	401	69.33	Chr2: 34797912–34798305
MAME_AL12	457	437	87.64	Chr9: 11906729–11907151
MAME_AL13	437	386	85.23	Chr1: 102583383–102583763
MAME_AL14	389	420	74.29	Chr1: 101991439–101991848
MAME_AL15	433	401	89.03	Chr2: 88493834–8494234
MAME_AL16*	419	175	77.14	Chr14: 10575636–1057880
MAME_AL17*	441	167	79.64	Ch4a: 15878798–15878962
MAME_AL18	423	388	87.89	Chr12: 9148656–9149041
MAME_AL19	398	326	76.69	Chr3: 28184617–28184940
MAME_AL20	464	424	67.45	Chr2: 96011318–96011720
MAME_AL21	511	193	86.53	Chr3: 75463218–75463407
MAME_AL22	500	440	84.55	Chr9: 8852091–8852522
MAME_AL23	439	528	89.20	Chr18: 8233399–8233921
MAME_AL24	530	400	82.75	Chr14: 616963–617351
MAME_AL25	460	218	81.65	Chr3: 19971531–19971741
MAME_AL26	389	292	79.11	Chr4: 12668275–12668562
MAME_AL27	473	341	85.92	Chr1: 24307993–24308325
MAME_AL28*	460	303	87.79	Chr5: 33104313–33104615
MAME_AL29	309	303	87.79	Chr1a: 6480559–6480885

maps to chromosome 2). This suggests that the BAC sequences that were available at the time were not a thorough sample of the genome and that the survey of the genome was more limited than intended. The results from mapping Fairy-wren and Zebra Finch loci highlight the added information the genome assembly provides. The ability to map anonymous loci from a fairy-wren, a species long diverged from Zebra Finches, to the Zebra Finch genome supports the conservation and use of anonymous loci as a means of quantifying genome-wide patterns of polymorphism, and also indicates that comparative mapping of markers to the Zebra Finch genome is a useful source of supplementary information on species-specific markers. Ironically, by mapping anonymous loci to the genome they lose their anonymity, so effort should be made to select loci for population surveys in a random way thus avoiding ascertainment bias.

Functional and comparative genomics

Ecological immunology

Birds have long played an important role in the study of immune system function. This is true of the Chicken, in particular because of its role as a domesticated species. Now more then ever, with the emergence of West Nile Virus (e.g. LaDeau *et al*. 2007) and avian flu (e.g. Li *et al*. 2004; Olsen *et al*. 2006), it is clear that birds represent an important vector for diseases that have direct impacts on human health. The Zebra Finch genome provides a critical point of comparison with the Chicken genome to expand our understanding of the avian immune system.

The major histocompatibility complex (MHC) has been an important focus of avian immunology (reviewed in Edwards *et al*. 1999; Hess and Edwards 2002). The Chicken MHC is remarkable in its highly streamlined, 'minimal essential', organisation (Kaufman *et al*. 1999). Beyond the order Galliformes (Kaufman *et al*. 1999; Shiina *et al*. 2004), however, little is known about the gene content and organisation of the avian MHC. Population studies of birds indicate that the structure of the avian MHC varies among lineages (e.g. Ekblom *et al*. 2003; Alcaide *et al*. 2007; Westerdahl 2007; Burri *et al*. 2008). Analysis of the Zebra Finch MHC, using the whole genome sequence and targeted sequencing of BACs, indicates that the Zebra Finch MHC differs markedly from the Chicken MHC. Most surprising was the finding that genes that are tightly linked in the Chicken MHC map to different chromosomes in the Zebra Finch genome (Balakrishnan *et al*. 2010*b*). The finding of MHC genes on multiple chromosomes has implications for the extent to which these genes might coevolve (Kaufman 1999). The Zebra Finch genome also offers the opportunity to move beyond analyses of MHC in studies of immune function and the role of the 'immunome' more broadly in ecology. Using the Zebra Finch genome Hellgren and Ekblom (2010), for example, have characterised the repertoire of β-defensins finding evidence of gene duplication and relaxed selection along the Zebra Finch lineage. It will be of great interest to begin to explore the roles of the diverse suite of immune-related genes, including toll-like receptors, CD1 genes, and defensins. Like MHC genes, these may even play a role in local adaptation or mate-choice, or both, or at a minimum will be of interest in terms of their rates and patterns of molecular evolution.

The genetics of adaptation

The Zebra Finch genome paves the way for improving our understanding of the genetics of adaptation in birds. Zebra Finch microarrays provide a tool for surveying gene expression across thousands of transcripts. Microarrays are slides on which thousands of cDNA probes, each representing a gene, are attached. Experimental RNA is extracted from birds under different physiological or ecological conditions, reverse transcribed to cDNA, after which this cDNA can be bound to the microarray. The intensity of the binding of the experimental cDNA with those genes or ESTs spotted on the microarray is a measure of transcript abundance, and can then be used to profile transcript abundance ('gene expression') under the experimental conditions. So far, two large Zebra Finch cDNA microarrays have been produced and each microarray was spotted with nearly 20 000 cDNAs from the EST collections described above (Wada *et al*. 2006; Replogle *et al*. 2008). These ESTs were also used to create an Affymetrix array, which uses shorter (20 bp) oligonucleotide probes (chemically synthesised probes; Naurin *et al*. 2008). With the genome in hand, we now have the means to understand better what these transcripts are and where they reside in the genome.

Thus far, Zebra Finch microarrays have primarily been used to survey patterns of gene expression in the Zebra Finch brain (Wada *et al*. 2006; Lovell *et al*. 2008; Dong *et al*. 2009; London *et al*. 2009; Tomaszycki *et al*. 2009). Replogle *et al*. (2008), however, also demonstrated the utility of Zebra Finch microarrays for study across a diversity of bird species by conducting comparative genomic hybridisation (CGH) experiments. In their CGH studies, genomic DNA (rather than cDNA) from each experimental sample (four oscine passerines, one suboscine passerine, and Chicken) was hybridised against an array with a reference sample of Zebra Finch DNA. The relative intensity of hybridisation of the sample and the reference essentially provides a measurement of the genetic similarity between the two species at each of the cDNAs on the array. Similarly, Naurin *et al*. (2008) successfully used their array with samples from Common Whitethroat (*Sylvia communis*), a passerine lineage ~25–50 million years divergent from the Zebra Finch. The results from both of these studies suggest that Zebra Finch cDNA and oligo-arrays should be useful for gene expression profiling and the detection of changes in copy number (gene duplications and losses) across passerine species.

A recent study by Cheviron *et al*. (2008) used Zebra Finch microarrays to study plasticity and adaptation in Rufous-collared Sparrows (*Zonotrichia capensis*). Rufous-collared Sparrows are neotropical passerines that inhabit the Pacific slope of the Andes. Their distributional range spans the Andean lowlands up an elevational gradient to a maximum altitude of 4600 m above sea level. To test whether high-elevation Sparrows displayed genomic adaptations to their elevation, Cheviron *et al*. (2008) compared gene expression in high-elevation, low-elevation and 'common garden' birds. The use of Zebra Finch microarrays allowed the identification of 333 transcripts that were differentially expressed among high- and low-elevation populations (Fig. 3). These genes included a statistical over-representation of genes involved in metabolic processes such as oxidative phosphorylation. In the common garden experiment, where high- and low-elevation birds were acclimated to a common environ-

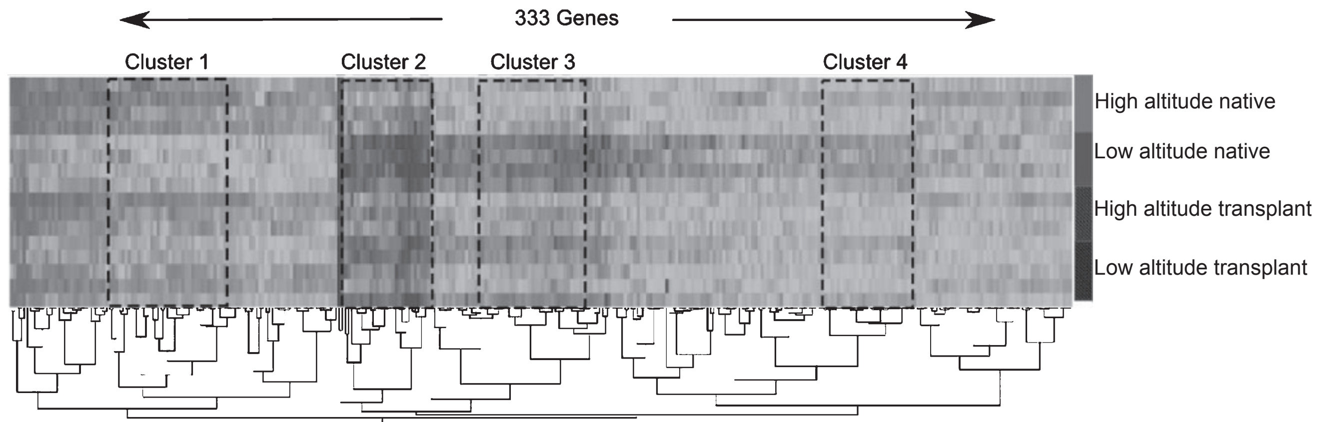

Fig. 3. Gene expression in Rufous-collared Sparrows (*Zonotrichia capensis*) as measured using Zebra Finch cDNA arrays (modified with permission from Cheviron *et al*. 2008). A total of 333 transcripts were differentially expressed between high- and low-altitude populations, as shown by differences in the intensity of shading. Lighter shading indicates higher expression. None of these genes, however, are differentially expressed in transplanted common garden populations, as indicated by the similar shading across these groups. Four clusters of genes are highlighted in dashed boxes. Within these clusters are genes with roles in metabolism, oxidative stress response and protein synthesis.

ment, gene expression differences among populations were not observed, suggesting that plasticity in gene expression was responsible for the differences among populations, rather than genetic adaptation. Complementary DNA arrays therefore represent a useful tool for studying adaptation in birds. Several studies are currently underway in which Zebra Finch cDNA microarrays are being used to study species other than Zebra Finches under the Songbird Neurogenomics Initiative (Replogle *et al*. 2008).

Complementary DNA arrays, however, are time consuming and expensive to build because DNA needs to be grown and extracted from genomic libraries. Oligonucleotide arrays for the Zebra Finch are now also commercially available, offering a lower cost alternative for avian transcriptomics. The most recent Zebra Finch oligo-array was designed with fairly long 60 bp probes and again will be useful, at a minimum, across passerine species. The next generation of transcriptomics technology, however, is already here as well and is accessible for studies of species of wild bird (Bonneaud *et al*. 2008). High-throughput sequencing platforms, such as Titanium 454 (Roche, Basel, Switzerland) and Solexa (Illumina, San Diego, CA, USA) offer the ability to directly sequence and quantify transcript abundance without the reliance on cross-species hybridisation to microarrays. The challenge of these approaches lies in the assembly and analysis of billions of base pairs of sequencing data that are typically generated in fairly short sequencing reads. The Zebra Finch genome is also a valuable asset in this context, providing a reference genome that will greatly facilitate the assembly of the short sequencing reads generated by next-generation sequencing.

Conclusions

The sequencing of the Zebra Finch genome represents a major step forward for avian ecology and evolutionary biology, a step that we imagine Richard Zann would have been very excited to see. The Zebra Finch provides a comparison point among birds with which we can generalise patterns of avian genomic evolution suggested by the genome of the Chicken. On the other hand, and perhaps more importantly, the Zebra Finch genome provides a resource that will encourage and improve studies of wild populations of birds. Indeed such studies, including those described above, are already being conducted but the real benefits of the Zebra Finch genome sequencing effort are still to come.

References

Åkesson, M., Hansson, B., Hasselquist, D., and Bensch, S. (2007). Linkage mapping of AFLP markers in a wild population of great reed warblers: importance of heterozygosity and number of genotyped individuals. *Molecular Ecology* **16**, 2189–2202. doi:10.1111/j.1365-294X.2007.03290.x

Alcaide, M., Edwards, S. V., and Negro, J. J. (2007). Characterization, polymorphism, and evolution of MHC class IIB genes in birds of prey. *Journal of Molecular Evolution* **65**, 541–554. doi:10.1007/s00239-007-9033-9

Arnold, A. P., and Clayton, D. F. (2002). Proposal for construction of a BAC library of the genome of the Zebra Finch (*Taeniopygia guttata*). Available at http://songbirdgenome.org/pdfs/NHGRI-ZF-BAC.pdf [Verified 29 July 2010].

Avise, J. C. (1994). 'Molecular Markers, Natural History and Evolution.' (Chapman and Hall: New York.)

Backström, N., Karaiskou, N., Leder, E. H., Gustafsson, L., Primmer, C. R., Qvarnström, A., and Ellegren, H. (2008*a*). A gene-based genetic linkage map of the collared flycatcher (*Ficedula albicollis*) reveals extensive synteny and gene-order conservation during 100 million years of avian evolution. *Genetics* **179**, 1479–1495. doi:10.1534/genetics.108.088195

Backström, N., Fagerberg, S., and Ellegren, H. (2008*b*). Genomics of natural bird populations: a gene-based set of reference markers evenly spread across the avian genome. *Molecular Ecology* **17**, 964–980. doi:10.1111/j.1365-294X.2007.03551.x

Backström, N., Forstmeier, F., Schliezeth, H., Mellenius, H., Nam, K., Bolund, E., Webster, M. T., Ost, T., Schneider, M., Kempanaers, B., and Ellegren, H. (2010). The landscape of recombination in the zebra finch *Taeniopygia guttata* genome. *Genome Research* **20**, 485–495. doi:10.1101/gr.101410.109

Balakrishnan, C. N., and Edwards, S. V. (2008). Nucleotide variation, linkage disequilibrium and founder-facilitated speciation in wild populations of the zebra finch (*Taeniopygia guttata*). *Genetics* **181**, 645–660. doi:10.1534/genetics.108.094250

Balakrishnan, C. N., Lee, J. Y., and Edwards, S. V. (2010*a*). Phylogeography and phylogenetics in the Nuclear Age. In 'In Search of the Causes of Evolution: From Field Observations to Mechanisms'. (Eds P. R. Grant and R. Grant.) (Princeton University Press: Princeton, NJ.).

Balakrishnan, C. N., Ekblom, R., Volker, M., Westerdahl, H., Godinez, R., Burt, D. W., Graves, T., Griffin, D. K., Warren, W. C., and Edwards, S. V. (2010*b*). Gene duplication and fragmentation in the zebra finch major histocompatibility complex. *BMC Biology* **8**, 29. doi:10.1186/1741-7007-8-29

Birney, E., Clamp, M., Kraspcyk, A., Slater, G., Hubbard, T., Curwen, V., Stabenau, A., Stupka, E., Huminiecki, L., and Potter, S. (2001). Ensembl: a multi-genome computational platform. *American Journal of Human Genetics* **69**, 219.

Bonneaud, C., Burnside, J., and Edwards, S. V. (2008). High-speed developments in avian genomics. *Bioscience* **58**, 587–595. doi:10.1641/B580706

Brainard, M. S., and Doupe, A. J. (2002). What songbirds teach us about learning. *Nature* **417**, 351–358. doi:10.1038/417351a

Brito, P., and Edwards, S. V. (2009). Multilocus phylogeography and phylogenetics using sequence-based markers. *Genetica* **135**, 439–455. doi:10.1007/s10709-008-9293-3

Burri, R., Niculita-Hirzel, H., Roulin, A., and Fumagalli, L. (2008). Isolation and characterization of major histocompatibility complex (MHC) class IIB genes in the Barn owl (Aves : *Tyto alba*). *Immunogenetics* **60**, 543–550. doi:10.1007/s00251-008-0308-0

Canestrelli, D., and Nascetti, G. (2008). Phylogeography of the pool frog *Rana* (*Pelophylax*) *lessonae* in the Italian peninsula and Sicily: multiple refugia, glacial expansions and nuclear-mitochondrial discordance. *Journal of Biogeography* **35**, 1923–1936. doi:10.1111/j.1365-2699.2008.01946.x

Cheviron, Z. A., Whitehead, A., and Brumfield, R. T. (2008). Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Molecular Ecology* **17**, 4556–4569. doi:10.1111/j.1365-294X.2008.03942.x

Clayton, D., Arnold, A. P., Warren, W. C., and Dodgson, J. (2005*a*). Proposal for construction of a physical map of the genome of the Zebra Finch (*Taeniopygia guttata*). Available at http://songbirdgenome.org/pdfs/NHGRI-ZF-map.pdf [Verified 22 July 2010].

Clayton, D. F., Arnold, A. P., Warren, W., Jarvis, E., Mello, C., Burt, D. W., and Ellegren, H. (2005*b*). Proposal to sequence the genome of the Zebra Finch (*Taeniopygia guttata*). Available at http://www.genome.gov/Pages/Research/Sequencing/SeqProposals/ZebraFinchSeq2.pdf [Verified 22 July 2010].

Couzin, J. (2002). NSF's ark draws alligators, algae and wasps. *Science* **297**, 1638–1639. doi:10.1126/science.297.5587.1638

Dawson, D. A., Burke, T., Hansson, B., Pandhal, J., Hale, M. C., Hinten, G. N., and Slate, J. (2006). A predicted microsatellite map of the passerine genome based on chicken-passerine sequence similarity. *Molecular Ecology* **15**, 1299–1320. doi:10.1111/j.1365-294X.2006.02803.x

Dong, S., Replogle, K. L., Hasadsri, L., Imai, B. S., Yau, P. M., Rodriguez-Zas, S., Southey, B. R., Sweedler, J. V., and Clayton, D. F. (2009). Discrete molecular states in the brain accompany changing responses to a vocal signal. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 11 364–11 369. doi:10.1073/pnas.0812998106

Doupe, A. J., and Kuhl, P. K. (1999). Birdsong and human speech: common themes and mechanisms. *Annual Review of Neuroscience* **22**, 567–631. doi:10.1146/annurev.neuro.22.1.567

Edwards, S., and Bensch, S. (2009). Looking forwards or looking backwards in avian phylogeography? A comment on zink and barrowclough 2008. *Molecular Ecology* **18**, 2930–2933. doi:10.1111/j.1365-294X.2009.04270.x

Edwards, S. V., Hess, C. M., Gasper, J., and Garrigan, D. (1999). Toward an evolutionary genomics of the avian mhc. *Immunological Reviews* **167**, 119–132. doi:10.1111/j.1600-065X.1999.tb01386.x

Ekblom, R., Grahn, M., and Hoglund, J. (2003). Patterns of polymorphism in the MHC class II of a non-passerine bird, the great snipe (*Gallinago media*). *Immunogenetics* **54**, 734–741.

Forstmeier, W. (2005). Quantitative genetics and behavioural correlates of digit ratio in the zebra finch. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **272**, 2641–2649. doi:10.1098/rspb.2005.3264

Gompert, Z., Forister, M. L., Fordyce, J. A., and Nice, C. C. (2008). Widespread mito-nuclear discordance with evidence for introgressive hybridization and selective sweeps in *Lycaeides*. *Molecular Ecology* **17**, 5231–5244. doi:10.1111/j.1365-294X.2008.03988.x

Goodwin, D. (1982). 'Estrildid Finches of the World.' (Cornell University Press: Ithaca, NY.)

Hansson, B., Åkesson, M., Slate, J., and Pemberton, J. M. (2005). Linkage mapping reveals sex-dimorphic map distances in a passerine bird. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **272**, 2289–2298. doi:10.1098/rspb.2005.3228

Hellgren, O., and Ekblom, R. (2010). Evolution of a cluster of immune genes (beta-defensins) along the ancestral lines of chicken and zebra finch. *Immunome Research* **6**, 3. doi:10.1186/1745-7580-6-3

Hess, C. M., and Edwards, S. V. (2002). The evolution of the major histocompatibility complex in birds. *Bioscience* **52**, 423–431. doi:10.1641/0006-3568(2002)052[0423:TEOTMH]2.0.CO;2

Hillier, L. W., Miller, W., Birney, E., Warren, W., Hardison, R. C., Ponting, C. P., Bork, P., Burt, D. W., *et al.* (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* **432**, 695–716. doi:10.1038/nature03154

Houtman, A. M., and Falls, J. B. (1994). Negative assortative mating in the white-throated sparrow, *Zonotrichia albicollis*: the role of mate choice and intra-sexual competition. *Animal Behaviour* **48**, 377–383. doi:10.1006/anbe.1994.1251

Jaari, S., Li, M.-H., and Merilä, J. (2009). A first-generation microsatellite-based genetic linkage map of the Siberian jay (*Perisoreus infaustus*): insights into avian genome evolution. *BMC Genomics* **10**, 1. doi:10.1186/1471-2164-10-1

Jarvis, E. D. (2004). Learned birdsong and the neurobiology of human language. *Behavioral Neurobiology of Birdsong* **1016**, 749–777. doi:10.1196/annals.1298.038

Jennings, W. B., and Edwards, S. V. (2005). Speciational history of Australian grass finches (*Poephila*) inferred from thirty gene trees. *Evolution* **59**, 2033–2047.

Karaiskou, N., Buggiotti, L., Leder, E., and Primmer, C. R. (2008). High degree of transferability of 86 newly developed Zebra Finch EST-linked microsatellite markers in 8 bird species. *Journal of Heredity* **99**, 688–693. doi:10.1093/jhered/esn052

Karolchik, D., Baertsch, R., Diekhans, M., Furey, T. S., Hinrichs, A., Lu, Y. T., Roskin, K. M., Schwartz, M., *et al.* (2003). The UCSC genome browser database. *Nucleic Acids Research* **31**, 51–54. doi:10.1093/nar/gkg129

Kaufman, J. (1999). Co-evolving genes in MHC haplotypes: the "rule" for nonmammalian vertebrates? *Immunogenetics* **50**, 228–236. doi:10.1007/s002510050597

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**, 923–925. doi:10.1038/44856

Kellner, W. A., Sullivan, R. T., Carlson, B. H., Thomas, J. W., and Prog, N. C. S. (2005). Uprobe: a genome-wide universal probe resource for comparative physical mapping in vertebrates. *Genome Research* **15**, 166–173. doi:10.1101/gr.3066805

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M., and Haussler, D. (2002). The human genome browser at UCSC. *Genome Research* **12**, 996–1006.

LaDeau, S. L., Kilpatrick, A. M., and Marra, P. P. (2007). West Nile virus emergence and large-scale declines of North American bird populations. *Nature* **447**, 710–713. doi:10.1038/nature05829

Lee, J. Y., and Edwards, S. V. (2008). Divergence across Australia's Carpentarian barrier: statistical phylogeography of the red-backed fairy wren (*Malurus melanocephalus*). *Evolution* **62**, 3117–3134. doi:10.1111/j.1558-5646.2008.00543.x

Li, K. S., Guan, Y., Wang, J., Smith, G. J. D., Xu, K. M., Duan, L., Rahardjo, A. P., Puthavathana, P., *et al*. (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* **430**, 209–213. doi:10.1038/nature02746

London, S. E., Dong, S., Replogle, K., and Clayton, D. F. (2009). Developmental shifts in gene expression in the auditory forebrain during the sensitive period for song learning. *Developmental Neurobiology* **69**, 437–450. doi:10.1002/dneu.20719

Lovell, P. V., Clayton, D. F., Replogle, K. L., and Mello, C. V. (2008). Birdsong "transcriptomics": neurochemical specializations of the oscine song system. *PLoS ONE* **3**, e3440. doi:10.1371/journal.pone.0003440

Lowther, J. K. (1961). Polymorphism in white-throated sparrow, *Zonotrichia albicollis* (Gmellin). *Canadian Journal of Zoology* **39**, 281. doi:10.1139/z61-031

Luo, M. Z., Yu, Y. S., Kim, H. R., Kudrna, D., Itoh, Y., Agate, R. J., Melamed, E., Goicoechea, J. L., *et al*. (2006). Utilization of a zebra finch BAC library to determine the structure of an avian androgen receptor genomic region. *Genomics* **87**, 181–190. doi:10.1016/j.ygeno.2005.09.005

Naurin, S., Bensch, S., Hansson, B., Johansson, T., Clayton, D. F., Albrekt, A. S., Von Schantz, T., and Hasselquist, D. (2008). A microarray for large-scale genomic and transcriptional analyses of the zebra finch (*Taeniopygia guttata*) and other passerines. *Molecular Ecology Resources* **8**, 275–281. doi:10.1111/j.1471-8286.2007.01979.x

Olsen, B., Munster, V. J., Wallensten, A., Waldenstrom, J., Osterhaus, A., and Fouchier, R. A. M. (2006). Global patterns of influenza A virus in wild birds. *Science* **312**, 384–388. doi:10.1126/science.1122438

Perfito, N., Zann, R. A., Bentley, G. E., and Hau, M. (2007). Opportunism at work: habitat predictability affects reproductive readiness in free-living zebra finches. *Functional Ecology* **21**, 291–301. doi:10.1111/j.1365-2435.2006.01237.x

Pryke, S. R., and Griffith, S. C. (2009*a*). Genetic incompatibility drives sex allocation and maternal investment in a polymorphic finch. *Science* **323**, 1605–1607. doi:10.1126/science.1168928

Pryke, S. R., and Griffith, S. C. (2009*b*). Postzygotic genetic incompatibility between sympatric color morphs. *Evolution* **63**, 793–798. doi:10.1111/j.1558-5646.2008.00584.x

Replogle, K., Arnold, A. P., Ball, G. F., Band, M., Bensch, S., Brenowitz, E. A., Dong, S., Drnevich, J., *et al*. (2008). The Songbird Neurogenomics (SoNG) Initiative: community-based tools and strategies for study of brain gene function and evolution. *BMC Genomics* **9**, 131. doi:10.1186/1471-2164-9-131

Shedlock, A. M., Janes, D. E., and Edwards, S. V. (2008). Amniote phylogenomics: testing evolutionary hypotheses with BAC library scanning and targeted clone analysis of large-scale DNA sequences from reptiles. In 'Phylogenomics'. (Ed. W. Murphy.) pp. 97–117. (Humana Press: Totowa, NJ.)

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

Smith, T. B. (1993). Disruptive selection and the genetic basis of bill size polymorpshism in the African finch *Pyrenestes*. *Nature* **363**(6430), 618–620. doi:10.1038/363618a0

Sorenson, M. D., Sefc, K. M., and Payne, R. B. (2003). Speciation by host switch in brood parasitic indigobirds. *Nature* **424**, 928–931. doi:10.1038/nature01863

Stapley, J., Birkhead, T. R., Burke, T., and Slate, J. (2008). A linkage map of the zebra finch *Taeniopygia guttata* provides new insights into avian genome evolution. *Genetics* **179**, 651–667. doi:10.1534/genetics.107.086264

Stapley, J., Birkhead, T. R., Burke, T., and Slate, J. (2010). Pronounced inter- and intrachromosomal variation in linkage disequilibrium across the zebra finch genome. *Genome Research* **20**, 496–502. doi:10.1101/gr.102095.109

Thomas, J. W., Caceres, M., Lowman, J. J., Morehouse, C. B., Short, M. E., Baldwin, E. L., Maney, D. L., and Martin, C. L. (2008). The chromosomal polymorphism linked to variation in social behavior in the white-throated sparrow (*Zonotrichia albicollis*) is a complex rearrangement and suppressor of recombination. *Genetics* **179**, 1455–1468. doi:10.1534/genetics.108.088229

Thorneycroft, H. B. (1966). Chromosomal polymorphism in white-throated sparrow *Zonotrichia albicollis* (Gmelin). *Science* **154**, 1571–1572. doi:10.1126/science.154.3756.1571

Thorneycroft, H. B. (1975). Cytogenetic study of white-throated sparrow, *Zonotrichia albicollis* (Gmelin). *Evolution* **29**, 611–621. doi:10.2307/2407072

Tomaszycki, M. L., Peabody, C., Replogle, K., Clayton, D. F., Tempelman, R. J., and Wade, J. (2009). Sexual differentiation of the zebra finch song system: potential roles for sex chromosome genes. *BMC Neuroscience* **10**, 24. doi:10.1186/1471-2202-10-24

Tuttle, E. M. (2003). Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. *Behavioral Ecology* **14**, 425–432. doi:10.1093/beheco/14.3.425

Wada, K., Howard, J. T., McConnell, P., Whitney, O., Lints, T., Rivas, M. V., Horita, H., Patterson, M. A. *et al*. (2006). A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 15 212–15 217. doi:10.1073/pnas.0607098103

Wakeley, J. (2008). 'Coalescent Theory: An Introduction.' (Roberts & Company: Greenwood Village, CO, USA.)

Wakeley, J., Nielsen, R., Liu-Cordero, S. N., and Ardlie, K. (2001). The discovery of single-nucleotide polymorphisms – and inferences about human demographic history. *American Journal of Human Genetics* **69**, 1332–1347. doi:10.1086/324521

Warren, W. C., Hillier, L. W., Graves, J. A. M., Birney, E., Ponting, C. P., Grutzner, F., Belov, K., Miller, W., *et al*. (2008). Genome analysis of the platypus reveals unique signatures of evolution. *Nature* **453**, 175–183. doi:10.1038/nature06936

Warren, W. C., Clayton, D. F., Ellegren, H., Arnold, A. P., Hillier, L. W., Kunster, A., Searle, S., White, S., *et al*. (2010). The genome of a songbird. *Nature* **464**, 757–762. doi:10.1038/nature08819

Westerdahl, H. (2007). Passerine MHC: genetic variation and disease resistance in the wild. *Journal of Ornithology* **148**(Suppl. 2), 469–477. doi:10.1007/s10336-007-0230-5

Zann, R. A. (1996). 'The Zebra Finch: A Synthesis of Field and Laboratory Studies.' (Oxford University Press: New York.)

Manuscript received 3 September 2009, accepted 12 January 2010

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.