Avian cytogenetics goes functional

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It is now over 10 years since the first avian genome (ICGSC, 2004) and the first complete avian karyotype (Masabanda et al 2004) were both published, however, until 2014, avian cytogenetics has focused heavily on descriptive studies (e.g. Griffin et al 2007; 2008; Skinner et al 2009; Volker et al 2010) with less attention to its functional relevance. Last year however saw two landmark efforts in the chromosomal studies of birds: a special issue of Chromosome Research in April and the announcement of recently completed sequences of multiple new avian genomes in Science and the BMC journals (taking the total number sequenced to over 50) in December. Studying the chromosomes of birds is, perhaps for the first time, telling us more about avian biology, function and evolution than it ever has.

What do we know so far? Karyotypic stability

The near-unique nature of the avian karyotype has remained a consistently reported feature of bird biology since the first chromosome preparations were made. Although many animal groups have microchromosomes, the small size and abundant number of chromosomes in avian species set birds apart genomically from other vertebrate groups. To the best of our knowledge, there are over 1000 published avian karyotypes, most comprehensively summarized by Christidis (1990), with several hundred added since this review. All of these karyotypes are partial however, with usually only 5-10 pairs of chromosomes easily distinguished, and the rest homogeneously classified. Moreover the vast majority of karyotypes hardly differ from each another, with rare exceptions including the stone curlew (*Burhinus oedicnemus*) (2n = 40), the beach thick knee (*Esacus magnirostris*) (2n = 40), several hornbills (2n = 42), kingfishers and hoopoes (*Upupa epops*) (2n > 120) at each end of the numerical spectrum (Christidis, 1990). Indeed, even since the advent of zoo-FISH, the identification of an interchromosomal rearrangement in a bird is a relatively uncommon event (Griffin et al., 2007).

Central to our understanding of avian biology and evolution is establishing the reasons *why* avian karyotypes are evidently so stable. Clues to such an enquiry might lie in those rare exceptions to the rule. For instance, the Falconiformes (falcons etc.) and Psittaciformes (parrots etc.) have noticeably undergone numerous evolutionary changes. Moreover it is noteworthy that when interchromosomal change occurs, it tends to recur. The best example of this is a fusion of the ancestral chromosomes 4 and 10; an event that appears to have occurred independently throughout evolution in chicken (*Gallus gallus*), greylag goose (*Anser anser)*, collared dove

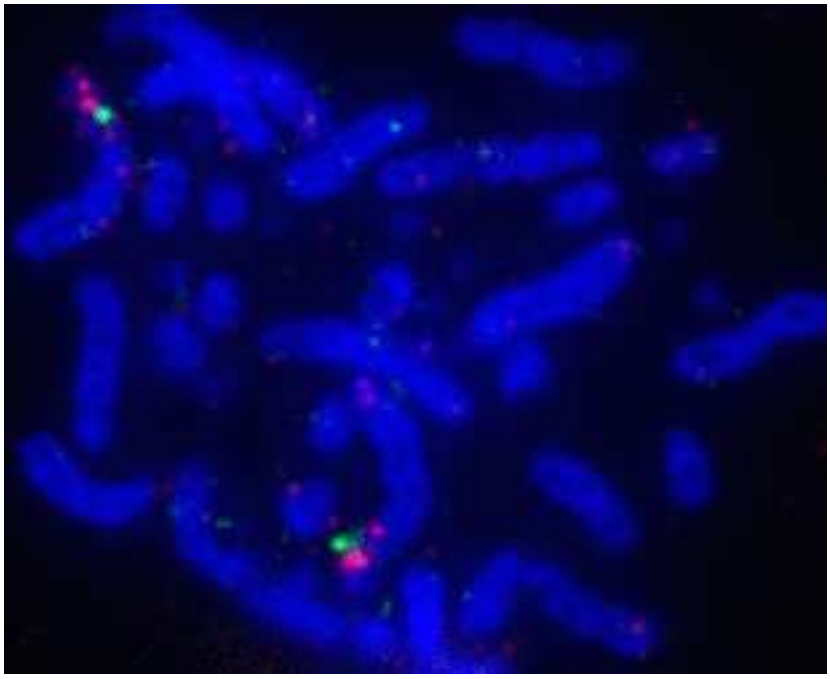
(*Streptopelia decaocto*) and probably other species also (Griffin et al., 2007). In this review, we examine some of the latest tools and preliminary solutions that are being used to understand the underlying mechanisms that lead to chromosome rearrangements in birds (and in eukaryotes in general).

If we accept that interchromosomal change occurs only rarely in birds then it is reasonable to assume that this happens usually only when there is an adaptive value to doing so. In most species, phenotypic diversity is usually associated with wholesale changes in karyotype structure. Aves as a phylogenetic class underwent a series of rapid speciation events beginning c.65MYA (million years ago) and ending c.50MYA. Chromosomal change is usually a cause or consequence of speciation (i.e. a species barrier) but until recently, the microchromosomes that constitute the majority of the avian karyotype, have not been amenable to study. The latest studies however have paved the way for a flurry of research activity that not only describes the avian karyotype in more detail, but might also provide functional clues as to its nature.

New molecular cytogenetic tools

Lithgow et al (2014) produced a set of chromosome paints and bacterial artificial chromosomes (BACs) that will start the process of characterizing the microchromosomes and their changes over evolutionary time. They reported the development of chicken microchromosomal paint pools and generation of pairs of specific microchromosome BAC clones with some success in zoo-FISH experiments. For instance they detected a fusion of the ancestral chicken chromosome 23 orthologue to a macrochromosomes in gyrfalcon (*Falco rusticolus*). McPherson et al. (2014) examined the Japanese quail (*Coturnix japonica*). Comparing chicken and turkey BAC clones on mitotic and meiotic chromosomes they demonstrated that high-resolution FISH is practicable. Ishishita et al. (2014) also assessed the distribution of centromeric repetitive sequences on both micro- and macrochromosomes. It is therefore now possible to achieve full, high-resolution characterization of all avian chromosomes in all species studied, including the elusive chromosome 16 and the D-group (smallest) chromosomes. There are several current strategies to fill the gaps; one of these is by the use of PacBio, a novel single-molecule real-time sequencing platform, targeting the sequence of smaller chromosomes using sorted chromosome preps, and assembling contigs into scaffolds and super-scaffolds from optical maps (Ganapathy et al., 2014).

Figure 1: FISH image of chicken chromosome BACs hybridized to peregrine falcon (*Falco peregrinus)* chromosomes. A fusion is apparent.



What have sequence assemblies taught us?

The progress of genome assembly in birds has been slow in comparison to other animal groups such as mammals. Following chicken (ICGSC, 2004) it took a further 6 years until the second and third avian genome sequences were published, namely those of the zebra finch (*Taeniopygia guttata* a model for neurological function, especially learned vocalization) (Warren et al., 2010) and turkey (*Meleagris gallopavo*) (Dalloul et al., 2010). More recently, the Pekin duck (*Anas platyrhynchos*) (Huang et al., 2013) was added along with two falcon species (*Falco peregrinus* and *Falco cherrug*) (Zhan et al., 2013). The availability of these assembled genomes provided the opportunity for comparative genomics at a chromosomal level. In 2010 we made the first comparison of two species using genome assembly information from the macrochromosomes (Völker et al., 2010). A similar comparison, more recently was made in chicken compared to duck (Rao et al., 2012) and then a three-way comparison (allowing studies of the direction of change) in chicken, turkey and zebra finch (Skinner and Griffin, 2012; Lithgow et al., 2014). The principal features of chromosomal change in birds are Homologous Synteny Blocks (HSBs), which are demarked by Evolutionary Breakpoint Regions (EBRs). While analyzing these features some

general patterns have started to emerge. The first is that, although interchromosomal change is rare, intrachromosomal changes are commonplace. Breakpoint re-use is also commonplace, significantly more so than in mammals, and there is some evidence of an association between chromosomal breakage and non-allelic homologous recombination (NAHR) (Völker et al., 2010).

Zhang et al. (2014) used a whole genome shotgun strategy to generate new whole genome sequences from 45 bird species representing many of the major clades and at least one representative from over 90% of all avian orders. Around 20 species had a high (50-fold or greater coverage) and these were the subjects of further cytogenetic studies. These included the common ostrich (*Struthio camelus*) and the budgerigar (*Melopsittacus undulatus*), which were further assembled using data from optical mapping experiments (Ganapathy et al., 2014). This had the effect of significantly increasing the assembly's N50 scaffold sizes to around 15Mb and were subsequently used, with those already assembled by chromosome (chicken, turkey, zebra finch and duck). Romanov et al. (2014) made use of novel whole genome sequence information from 21 avian genome sequences available on an interactive browser (Evolution Highway). By focusing on the six best-assembled genomes (chicken, turkey, duck, zebra finch, ostrich budgerigar), a putative karyotype of the avian ancestor (probably a bipedal feathered dinosaur) was assembled for each chromosome. The evolutionary events were reconstructed that led to each of the six species' genome organization. Intra- and inter- chromosomal changes appear best explained most parsimoniously by a series of inversions and translocations with common breakpoint reuse. Microchromosomes represent conserved blocks of synteny in most of the 21 species and a series of interchromosomal changes in the ostrich were also described that would not have been predicted by karyotype analysis alone. These results suggest that mechanisms exist to preserve a static overall avian karyotype/genomic structure, including the microchromosomes, with rare interchromosomal change (e.g. in ostrich and budgerigar lineages) this is discussed in depth in the next section. Of the species examined, it seemed that chicken had the least number of chromosomal rearrangements compared to the dinosaur ancestor. From Evolution Highway it is also possible to assess rates of chromosomal evolution in birds. Zhang et al. (2014) suggest that birds have a lower chromosomal rearrangement rate than mammals but nonetheless can undergo "bursts" of rearrangement, e.g. during the evolution of vocal learning. This finding corroborates those of Romanov et al. (2014) that identified the zebra finch and budgerigar as the two species with the most chromosomal rearrangements from the avian

ancestor.

If we accept that chicken and its Galliform relatives underwent the least number of chromosomal changes whilst diverging from the ancestral bird, we also must consider whether they also have undergone the fewest phenotypic changes. In other words is the dinosaur avian ancestor more like a land fowl than any other bird? The most ancient near-certain fossil representative of modern birds (Neornithes) was almost certainly aquatic (for example, *Vegavis*, a genus of birds from the Late Cretaceous epoch) and has been identified as a Galloanseres. Indeed, the earliest known bird-like creatures in the fossil record (e.g. the Ornithurae *Gansus*) were either fully aquatic or at least amphibious and it has been suggested that, due to the fact that they had webbed feet (as well as other traits), they were more like ducks (Romanov et al 2014). On the other hand, most authors agree that the dinosaur ancestors of birds were terrestrial, feathered, bipedal, relatively small and with limited flying ability - not unlike a chicken. At best we can determine therefore, the ancestral birds were most likely more phenotypically associated with the Galloanseres and the confusion of whether they were more akin to water- or land fowl may be due to interpretations based on depositional sampling biases, limited understanding of functional anatomy, and whether the individuals that have been discovered are actually fully representative of the groups to which they belonged. Chromosomal evidence provides an independent record of the functional material of inheritance in living birds and, as such, can complement a fossil record that is always likely to be incomplete.

Of all species studied so far it seems clear that the rearrangement of chromosomes is non-random (Pevzner and Tesler, 2003; Larkin et al., 2009). The reasons for this non-random nature warrant deeper investigation. According to mammalian evidence, evolutionarily conserved HSBs appear to evolve in different ways from the dynamic and ever-changing EBRs; whether this is true of birds remains to be seen. In mammals, chromosomal breakpoints are correlated to sequences of segmentally duplicated or repetitive DNA (Larkin et al., 2009; Bovine Genome Sequencing and Analysis Consortium et al., 2009; Groenen et al., 2012; Ruiz-Herrera et al., 2012) and species-specific EBRs are correlated with regions enriched for transposable elements (TEs) (Bovine Genome Sequencing and Analysis Consortium et al., 2009; Groenen et al., 2012). In mammals, EBRs and HSBs largely contain genes with notably different functional ontologies, e.g. organismal development in HSBs (Larkin et al., 2009) and lineage-specific biology and adaptive

features in EBRs (Larkin et al., 2009; Bovine Genome Sequencing and Analysis Consortium et al., 2009; Groenen et al., 2012). It has been suggested therefore that chromosome rearrangements and the respective gene ontologies contained within HSBs and EBRs help to explain lineage-specific phenotypes in mammals. Mammalian and avian genomes are very different however (not least because of the interchromosomal stability of avian genomes) and thus the question remains about whether the patterns that have been observed in mammals will apply to birds also. Birds have less repetitive DNA through the elimination of repetitive sequences (International Chicken Genome Sequencing Consortium, 2004; Shedlock, 2006; Zhang et al., 2014) so that the avian genome is constrained by size, primarily because of gene loss as well as lineage specific erosion of repetitive elements and large segmental deletions. In addition to their karyotypic stability, bird genomes also have a very high degree of evolutionary stasis at nucleotide sequence and gene synteny levels. Nonetheless, one of the key findings was the detection of non-neutral evolutionary changes in functional genes as well as non-coding regions. Many of these changes coincide with adaptations to different lifestyles and niches and display homoplasy (Zhang et al., 2014).

The non-random nature of chromosome rearrangement in birds, the reasons for the apparent interchromosomal (but not intrachromosomal) stability of avian karyotypes (see next section), the role of TEs (transposable elements) and NAHR, the relationship to phenotype, the question of whether spatial organization of ancestral gene networks is maintained in bird and other reptile lineages, the question of whether lineage-specific EBRs alter gene order in networks that had adaptive value, all require further investigation. Harnessing the data from over 50 avian genomes (undoubtedly with many more on the way) and employing tools such as Evolution Highway will give us unprecedented insight into avian chromosome evolution and its relationship to avian biology.

Why is the avian karyotype structure conserved inter- but not intra- chromosomally?

Burt's "fission-fusion" hypothesis suggested that most avian microchromosomes became fixed in the common dinosaur ancestor with karyotype of ~2n = 60 including 20 microchromosome pairs (Burt 2002). The remainder, including the smallest was, is it suggested, created by further fission. Romanov et al (2014) suggested that a basic pattern of 2n = 80 (~30 microchromosome pairs) was fixed before the Palaeognathae-Neognathae divergence 100 MYA. The subsequent paucity

of intermicrochromosomal rearrangements between most Neognathae suggests an evolutionary advantage either to retaining this pattern or a lack of opportunity for change. For instance, an explanation for such evolutionary stasis might be that the underlying mutational mechanisms of chromosomal changes being fundamentally different in birds compared to other amniotes through a lack of adaptive value, rather than purifying selection, slowing down the rate of change. Much of this could be explained, in part, by a paucity of copy number variants (including segmental duplications), recombination hotspots, transposable elements and/or endogenous retroviruses, however this would not explain why interchromosomal change is rare but intrachromosomal change is common, particularly in groups that have undergone rapid speciation such as Passeriformes.

The rate of chromosome rearrangement (and subsequent speciation) depends on: 1) the mutation rate and 2) the fixation rate (Burt et al. 1999). The first of these is related to the frequency of homologous sites (Burt, 2002). Repeat structures in general (e.g. CNVs), and transposable elements in particular, provide substrates for chromosomal rearrangement. In a genome constrained by size, the opportunity for mutation is reduced and only fission (or intrachromosomal change e.g. inversion) can occur. This provides an explanation why a) avian genomes are more fragmented than any other vertebrate (birds have the most chromosomes) and b) why there have been fewer interchromosomal rearrangements. There might also be advantages to retaining multiple chromosomes in a karyotype through the generation of variation, the driver of natural selection. That is, a karyotype with more chromosomes leads a greater number of genetic variants that the gametes produce and an increase in recombination rate due to the fact that there needs to be at least one obligatory chiasma per chromosome. Burt (2002) proposed that a higher recombination rate has also led to the features that we most associate with microchromosomes (high GC-content, low repeats, high gene-density etc.) and resulted in the formation and fixation of the archetypal avian karyotype with both macro- and microchromosomes and little interchromosomal rearrangement. Such as constraint however does not preclude rearrangement within the individual chromosomes. Romanov et al (2014) and King (1995) argue that an increase in intrachromosomal rearrangement correlates with bursts of speciation in birds, perhaps mediated by an increase in localized repeat content.

Some birds nonetheless have a significantly different karyotype from the standard 2n = ~80. This

can occur within one closely related group e.g. Adélie penguin (*Pygoscelis adeliae*) (2n = 96) and the emperor penguin (*Aptenodytes forsteri*) (2n = 72) (but both associated with high degrees of intermicrochromosomal rearrangement) thereby suggesting that similar mechanisms can both reduce or increase chromosome number in relatively short time frames. Comparisons of chromosomal change in the zebra finch and the budgerigar suggest that rearrangement rates are similarly high in both groups to which they belong (Passeriformes and Psitacciformes respectively) but that the latter is capable of fixing interchromosomal rearrangements, while the former is not. The mechanisms underpinning these differences are, as yet, unknown but studies of the gene ontology terms of species specific EBRs might provide clues. As more avian genomes with better assemblies are analyzed, this may indicate adaptive phenotypic features associated with specific gene ontologies typical of individual orders, families or genera.

The sex chromosomes

Worthy of especial consideration is the conserved sex chromosome ZW system that is present in all birds apart from the Palaeognathae. Their independent origin from the XY system does not escape the fact that similar mechanisms appear to have run in parallel, for instance genes on the Z chromosome (like the mammalian X) have undergone selection for male-advantage functions. Like the Y chromosome, the W is small (albeit medium-sized by avian standards), heterochromatic and gene poor. Graves (2014) suggests that the W chromosome is at a more advanced stage of differentiation than the Y chromosome as it has accumulated more LINEs and lost more genes during its evolution. Pokorná et al (2014) considered multiple sex chromosomes and meiotic drive in a range of amniotes. This study noted that the single ZW system in birds contrasts with that of other reptile and amniote groups; they raised a very exciting hypothesis that this contrast may possibly be related to the differential involvement of sex-specific sex chromosomes in female meiosis (females being the heterogametic sex). Early in the assembly of the chicken genome, the quality of the build of both the Z and W sex chromosomes was very poor and limited studies existed on sex determination. Since this, the Z chromosome was painstakingly assembled and sequenced BAC by BAC (Bellott et al., 2010), and is now one of best-assembled chromosomes in the chicken genome. The same is now expected for the W sex chromosome, which currently is very poorly assembled (Chen et al., 2012). Zhou et al. (2014) conclude that the ancestral sex chromosome organization is closer to that of the Palaeognathae (ostrich and emu) and demonstrated that there is less degradation of the sex chromosomes and

a closer synteny with non-avian reptile species.

Copy number variation

Redon et al. (2006) first highlighted the impact of copy number variation (CNV) in the human genome. This seminal study heralded a new era in cytogenetics and has subsequently been applied to many other species and groups including birds. Skinner et al. (2014) provided a global overview of apparent cross-species CNVs in birds using cross-species array CGH. Griffin and Burt (2014) point out issues of definition in that "copy number variation," strictly speaking, refers to polymorphisms *within a species*. The question arises therefore whether results of cross species array CGH represent genuine variation in copies of orthologous genes between species. Skinner et al. (2014) stated that "difference in gene copy number between species is a question of gene duplication, segmental duplications etc. and may be driven by expansion and contraction of paralogs within different gene families." Nonetheless, this paper provided a broad appraisal of apparent cross-species CNVs in 16 avian species. Microchromosomes appear to have more apparent CNVs than macrochromosomes. Indeed, in species with microchromosomal fusions such as Falconiformes, the fused "former microchromosomes" still retained their ancestral features such as a higher degree of cross-species CNVs. Skinner et al (2014) reported that about 50% of the apparent cross-species CNVs overlap with known chicken-specific CNVs. In terms of gene ontology there appears to be a general enrichment in immune response and antigen presentation genes as well as 5 CNVRs perfectly correlated with the unique loss of sexual dichromatism. More specifically, there were also suggestions of CNVs involved in diet in turkey (proteolytic digestion/degradation of trypsin inhibitors), and correlation of the unique migratory behaviour of common quail among fowl through the following genes: OBSCN associated with hypertrophy of myofribrils, MAPK8IP3 implicated in respiratory gaseous exchange [Skinner et al., 2014]. There were also suggestions of an association with muscle activity in falcons though the gain of MYOZ3, preferentially expressed in fast-twitch myofibers and skeletal muscle and an association between immune function in the common quail (Coturnix coturnix) and silver pheasant (Lophura nycthemera) (LEAP2 and ITCH genes) as well as homeotic genes in common pheasant and California quail (SCML2 and DLX5). Finally, Skinner et al. (2014) identified cross-species CNVs associated with brain development and neuronal function in turkey (e.g. loss of CTXN1), common quail (gain of LRFN5) and duck (e.g., DLGAP2).

Conclusions

The most recent advances in avian cytogenetics have culminated in great promise not only for the study of bird karyotypes, but also for providing insight into the mechanisms of chromosome evolution in general. New avenues for investigation include gene regulation; for instance it will become necessary to map accurately the physical location of poly-adenylation and transcription start sites, important reference points that define promoters and post-transcriptional regulation. It will also become possible to sequence full-length transcripts, to allow accurate identification of alternate splicing events and their controlling elements. The "ENCODE" (Encyclopedia of DNA Elements) project has helped to define functional elements of the human genome, including those aforementioned as well as other chromatin signals, e.g. active chromatin, enhancers, insulators, methylation domains, etc. An effort of "agENCODE" is underway to include agriculturally important birds such as chicken, turkey, duck, quail and perhaps ostrich. The study of cytogenetics will be essential here in helping to define higher order structures in nuclear organization that show regulatory interactions within and between chromosomes. Finally reconstruction of evolutionary events allows us to study genome organization and function not only in extant but, by extrapolation, in extinct species also. Reconstruction of avian-reptilian ancestral karyotypes will allow us to define chromosomal rearrangements in long-dead species that have captured the public imagination. Here be dragons!

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