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Comparative cytogenomics between chicken and duck: wider insights into genome evolution and organisation

A thesis submitted to the University of Kent for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of

Science, Technology and Medical Studies

2009

Benjamin Matthew Skinner

Department of Biosciences

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

B. M. Sk

Benjamin Matthew Skinner
30 July 2009

Incorporation of published work

This thesis incorporates published work from four papers (see appendix). The text that I contributed to these papers remains intact in the thesis. Where other authors have contributed material, this has been rewritten. I am the author of all other material in the thesis.

1) The evolution of the avian genome as revealed by comparative molecular cytogenetics (Griffin *et al.*, 2007).

Much of the information within this review is contained in chapter 1. All figures and text on comparative chromosome painting, nuclear organisation and telomeres are my work. The text on chromosome numbers in birds, introduction and conclusions involved other authors, and this material was revised accordingly.

2) Practicable approaches to facilitate rapid and accurate molecular cytogenetic mapping in birds and mammals (Morris *et al.*, 2007)

My contribution to this paper was method development. The method forms a significant part of chapter 3, but none of the results presented in this thesis form part of the published paper. No aspect of the karyotyping program forms part of this thesis.

3) Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis (Skinner *et al.*, *in press*)

4) An Appraisal of Nuclear Organisation in Interphase Embryonic Fibroblasts of Chicken, Turkey and Duck (Skinner *et al.*, *in press*)

As principle author on these manuscripts, I wrote the text and produced the figures, with critical comments and revisions from other authors. These works form significant contributions to chapters 4 and 5 respectively.

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To my family, for their support and understanding.

To Darren, who gave me the opportunity to study for a PhD, has provided constant support and guidance through it, and has been a friend as well as a supervisor.

Table of Contents

Table of Figures

Table of Tables

Abbreviations

aCGH	array comparative genomic hybridisation
APL	Pekin duck, *Anas platyrhynchos*
BAC	Bacterial artificial chromosome
BGC	Biased gene conversion
BSA	Bovine serum albumin
CNV(s)	Copy number variation (variants)
CNVR	Copy number variable region
DAPI	4,6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagles' media
DOP-PCR	Degenerate oligonucleotide primed PCR
EST	Expressed sequence tag
FISH	Fluorescent *in-situ* hybridisation
GGA	Chicken, *Gallus gallus*
IUCN	International Union for Conservation of Nature
LBC	Lampbrush chromosome
LINE	Long interspersed nuclear repeat
LPS	Lipopolysaccharide
LTR	Long terminal repeat
MGA	Turkey, *Meleagris gallopavo*
Mya	Million years ago
Myrs	Million years
NAHR	Non-allelic homologous recombination
PCR	Polymerase chain reaction
QTL	Quantitative trait locus
RH	Radiation hybrid
SD	Segmental duplication
SINE	Short interspersed nuclear repeat
SNP	Single nucleotide polymorphism
SSC	Sodium saline citrate
WGD	Whole genome duplication
YAC	Yeast artificial chromosome

Abstract

Genome organisation can be considered at a number of levels from the karyotype to gene order, copy number variation and the organisation of chromosome territories and loci within the interphase nucleus (nuclear organisation). Detailed studies of these areas in birds however are limited. As the only bird with a published genome sequence, and for its importance in areas such as agriculture, developmental biology and evolutionary studies, the chicken (*Gallus gallus*) is the most studied bird. Comparative genomics provides a powerful method for transferring information from the chicken to other, less well studied species. An obvious target for comparative genomic studies in birds is the Pekin duck (*Anas platyrhynchos*), for its agricultural significance, resistance to avian influenza, and evolutionary relationship to chicken.

This thesis reports comparative genomic studies in duck and other birds. A method for easy assignment of physical markers to chromosomes was established through the measurement of relative sizes of chicken, duck and turkey chromosomes. Physical mapping in the duck revealed previously undescribed chromosomal rearrangements, provides further evidence for conserved synteny among avian microchromosomes and yields an improved definition of the duck karyotype. A detailed study of nuclear organisation was carried out in chicken fibroblasts; this baseline was used to investigate patterns of nuclear organisation in turkey (*Meleagris gallopavo*) and duck fibroblasts, and in activated chicken and duck macrophages. Finally, microarray based comparative genomic hybridisation studies of copy number variation (CNV) were conducted in 10 bird species to complement the lower resolution cytogenetic mapping. These suggest that CNVs are less frequent in birds than in mammals, yet more commonly associated with genes. This work improves our understanding of avian genomics and evolution.

1. Introduction: The Genomics and Evolution of Birds

Birds (class Aves) are bipedal homeothermic vertebrates. They are characterised by the ability to fly, feathers, a toothless beak and oviparity. Although these features have appeared individually in other species, the combination is unique to birds. Indeed, birds are extremely unusual in many ways when compared with other vertebrates.

Birds are the most diverse of tetrapod vertebrate groups; there are approximately 9,990 extant species of birds (International Union for Conservation of Nature (IUCN) Red List, 2008), and 22,000 subspecies (Avibase - World Bird Database, http://avibase.bsc-eoc.org/avibase.jsp), of a counted 57,739 vertebrate species (Baillie *et al.,* 2004) – only fishes have more species (around 28,500) and there are twice as many bird species as mammalian species (Baillie *et al.,* 2004). Birds occupy a wide range of habitats, and show great diversity in feeding and locomotion styles, phenotype and sizes. The smallest bird in the world, the bee hummingbird (*Mellisuga helena)* is approximately 5cm in length, while the largest, the ostrich (*Struthio camelus*) stands over two metres tall (Avibase). It is worth dwelling on the tremendous variety and diversity seen among birds, as it makes the subsequent discussion of their genomes all the more intriguing.

Considering lifestyle, there are some birds that are flightless – the ratites (ostrich, emu etc) and swimming birds, such as penguins. These birds show evidence for a loss of flight as an adaptation to their habitats, yet still have very different means of locomotion. While ostriches are capable of running at 74km/h (incidentally the fastest land bird), penguins are clumsy on land, with wings adapted for "underwater flying" (Gosler, 2007). Some penguins are also able to filter salt water through a nasal gland, effectively sneezing the salt out and drinking fresh water (Schmidt-Nielsen, 1958). Of the flying birds, there are many different lifestyles too. Some remain in the air for nearly all of their lives, such as swifts, while others fly in short bursts, such as hummingbirds and African sunbirds (Geerts and Pauw, 2009). Others can fly, but spend much of their time swimming on water, for example ducks and other waterfowl. Besides these differences in lifestyle, there are often striking sexual dimorphisms, of which the peafowl (*Pavo cristatus*) is a prime example (Geist, 1977).

All birds are oviparous (egg-laying). This can be explained by the effect of vivparity (live-bearing) on flight, yet even flightless birds are oviparous. This means Aves is the only vertebrate class that does not have both oviparous and viviparous members (Gosler, 2007). Metabolically, birds are more active than mammals; they have a typical lifetime energy expenditure approximately four times higher than that of an equivalently sized mammal. Their blood glucose levels are also 3-4 times those of mammals, a level that would class them as diabetic if human, and their body temperature is higher than that of mammals (39-41°C). Despite this, birds tend to live longer than mammals of equivalent size (Holmes, 2003).

Birds then, are unusual and interesting in many respects. Of the many attributes distinguishing birds from other vertebrates, this thesis is concerned with their genomes. The remainder of this chapter will outline what is known about avian genomes (and why they are special), with reference to the chicken genome, and will describe the current thinking on avian genome evolution.

1.1. Features of Avian Genomes

Although birds are highly diverse, avian genomes studied to date appear much less so. It must be emphasised that the number of species for which we have detailed genomic information is still low in comparison to the total number of avian species, and those studies that have been performed predominantly focus on a few orders (e.g. Galliformes) with some scientific, agricultural or ecological importance. Consequently, what appear to be 'general features' of avian genomes may only represent the current sampling bias. With this caveat in mind, low resolution studies of the genome, from measurements of chromosome number to basic banding techniques have shown that large scale chromosomal events are relatively rare in most avian lineages studied. Even in those lineages which show the most rearrangement, the overall rate of change still appears lower than in, for example, mammals having similar divergence times.

Birds appear to have characteristically small genomes, approximately one third the size of a typical mammalian genome (Burt, 1999) which has, it is suggested, evolved in response to the energy conservation requirements associated with the evolution of flight (see section 1.6) (Hughes and Piontkivska, 2005).

A further typical avian feature is the gross organisation of the genome (i.e. the karyotype), which is readily identifiable to a relatively trained eye. The high diploid number, in combination with the large number of microchromosomes appears a uniquely avian feature. That is, although many reptiles (including lizards, snakes and crocodiles) are known to have microchromosomes, and karyotypes of particular species of turtle (where 2n=66) are quite similar to avian ones, the "so many, so small" pattern seems distinct among birds. Like flight, feathers and a small genome, this characteristic karyotype, once it had appeared in birds, seems to have remained relatively constant (with few exceptions) to the present day. A fuller account of genome evolution in vertebrates and how avian genomes may have evolved is presented in section 1.6.

1.1.1. Utility of Comparative Genomics

All studies in genomics are attempts at different levels of resolution to understand the characteristics emergent from the genome of an organism. These increasing levels of resolution become more time-consuming, resource intensive and costly. Hence, the number of species for which fully sequenced genomes are available is still low (indeed, it is arguable whether any 'fully' sequenced genomes have been generated, given the difficulties inherent in sequencing areas of repetitive content). At the time of writing, 916 genome projects – prokaryotic and eukaryotic – are listed as having a completed genome sequence, with 3454 more genomes in the process of being sequenced (Genomes Online database, Liolios *et al.*, 2008) – a tiny fraction of the total number of species. Yet, with each stage of decreasing resolution, the number of species for which information is available increases.

Comparative genomics, then, is the process of transferring information down this knowledge gradient, from species with a higher level of resolution to those with a lower level of resolution, allowing the knowledge from one species to improve the understanding of others. It has facilitated studies in many disciplines, from evolutionary biology, to developmental studies, to agriculture.

There are many iterations of the terms "gene mapping" or "genomics" and the following diagram (Figure 1.1) illustrates these and how they relate to one another. While the karyotype gives the most global overview of the genome, it is the sequence that provides the opportunity for more detailed analyses. The use of comparative genomics and what it has revealed about avian genomes will be detailed in the following sections.

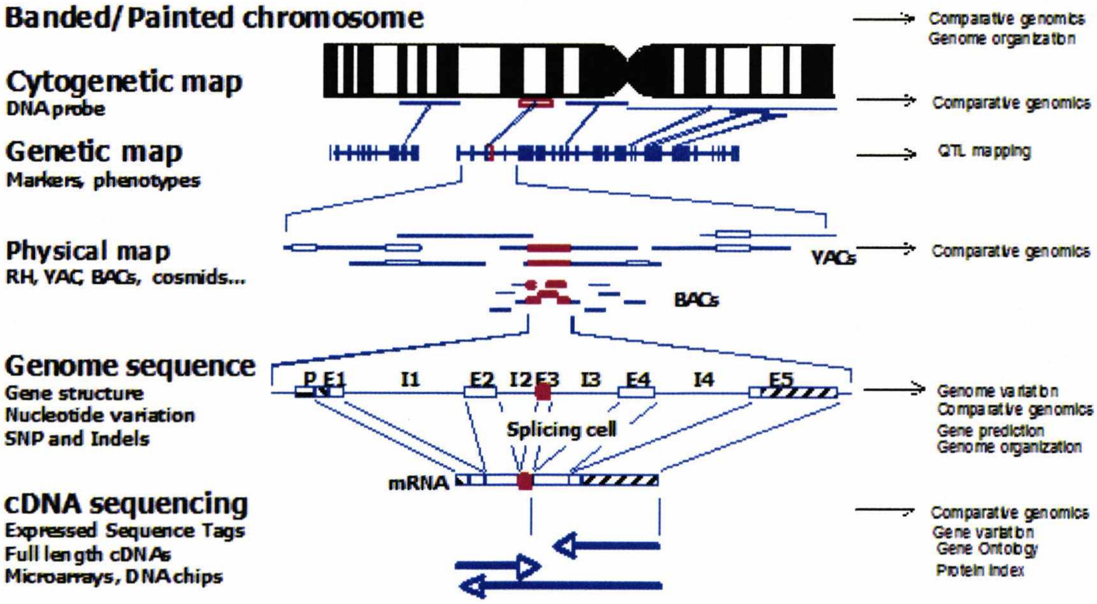


Figure 1.1: Iterations of the term "genome mapping" and (right hand side) the areas of genomic research in which they can be applied. Note the regular recurrence of "comparative genomics." RH, Radiation Hybrid mapping; YAC, Yeast Artificial Chromosome; BAC, Bacterial Artificial Chromosome (see section 1.4.2); SNP – Single Nucleotide Polymorphism (see section 1.4.4.2).

1.1.2. Avian Karyotypes

The karyotype of an organism provides the lowest resolution of information about the genome, yet can provide a lot of data. The number of chromosomes is a direct measure of the number of linkage groups that would be expected upon a sequencing effort. Comparisons of chromosome number, fundamental number and chromosome morphology with related species allow the elucidation of basic evolutionary processes – chromosomal fusions or fissions and polyploidy being basic examples. One of the fundamental requirements of karyotyping is knowledge of chromosome number in a species.

1.1.2.1. Chromosome Number in Birds

The most complete account of chromosome number in birds is presented by Christidis (1990), who described chromosome number in 723 species of birds, plus partial karyotypes. Rodionov (1997) suggested that the number was closer to 800, and cited a number not mentioned by Christidis. The diploid number is very consistent, with around 63% of birds where 2n=74-86 and 24% with 2n=66-74 (Christidis, 1990). This contrasts with the more rapid rate of change in mammals (Wienberg, 2004); large scale changes in chromosome number are commonplace in the genome evolution of mammals and the best known examples include comparisons of the Chinese and Indian Muntjacs, *Muntiacus reevesi* (2n=46) and *Muntiacus muntjak* (2n=6 in females and 7 in males (Yang *et al.,* 1997). The number of chromosomes in the bird species that have been examined appears both higher and more constrained than in most other vertebrate groups (Figure 1.2).

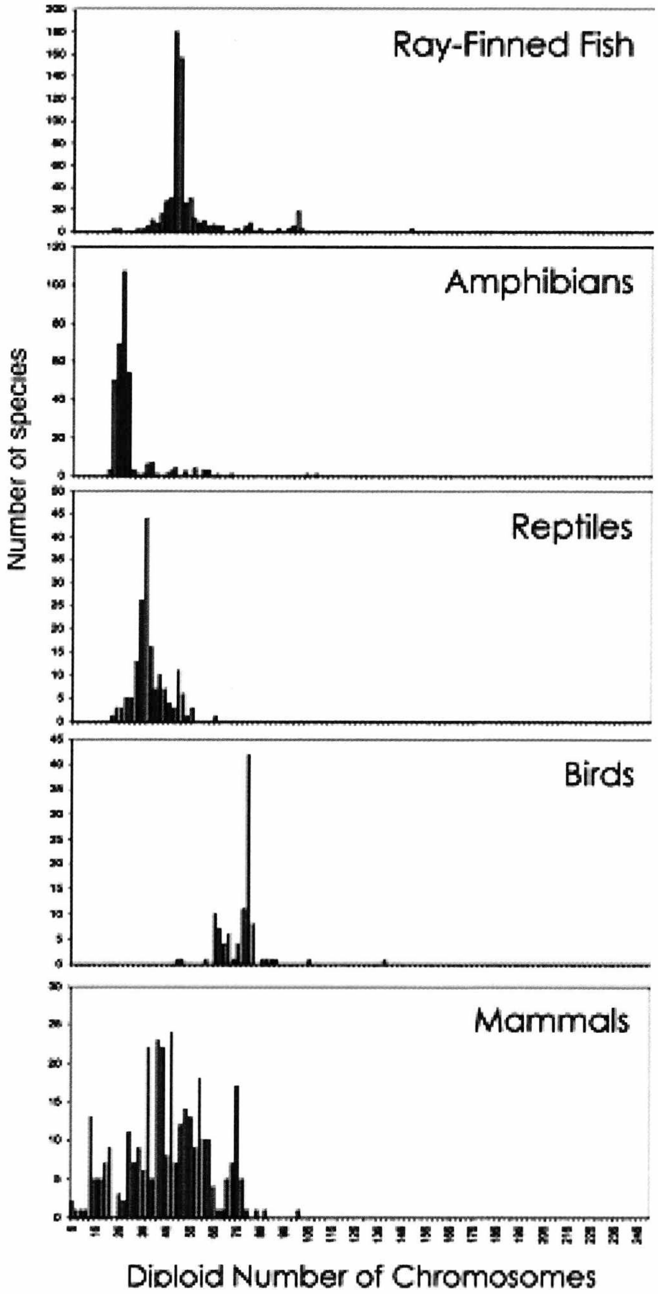


Figure 1.2: Diploid numbers of species and subspecies within vertebrate groups; birds sampled to date have both high and constrained chromosome numbers (from Mank and Avise, 2006, underlying data from Gregory, 2005).

Examples of birds with significantly fewer chromosomes than the average include the Laridae (gulls and terns, 2n=66-70), the Pelecaniformes (pelicans etc, 2n=66-70 with one known exception, the Little Cormorant, *Phalacrocorax niger)* (Christidis, 1990) and the Psittacidae (parrots, 2n=60-72). Interestingly, the Psittacidae are an example of where clear differentiation between macro- and micro chromosomes can be seen; for instance the Crimson Rosella, *Platycerus elegans*, has seven pairs of macrochromosomes (including Z and W), the remainder being at least 10 times smaller than that the smallest pair of macro-autosomes (Christidis, 1990). In the Falconiformes chromosome number varies from 2n=50-72 in all but the Cathartidae (new world vultures), Sagittariidae (Secretary bird) and selected Falconidae (falcons and caracaras). Indeed, the Falconidae show the most variation among the Falconiformes with *Falco jugger, F. peregrinus* and *F. subbuteo* (Laggar, Peregrine and Hobby Falcons respectively) having 2n=50 and the Crested Caracara, *Polyborus plancus,* having 2n=84-86 (Christidis, 1990).

For the sake of completeness it is appropriate to mention the extremes at both ends of the spectrum. Smallest among known diploid chromosome numbers are the trumpeter hornbill *Ceratogyman bucinator* (2n=40), the stone curlew *Burhinus oedicemus* (2n=42), the beach thick knee *Burhinus magnirostris* (2n=42), and the black and white casqued hornbill *Ceratogymna subcilindrica* (2n=42) (Christidis, 1990). On the other end of the scale, the hoopoe *Upupa epops* has a diploid number of 126. The greatest number of reported chromosomes in a bird however, is either the common kingfisher *Alcedo atthis* where 2n=132 or 138 or the Gray or Southern Go-away-bird *Corythaixoides concolor* (2n=136-142) (Christidis, 1990). These birds are rare examples, however, since the next highest number is cited as 108-110 in several unrelated species (Christidis, 1990).

1.1.2.2. General Features of Avian Karyotypes

The high degree of karyotype conservation seen in the bird groups so far studied makes it possible to describe a karyotype typical of these birds; this is very similar to that of the chicken (2n=78), the first bird for which a fully defined karyotype was published (Masabanda *et al.,* 2004). Hence the chicken will be used as an example in the description of the genomic features of known avian karyotypes. The chicken (*Gallus gallus domesticus;* GGA) is the domesticated descendent of the Red Junglefowl (*Gallus gallus*), on which most genomic and hence cytogenetic studies have been performed. Its role in agriculture and as a classical embryology model – as well as a model for human disease – have made it the primary species from which nearly all comparative vertebrate genome analyses in birds have ensued.

1.1.2.3. Macro- and Microchromosomes

A convenient starting point is in the terminology. The terms macro- and microchromosome have been in common use for many years when describing avian chromosomes; however, they can be somewhat misleading. Closer inspection shows that there is usually no clear dividing line between the smaller and the larger chromosomes (e.g. Figure 1.3), which may explain why different authors give different accounts of the relative numbers of macro- and micro-chromosomes. Yet, although in most avian species for which data are available there is no distinct size boundary between the macro- and microchromosomes, there are other genomic features that distinguish them. Microchromosomes tend to be GC rich, hyperacetlyated, late replicating and contain a higher density of CpG islands and genes than the macrochromosomes (Burt, 1999).

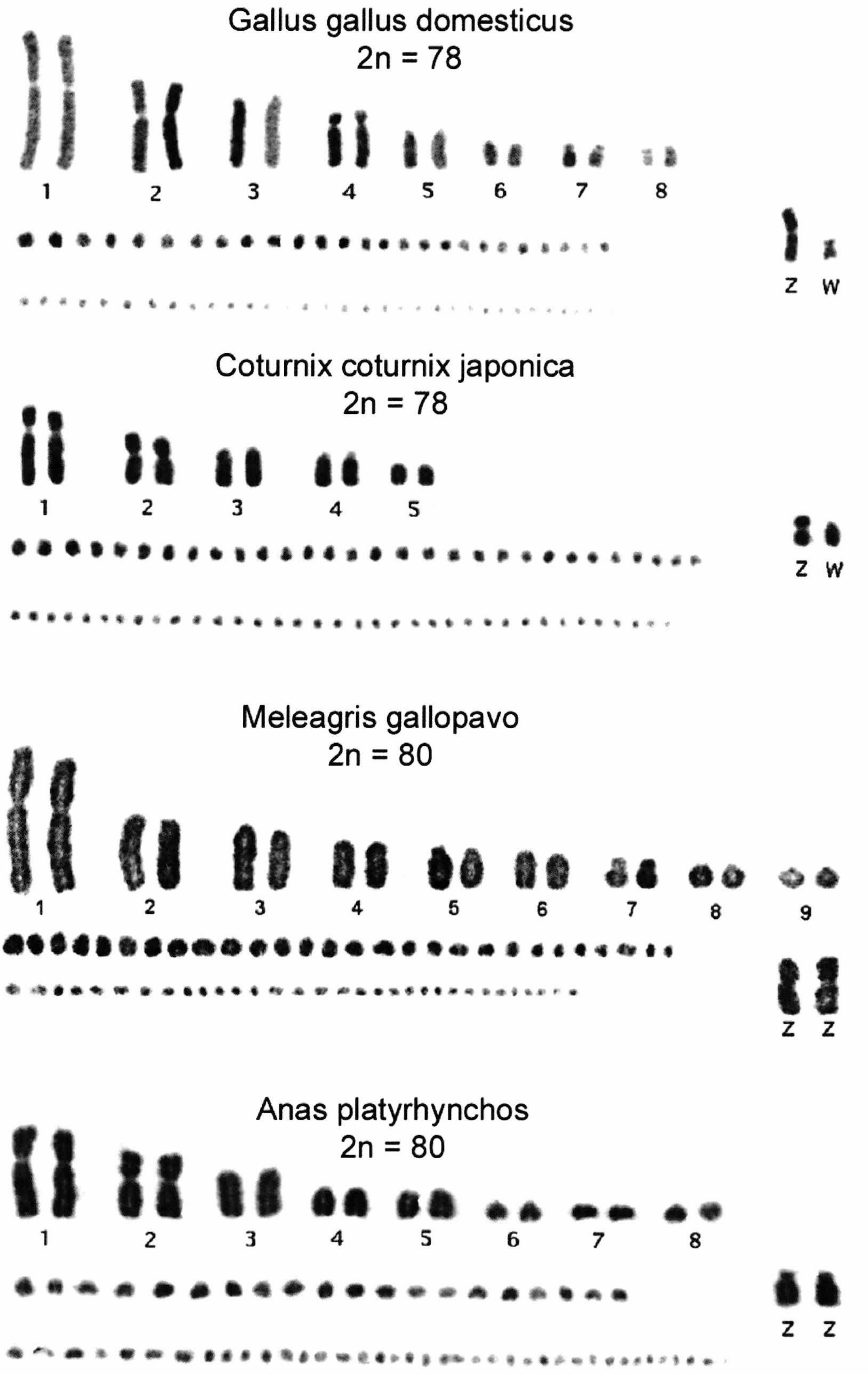


Figure 1.3: Example avian karyotypes (Giemsa stained chromosomes). From the top, chicken, Japanese quail, turkey, duck. Modified from Schmid *et al.* (2005).

In an attempt to reconcile these descriptions, Masabanda *et al.* (2004) suggested a different classification system. This was related to the ability to resolve the chromosomes in a flow karyotype and to the newly emerging chicken genome sequence. They assigned group A as the chromosomes that could be resolved in a flow karyotype (which included chromosome 10 which, although slightly smaller that 11 and 12, was resolved alone where 11 and 12 sorted together). Groups B and C comprised the remainder of the chromosomes (11-32) that had known markers from the genome project assigned to them at the time of writing. The groups were separated by the NOR chromosome which, despite its relatively small size, had previously been assigned number 16. Even five years after the original publication, the smallest "group D" chromosomes (33-38) have yet to be anchored to the genome assembly; it was even only recently that sequence information was assigned to the entirety of GGA25 (Douaud *et al.,* 2008) from chrUn (120Mb of sequence not assigned to a chromosome or linkage group). This raises the question of whether this is simply due to their small size, or a more fundamental biochemical reason; for example, there has been a suggestion that there is a cloning or sequencing bias against the smallest chromosomes due to high G+C content and a large proportion of repeats (Douaud *et al.,* 2008). GGA25 does indeed show a G+C content of 52.4%, among the highest for a chicken chromosome (Hillier *et al.,* 2004), and also a high proportion of minisatellite markers (Groenen *et al.,* 2000).

1.1.2.4. Chromosome Banding

The classical way of studying any karyotype is through chromosome banding. Chromosome banding relies upon the selective binding of dyes to DNA regions of different composition. One of the most well known and common banding techniques, G-banding, uses trypsin digestion of chromatin associated proteins followed by Giemsa staining. Giemsa, a mixture of ethylene blue and eosin, binds to AT rich areas preferentially, resulting in a unique banded appearance for each chromosome pair. G-banding has been refined in human cytogenetics especially, with 300-400 bands routinely visible in the genome to the trained eye (ISCN, 2005). In humans, at least, G-positive (dark) bands are areas of heterochromatin, non-coding DNA, and tend to be late replicating and AT rich, while the light bands are areas of euchromatin, coding

DNA, and are normally early replicating and GC rich. The molecular basis of chromosome bands however is still the subject of some debate. GC content, SINE/LINE elements, CpG island density, condensation level, and replication timing have all been suggested as correlates (Comings and Wyandt 1976, Holmquist *et al.*, 1982, Korenberg and Engels 1978,Korenberg and Rykowski 1988, Chen and Manuelidis 1989, Craig & Bickmore 1994, Bak *et al.*, 1981, Sumner *et al.*, 1993, Sen and Sharma 1985, Manuelidis and Ward 1984, Goldman *et al.*, 1984, Bernardi 1989).

Other mammals typically can also be successfully G-banded, and chromosome identification in these cases is more a function of observer experience than technique limitation. For other vertebrate classes, G-banding is less apparent; some fishes have been successfully banded, others show no banding, a pattern also seen in reptiles and amphibians (e.g. de Brito Portela-Castro *et al.*, 2008, de Carvalho *et al*., 2005). The general reason for this is the lack of a distinct AT- or GC isochore structure within their genomes. Birds also show fewer distinct bands than mammals, due in part to the small size of avian microchromosomes. Other banding techniques include R-banding (reversed G-banding), C-banding (staining constitutive heterochromatin), and Q-banding (a fluorescent banding technique using quinacrine, which gives a similar banding pattern to G-bands). A commonly used alternative to G-banding is DAPI-actinomycin-D banding, which provides a similar banding pattern without the need for precise trypsin digestions (e.g. Mayr *et al.*, 1983).

Banding, of whichever method, provides more information than simple chromosome staining when looking between species. The pattern of bands can allow inter- and intra-chromosomal rearrangements to be identified. A recent example is the use of G-banding as a means of identifying the sex chromosomes of a side-necked turtle (*Emydura macquarii*), and the suggestion that they evolved by way of translocation (Martinez *et al.*, 2008). While not a new technique, the generation of banded karyotypes is still an important ongoing process, even in orders that are comparatively well studied (Pieńkowska-Schelling *et al.*, 2008). In general terms however, G-band information is limited in birds firstly because bands on the group A chromosomes are less distinct than in mammals (perhaps due to less distinct differences between the molecular correlates of G-banding along the genome) and secondly because the group B-D chromosomes are too small to visualise any banding pattern (Ladjali-Mohammedi *et al.,* 1999). Auer *et al.* (1987) published a chicken karyotype up to GGA18 based on enhanced fluorescence banding, but this resolution is beyond the scope of G-banding. In defining the full karyotype molecular cytogenetic approaches had to be used to develop chromosome-specific FISH probes for each chicken chromosome (Griffin *et al.,* 1999, Masabanda *et al.,* 2004).

1.1.2.5. Avian Sex Chromosomes

In contrast to mammals, birds have a ZW sex chromosome system. That is, the female is heterogametic (ZW) while the male is homogametic (ZZ). It is worth noting that of the vertebrates, only birds and mammals have been observed to have a consistent sex determination system; other reptiles have ZW, XY or temperature dependent systems, as do fishes and amphibians (Schmid *et al.,* 2001; Sola *et al.,* 1981). Additionally, the sex chromosomes in vertebrates have in many cases evolved from different ancestral autosomes; for example, there is no homology between the placental mammalian XY and avian ZW (Nanda *et al.,* 1999, Nanda *et al.,* 2000, Nanda *et al.,* 2002), although there is between the monotreme X and the avian Z (Veyrunes *et al.*, 2008). Nor indeed is there homology between all reptile and avian Zs; while the gecko lizard (*Gekko hokouensis*) Z has linkage homology with chicken Z (Kawai *et al.,* 2008), the same chicken Z probes are found on an autosome of the Chinese soft-shelled turtle, *Pelodiscus sinenis* (Matsuda *et al.,* 2005).

The Z and W are differentiated in most modern birds studied, the exception being the ratites, in which Z and W are of equal size and morphology (and possibly gene content; Shetty *et al.,* 1999, Saitoh *et al.,* 1992, Fridolfsson *et al.,* 1998). The ratite W is considered to be the more primitive form, and in neognathous birds the W appears much smaller than the Z and mainly heterochromatic. Few genes have been discovered on the W, at least in chicken, and the galliform W seems to be composed largely of two repeat families (Saitoh, 1992). There is a small pseudoautosomal region on Z and W with an obligate crossing-over at meiosis, though most of the W chromosome does not recombine.

The Z chromosome appears to be an ancient conserved chromosome, based on conserved synteny in birds and synteny (with autosomes) in the Chinese soft-shelled turtle and Japanese four-striped rat snake (Shetty *et al.*, 1999, Matsuda *et al.*, 2005). The avian Z is subject to some of the most extensive intrachromosomal rearrangements described within avian lineages (Shibusawa *et al.*, 2004a, b). The W chromosome appears to have evolved from an ancestral Z form (Shetty *et al.*, 1999). The metacentric Z chromosome of the chicken appears as a submetacentric chromosome in many of the other Galliformes, as well as in the Anseriformes, and there are indications of heterochromatin accumulation in the q arms of chicken and the blue-breasted and Japanese quails (Shibusawa *et al.*, 2004b). Conserved linkage between chicken chromosome Z and turtle chromosome six and the short arm of the Japanese rat snake (*Elaphe quadrivirgata*) chromosome two (Kawai *et al.*, 2008), suggests that chicken (apart from the heterochromatin on the q arm) represents the ancestral form. The identification and understanding of inversions, centromere relocations and heterochromatin accumulation have yet to be determined; this is in part due to the lack of markers in the sex chromosomes on the chicken genome assembly.

An important unresolved question is the role of the sex chromosomes in sex determination in birds. It remains unclear whether the presence of the female-specific W chromosome triggers female development, or if maleness is a Z chromosome dosage effect (Ellegren, 2001). Data has been presented that supports both models. A recent study even revealed a temperature dependent effect, in Australian brush-turkey (*Alectura lathami*) embryos (Goth *et al.*, 2005), though is still unclear whether this is solely due to differential mortality (as seems likely) or whether actual temperature dependent sex determination mechanisms are present (Goth, 2007). It is known that in mammals it is the presence of the Y chromosome that triggers male development – triggered by the *SRY* gene on the Y chromosome, although other Y-specific genes are also important for testis development (Sinclair *et al.*, 1990). Raymond *et al.*, (1999) found expression of the *DMRT1* (Doublesex- and Mab-3-Related Transcription factor) gene, which maps to the chicken Z chromosome (Nanda *et al.*, 1999), in the genital ridge and in the Wolffian ducts (progenitors of the male-specific internal reproductive structures) of stage 25 chicken embryos (4.5-5 days post fertilisation).

This stage precedes sexual differentiation, suggesting that DMRT1 acts upstream of the protein cascade involved in male development (Ellegren, 2001). The *DMRT1* gene becomes testis-specific after the onset of sexual differentiation and is expressed exclusively in the testes of adult birds (Shan, 2000).

Data on *DMRT1* expression in birds is similar to that seen in reptiles (alligator), mammals (human and mouse) and fish (trout and tilapia). In alligator, where sex determination is temperature dependent, *DMRT1* expression is upregulated at male-producing temperatures (Smith *et al.*, 1999). *DMRT1* expression is found in the genital ridge in both human and mouse embryos of both sexes prior to sexual differentiation, but becomes restricted to the seminiferous tubules of the testis as gonadogenesis proceeds (Raymond *et al.*, 1999). Furthermore, mouse XY $\mathrm{Dmrt1}^{-/-}$ knock-outs fail to produce differentiated testes and also experience germ cell death (Raymond *et al.*, 2000), suggesting that *DMRT1* plays a key role in mammalian testis differentiation. In fish, *DMRT1* expression is also specific to testicular differentiation (Marchand, 2000). While this would suggest that the role of *DMRT1* might be related between organisms that have either a genetic or environmental basis of sex determination (Ellegren, 2001), some evidence has been presented that indicates *DMRT1* may not be involved in reptilian sex determination. The *DMRT1* gene maps to an autosome pair in the Japanese wrinkled frog (*Rana rugosa*), as well as in the Chinese soft-shelled turtle and three species of snake (Uno, 2008). Further evidence for a Z-dosage based effect in birds has been suggested by the high conservation of gene content on the Z in several different families (Nanda *et al.*, 2008), and that birds do not undergo global Z inactivation – rather dosage compensation appears to have evolved on a gene-by-gene basis (Melamed and Arnold, 2007).

Evidence for W-linked sex determining genes is less clear. However, one candidate gene is *HINTW* (encoding histidine triad nucleotide binding protein), a W specific gene distinct from its Z linked counterpart *HINTZ* (Hori *et al.*, 2000). There is evidence showing that *HINTW* has undergone rapid positive selection (Ceplitis and Ellegren, 2004) and has a high gene copy number (Backstrom *et al.*, 2005). Yet among the ratites, *HINTW* and *HINTZ* appear not to be differentiated; consequently, the possible role in sex determination is still uncertain (Smith, 2007).

1.1.2.6. Lampbrush chromosomes

Lampbrush chromosomes are found during oogenesis in most animals; the exceptions being mammals and some insects (Callan, 1986). Lampbrush chromosomes (LBCs) are more than 30 times longer than the corresponding mitotic metaphase chromosomes (Mizuno and Macgregor, 1998), and form during the diplotene stage of meiotic prophase I in oogenesis (Callan, 1986). They are characterised by a distinctive chromosome-loop structure, as well as intensive RNA transcription sites associated with lateral loops (Macgregor, 1987).

Avian macrochromosomes at the lampbrush stage have been described in detail, in chicken (Chelysheva *et al.*, 1990), Japanese quail (Rodionov and Chechik, 2002) and chaffinch, (Saifitdinova *et al.*, 2003), and the differences between mitotic metaphase chromosomes and LBCs have been studied (Derjusheva *et al.*, 2003, Saifitdinova *et al.*, 2003). The utility of LBCs in avian genomics lies in the resolution they provide when examining the microchromosomes. For example, comparison of chicken and Japanese quail LBCs revealed that while chicken microchromosomes are predominantly acrocentric, quail microchromosomes are predominantly metacentric (Galkina *et al.*, 2006, Rodionov and Chechik, 2002).

Each LBC contains an array of compact chromatin granules, termed chromomeres. Since the chromomeric pattern seen on LBCs is not oocyte-specific, LBCs provide a platform for higher-resolution physical mapping than achievable with even the most elongated metaphase spreads. An example of this for comparative genomics is the use of turkey chromosome paints on chicken lampbrush chromosomes to confirm a centromeric breakpoint of the ancestral chromosome 2 in the turkey lineage, which remained intact in the chicken lineage (Griffin *et al.*, 2008).

1.1.3. Repetitive Content of Avian Genomes

The repetitive content of the chicken genome, is estimated to be about 8-15% (Wicker *et al.*, 2005). There are two main classes of repeats known in the chicken genome; short tandem repeats (e.g. centromeric or telomeric repeats) and those derived from transposable elements, further divided into Class 1 (involving an mRNA intermediate) and Class 2 elements (with mobility of the DNA directly). The Class 1 elements are also subdivided into those with long terminal repeats (LTRs), and those without (long and short interspersed nuclear elements, LINEs and SINEs).

The most common repeat element in the chicken genome, and presumably other birds, is the CR1 repeat family, LINEs of ~4.5kb, present in the chicken genome in about 100,000 copies (Vandergon *et al.,* 1994). Most of the copies in chicken are non-functional due a 5' truncation; ~99% of chicken CR1 are truncated to 1.2kb (Hillier *et al.*, 2004), and over 98% of the CR1 elements studied by Wicker *et al.,* (2005) were less than 2kb in length. The CR1 elements contribute about 3.1% of the chicken genome (Wicker *et al.,* 2005), and are found across the entire genome, though at a higher concentration on the macrochromosomes and at some sub-telomeric "hotspots" (Coullin *et al.,* 2005; Figure 1.4) which tend to be GC rich (Olofsson *et al.,* 1983).

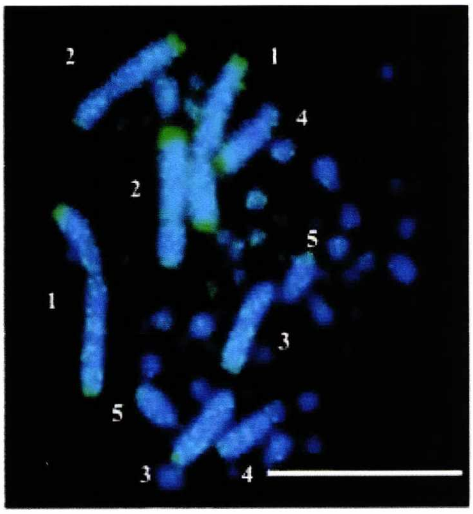


Figure 1.4: Pattern of CR1 repeat elements on chicken chromosomes. A higher density can be seen on the macrochromosomes, plus sub-telomeric hotspots. From Coullin *et al.* (2005).

This pattern has also been seen in other orders – other Galliformes showed the same CR1 "banding" as chicken, and similar patterns occur Anseriformes, Passeriformes and Falconiformes (Coullin *et al.,* 2005), supporting a possible epigenetic function. The next most common repeat element is a Class 2 element (*Galluhop*), which comprises 0.51% of the genome, consisting of a ~1250bp sequence or a more common (by about ten-fold) truncated 530bp version (Wicker *et al.*, 2005).

1.1.3.1. Short tandem repeats

As in mammals, avian telomeres are composed of a repeat sequence, 5'-$(TTAGGG)_n$-3', a pattern conserved throughout vertebrate evolution over 400 million years (Meyne *et al.*, 1989). Telomeric sequences comprise 4% of the chicken genome, making them ten times more prevalent than in mammals (in contrast, the prevalence in humans is 0.3%) and with a length range of 0.5-2Mb (at least in chicken; Delany, 2000). Telomere array organisation studies by Delany (2000) in chicken divided them into three classes based on telomere size, chromosome location and stability. Class I are interstitial, 0.5-10kb, and show no evidence of telomere shortening. Class II are terminal, 10-40kb, and show age related shortening. Class III are terminal, 40kb-2Mb, and also do not show shortening. The centromeric and sub-telomeric regions may also contain a number of classes of larger tandem repeats. Of these, the most common in chicken is the 41-42bp *CNM* element (Matzke *et al.*, 1990). These repeats have been isolated from chicken, turkey and Japanese quail centromeric sequences, and their low sequence identities suggest that they have undergone substantial divergence (Yamada *et al.*, 2002).

1.1.3.2. Microsatellites

Microsatellites are polymorphic loci in nuclear and organelle DNA that consist of repeating units of 1-4 base pairs. They have developed over the last decade into important markers in a number of genetic areas, including genome mapping and medical, evolutionary, and ecological genetics (Ellegren, 2004). Comprehensive studies of the absolute numbers of microsatellites in various genomes are needed to address whether microsatellite abundance is directly a function of genome size (Burt, 1999); microsatellites predominantly occur in noncoding DNA, and if the number of such sequences acts as the sole constraint for the evolution of simple repeats, the absolute numbers of repeats in the genome should be closely correlated with DNA content. Hybridisation experiments with divergent taxa have indicated this to be the case (Hamada, 1982).

It would, given a small genome size of birds, be expected that small avian genomes contain significantly fewer microsatellites than most mammals, and there are indications that this may be the case for dinucleotide motifs (Hamada, 1982). Primmer *et al.* (1997) analyzed the occurrence, frequency, and distribution of microsatellites in three bird species, chicken, white-backed woodpecker (*Dendrocopus leucotos)* and barn swallow (*Hirundo rustica)*. Their data from showed that microsatellite repeats generally occur less frequently in birds compared with other vertebrates (one every 20-39 kb in birds versus one every 6kb in humans). In contrast to mammalian microsatellites, bird microsatellites do not appear to be associated with SINEs. Both SINEs and LINEs seem less abundant in birds than in mammals (Hillier *et al.,* 2004) and none characterised so far terminate with a poly(A) tail. In mammals, poly(A) tails derived from retrotransposition provide a resource for the evolution and expansion of more complex repeats (Eickbush, 1992). Hence, low frequencies of microsatellites in birds may be at least partly attributable to a lack of poly(A) tails.

1.2. An Outline of Avian Karyotype Evolution

Avian species first appear in the fossil record in the Jurassic approximately 150 million years ago – these being the 11 fossils of *Archaeopteryx* (holotype described by von Meyer, 1861). A putative fossil bird dating to the Triassic, 220 Mya, *Protoavis*, is generally considered to be a chimera of non-avian archosaur fossils (Zhou, 2004). Mitochondrial evidence suggests the common ancestor of birds (synapsids) and mammals (diapsids) diverged 310 million years ago, while the common ancestors of birds and crocodilians may have diverged 210-250 million years ago. The divergence of turtles appears less certain; however they too are thought to have diverged from the bird lineage over 210 million years ago. Recent molecular evidence from both mitochondrial and nuclear sources places birds, crocodilians and turtles in the same group (Archosaurs) with lizards and snakes (Lepidosaurs) separate. Matsuda *et al.* (2005), through the isolation of cDNA libraries from soft-shelled turtles and comparison with chicken sequences, provided compelling evidence that there was highly conserved linkage homology between birds and turtles (specifically chickens and soft-shelled turtles); moreover chicken and turtle chromosomes 1-5 (as well as turtle 6 and chicken Z) appear to be precise counterparts of one another. Of these chromosomes the Z chromosome is thought to be an ancient sex chromosome and sequence comparisons from the human and chicken genome projects reveals a remarkable degree of synteny of chicken chromosome 4q and (coincidentally) human chromosome 4 (Chowdhary and Raudsepp 2000, Hillier *et al.*, 2004). That is, although unsurprisingly, there is extensive inter-chromosomal rearrangement between all other chromosomes, there is none between human chromosome 4 and chicken chromosome 4q other than a small segment of another chicken chromosome in the p-terminus of human chromosome 4. Taken together then, the ancestral avian chromosomes 1, 2, 3, 5 and Z seem to have appeared at least 210 million years ago with the ancestral chromosome 4 appearing at least 310 million years ago.

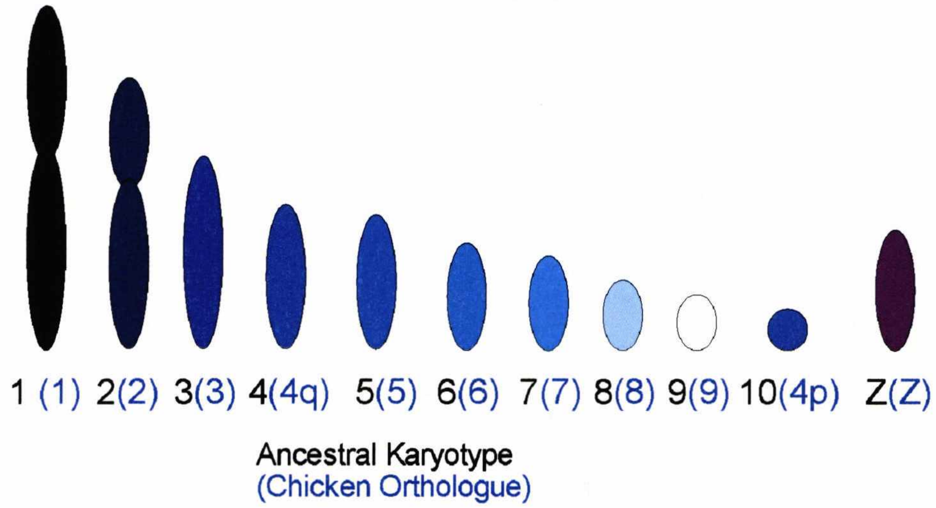


Figure 1.5: Depiction of the ancestral avian macrochromosome karyotype and chicken orthologues.

Attempts to depict the ancestral karyotype of birds by examining banding patterns date back to at least 1982 (Stock and Bunch, 1982). The ancestral karyotype for the Galliformes as predicted by Shibusawa *et al.* (2004) by comparative chromosome painting appears to be conserved throughout many of the avian lineages studied to date. Hence, for the purposes of this introduction reference will be made to chromosome rearrangements in relation to the putative avian ancestor, rather than the chicken. The chicken chromosome 4p has been shown to be, most likely, a fusion of the ancient ancestral chromosome 4 to another ancestral chromosome (Hillier *et al.*, 2004). Banding comparisons of hybridised paints indicate that this chromosome is chromosome 9 in turkey (Griffin *et al.*, 2008), and 9-13 in duck (Fillon *et al.*, 2007). For the purposes of this introduction, the orthologue of chicken chromosome 4p will be referred to as "ancestral chromosome 10". Taking the studies as a whole it seems clear that the pattern of the chicken orthologues of chromosomes 1, 2, 3, 4q, 5, 6, 7, 8, 9, 4p and Z likely represent the ancestral chromosomes 1-10+Z for all birds, illustrated in Figure 1.5. The timing of appearance of extant chromosomes 6-9 remains to be determined (e.g. by means similar to that employed by Matsuda *et al.* (2005); it is reasonable however to suggest that they appeared at a similar point to their larger counterparts. The W chromosome is assumed to have evolved around 120 Mya by previously described mechanisms of sex chromosome divergence from a homomorphic sex chromosome pair (Marshall Graves and Shetty 2001). As will become clear in subsequent sections, it seems to be chromosomes 1, 2, 4 and 10 that are more prone to inter-chromosomal rearrangements (fissions and/or fusions) and the Z more prone to intra-chromosomal rearrangements. Some selected orders and families are known to have multiple rearrangements (mostly microchromosomal fusions) and these are also reviewed in detail presently.

The appearance of the microchromosomes seems to have been a gradual rather than sudden event. Molecular and fossil data suggest that the divergence of all the major amniotes (reptiles, birds and mammals) occurred around 300-310 million years ago. The presence of microchromosomes in birds, lizards, snakes, crocodiles and turtles but not in mammals or amphibians suggests that either the first appearance of microchromosomes was after this time, or that ancestral microchromosomes underwent fusions in both the amphibian and mammalian lineages.

1.2.1. Comparative Genomics using FISH

The principal application of FISH (fluorescent *in-situ* hybridisation) is gene mapping. Either whole chromosome paints or individual clones from one species can be mapped on to the chromosomes of another species, thus increasing the information available for both. Extensive sequence information is not required and thus large numbers of species can be analysed once chromosome paints or individual clones from one reference species (e.g. human or chicken) have been isolated. Reciprocal chromosome painting (taking chromosome paints for species A and painting them onto species B, then taking the chromosome paints for species B and painting them back onto species A has also become commonplace in comparative genomics (e.g. Nie *et al.,* 2008, Mlynarski *et al.,* 2008). Before describing cross-species FISH in birds in detail, it is appropriate to consider the utility of such resources in other vertebrate groups.

1.2.1.1. Cross-species FISH in mammals

The first whole chromosome paints available were the human paints, which long remained the only source of paints covering individual chromosomes of a whole mammalian karyotype. Initially developed and used for the study of translocations in human patients (e.g. Kraker *et al.,* 1992), these resources were soon used in comparative genomic studies. Especially, comparative chromosome painting has been used to reconstruct likely mammalian ancestral karyotypes (for example, Robinson and Ruiz-Herrera, 2008, Nash *et al.,* 2008, Stanyon *et al.,* 2008) and thereby elucidate patterns of mammalian genome evolution.

Among the mammalian species investigated via comparative chromosome, some studies of interest stand out. One of these is the painting of Indian muntjac deer, *Muntiacus muntjak,* chromosomes (Yang *et al.,* 1997a). The Indian muntjac has a dramatically reduced chromosome number (2n=6, 7) compared with other related deer. The comparative painting data revealed that this was mainly due to fusions of entire chromosome blocks, which was later confirmed by comparative reciprocal painting between the Indian muntjac, Chinese muntjac, *Muntiacus reevesi,* and brown brocket deer, *Mazama pandora* (Yang *et al.,* 1997b) (Figure 1.6).

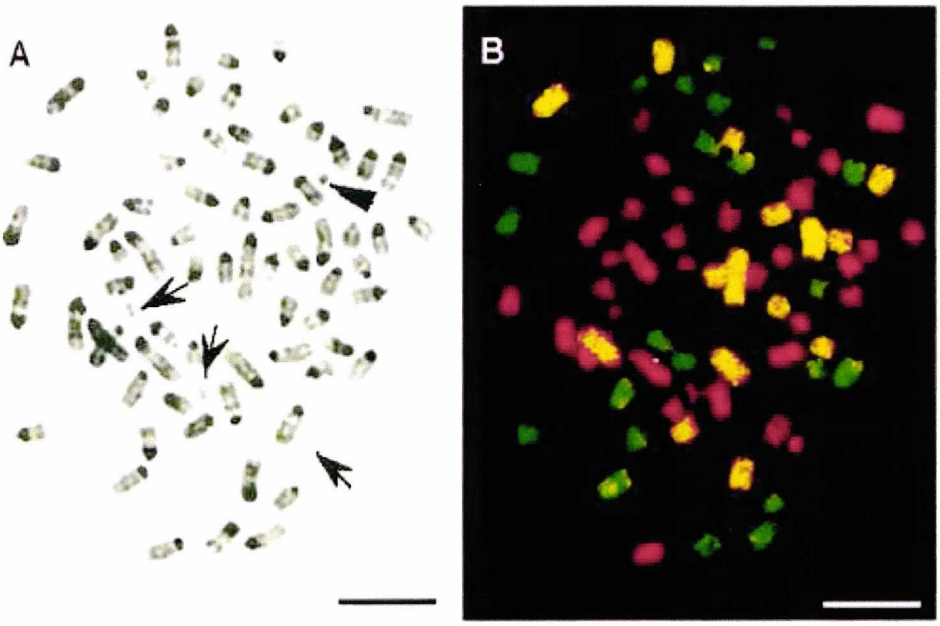


Figure 1.6: A) DAPI banded brown brocket deer metaphase (2n=70+3B). B) Same metaphase with Indian muntjac (2n=6,7) chromosome paints. Differences in chromosome number are seen to be due mainly to chromosomal fusions in the Indian muntjac. From Yang *et al.* (1997b).

Some of the comparative painting data collected for mammals is being aggregated and presented interactively online in the Chromosome Homology Mapping Atlas (http://www.chromhome.org).

An interesting finding from comparative gene mapping in echidna and platypus is that four of the platypus X chromosomes and one Y chromosome have homology with the avian Z (Rens *et al.,* 2007). This indicates that the sex chromosomes of the monotremes evolved from a separate pair of ancestral autosomes to those of marsupial and placental mammals, and therefore places mammalian sex chromosome evolution after this divergence, 166 million years ago.

1.2.1.2. Cross-species FISH in other vertebrates

Comparative painting in other vertebrates is limited; there have been no major comparative analyses in fishes. Those studies that exist are primarily interested more in the analysis of repetitive sequences or microsatellites within populations (for example, Harvey *et al.* (2003), Artoni *et al.* (2006)). This is similarly true of amphibians and reptiles, where the first whole chromosome painting study was reported by Mühlmann-Díaz *et al.* (2001), using a yellow-bellied slider turtle (*Trachemys scripta*) chromosome 1 paint on chromosomes from four other turtle species. The study revealed that this chromosome has been cytogenetically stable during turtle evolution. More recently comparative genomic hybridisation (CGH) has been used to identify sex chromosomes in the dragon lizard (Ezaz *et al.,* 2005). In this case, differentially labelled male and female genomic DNA were hybridised to lizard chromosomes, and unequal hybridisation ratios on specific chromosomes indicated the sex chromosomes.

1.2.1.3. Cross-species FISH in birds

The patterns of chromosomal evolution outlined below have been constructed from comparative chromosome painting and other comparative FISH mapping studies. There are thought to be three major events involved in bird evolution: 1) the divergence of Palaeognathae (extant orders comprise the Struthioniformes and Tanamiformes) and Neognathae (others) approximately 100-120 million years ago; 2) the divergence from the Neognathae of the Galloanserae (e.g. chicken, turkey, goose, duck etc.) approximately 100 million years ago; and 3) the divergence of the remainder of the Neognathae into the higher land and higher water birds approximately 70-80 million years ago. These divergences can be seen in Figure 1.7. Recent molecular evidence has challenged some older phylogenies, reordering some relationships within the Neoaves (Hackett *et al.*, 2008), but has confirmed the major divergences outlined subsequently.

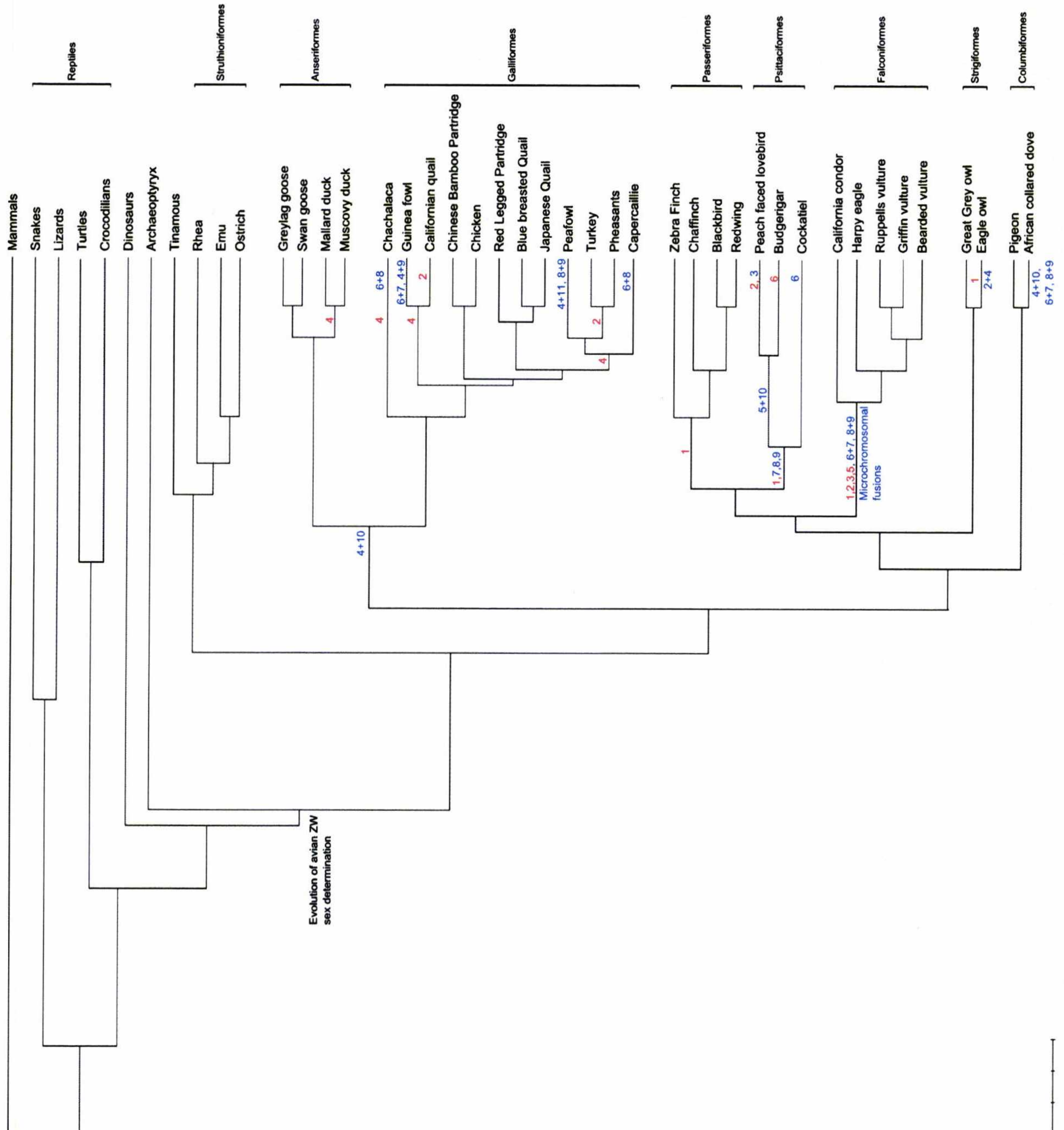
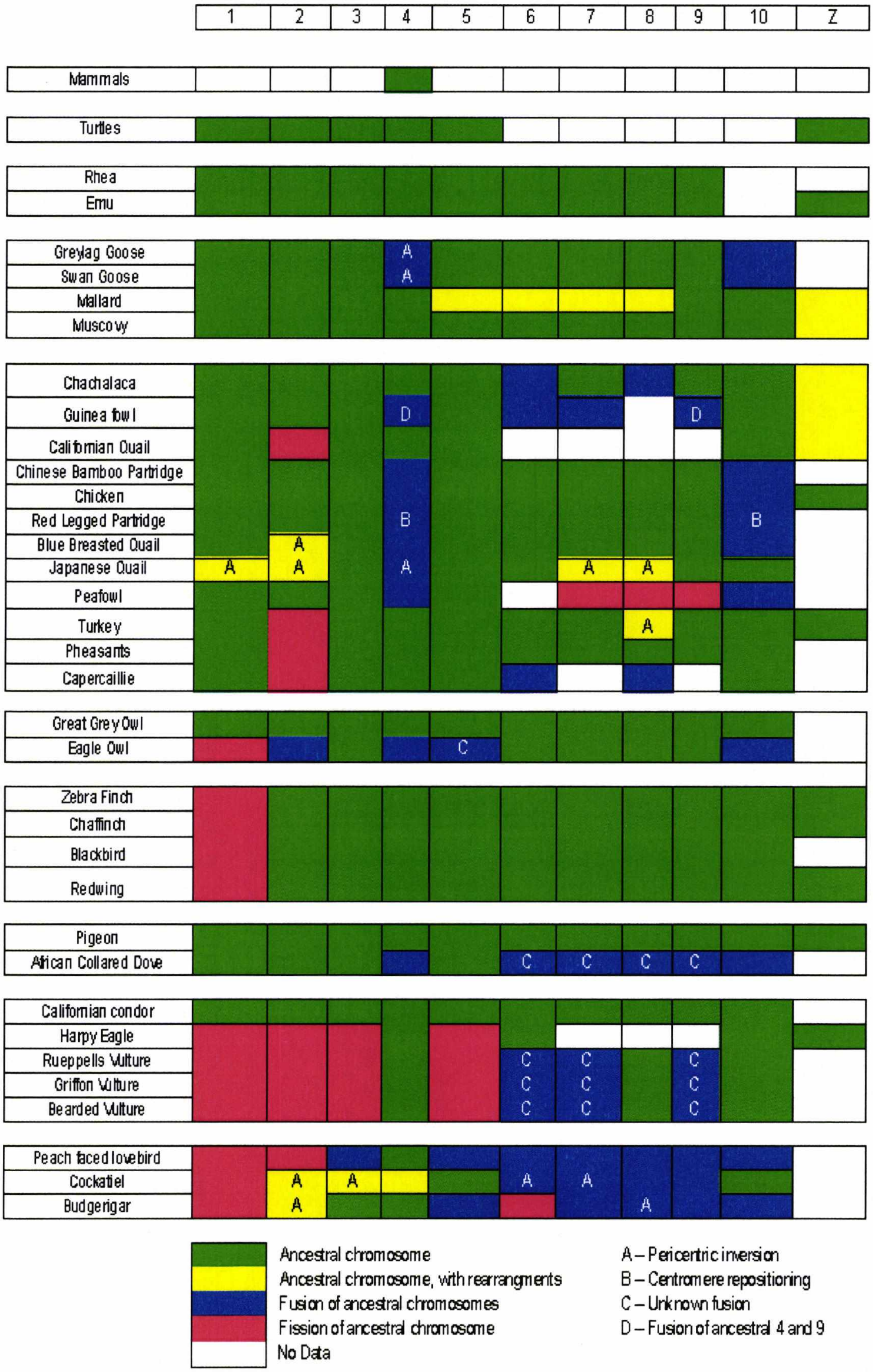


Figure 1.7: Phylogenetic tree of species for which comparative genomic data exists. The tree has been collated from consensus studies of DNA hybridisation studies, mitochondrial DNA sequencing and comparative protein sequencing. The phylogeny of the Neoaves is hypothetical based on Hackett *et al.* (2008). Only interchromosomal changes are shown, fissions are represented in red, fusions are represented in blue. All numbers correspond to the ancestral avian karyotype.



	1	2	3	4	5	6	7	8	9	10	Z
Mammals	⬜	⬜	⬜	🟩	⬜	⬜	⬜	⬜	⬜	⬜	⬜
Turtles	🟩	🟩	🟩	🟩	🟩	⬜	⬜	⬜	⬜	⬜	🟩
Rhea	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	⬜
Emu	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜	🟩
Greylag Goose	🟩	🟩	🟩	🟦 A	🟩	🟩	🟩	🟩	🟩	🟦	⬜
Swan Goose	🟩	🟩	🟩	🟦 A	🟩	🟩	🟩	🟩	🟩	🟦	⬜
Mallard	🟩	🟩	🟩	🟩	🟨	🟨	🟨	🟨	🟩	🟩	🟨
Muscovy	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟨
Chachalaca	🟩	🟩	🟩	🟩	🟩	🟦	🟩	🟦	🟩	🟩	🟨
Guinea fowl	🟩	🟩	🟩	🟦 D	🟩	🟦	🟦	⬜	🟦 D	🟩	🟨
Californian Quail	🟩	🟥	🟩	🟩	🟩	⬜	⬜	⬜	⬜	🟩	🟨
Chinese Bamboo Partridge	🟩	🟩	🟩	🟦	🟩	🟩	🟩	🟩	🟩	🟦	⬜
Chicken	🟩	🟩	🟩	🟦	🟩	🟩	🟩	🟩	🟩	🟦	🟩
Red Legged Partridge	🟩	🟩	🟩	🟦 B	🟩	🟩	🟩	🟩	🟩	🟦 B	⬜
Blue Breasted Quail	🟩	🟨 A	🟩	🟦	🟩	🟩	🟩	🟩	🟩	🟦	⬜
Japanese Quail	🟨 A	🟨 A	🟩	🟦 A	🟩	🟩	🟨 A	🟨 A	🟩	🟩	⬜
Peafowl	🟩	🟩	🟩	🟦	🟩	⬜	🟥	🟥	🟥	🟦	⬜
Turkey	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟨 A	🟩	🟩	🟩
Pheasants	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜
Capercaillie	🟩	🟥	🟩	🟩	🟩	🟦	⬜	🟦	⬜	🟩	⬜
Great Grey Owl	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜
Eagle Owl	🟥	🟦	🟩	🟦	🟦 C	🟩	🟩	🟩	🟩	🟦	⬜
Zebra Finch	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Chaffinch	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Blackbird	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜
Redwing	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
Pigeon	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
African Collared Dove	🟩	🟩	🟩	🟦	🟩	🟦 C	🟦 C	🟦 C	🟦 C	🟦	⬜
Californian condor	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	⬜
Harpy Eagle	🟥	🟥	🟥	🟩	🟥	🟩	⬜	⬜	⬜	🟩	🟩
Rueppells Vulture	🟥	🟥	🟥	🟩	🟥	🟦 C	🟦 C	🟩	🟦 C	🟩	⬜
Griffon Vulture	🟥	🟥	🟥	🟩	🟥	🟦 C	🟦 C	🟩	🟦 C	🟩	⬜
Bearded Vulture	🟥	🟥	🟥	🟩	🟥	🟦 C	🟦 C	🟩	🟦 C	🟩	⬜
Peach faced lovebird	🟥	🟥	🟦	🟩	🟦	🟦	🟦	🟦	🟦	🟦	⬜
Cockatiel	🟥	🟨 A	🟨 A	🟨	🟩	🟦 A	🟦 A	🟦	🟦	🟩	⬜
Budgerigar	🟥	🟨 A	🟩	🟩	🟦	🟥	🟦	🟦 A	🟦	🟦	⬜

🟩 Ancestral chromosome
🟨 Ancestral chromosome, with rearrangments
🟦 Fusion of ancestral chromosomes
🟥 Fission of ancestral chromosome
⬜ No Data

A – Pericentric inversion
B – Centromere repositioning
C – Unknown fusion
D – Fusion of ancestral 4 and 9

Figure 1.8: A representation of rearrangements from the ancestral macrochromosome karyotype in species thus far studied using comparative chromosome painting.

1.2.2. The First Divergence - Paleognathae

Modern day representatives of the Palaeognathae comprise only two extant orders; the flightless Struthioniformes (ratites, e.g. emu, ostrich, rhea, cassowary, kiwi) and the Tinamiformes (tinamous). One of the first successes of avian comparative painting was the confirmation of what had been expected from classical studies; at least for the macrochromosomes, synteny is remarkably conserved. This was revealed by experiments using chicken whole chromosome paints from chromosomes 1-9 + Z on emu (*Dromaius novaehollandiae*) metaphases (Shetty *et al.,* 1999). Only one chromosome showed evidence for an interchromosomal rearrangement, this being chicken chromosome 4 which in emu is represented by the ancestral chromosomes 4 and 10. A similar pattern was more recently noted by Guttenbach *et al.* (2003) in the American Rhea (*Rhea americana*). Given that the chickens share a very similar karyotype (at least for the macrochromosomes) with the ratites it seems clear that the first divergence was either not accompanied by a major autosomal change or was accompanied by a change in the smaller chromosomes that has yet to be discovered. The diploid number of 2n=80-82 in all extant ratites suggest the former to be more likely. A unique feature of the ratites is that they have homomorphic sex chromosomes, indicative of an ancestral autosomal origin and sex chromosome differentiation after the divergence of this group (Guttenbach *et al.,* 2003).

1.2.3. The Second Divergence - Galloanserae

The Galliformes are an order comprising the land fowl (i.e. grouse, pheasants, quails etc.) and contain the species for which the most genomic and sequencing information is available. Moreover, due to their status as agricultural birds, they are among the most studied avian orders in many other areas of science and, since the availability of chicken chromosome paints (Griffin *et al.,* 1999; Masabanda *et al.,* 2004), have been natural targets for comparative studies. Indeed the largest body of comparative genomic studies have been done with reference to this order. As mentioned, all birds examined other than the ratites have heteromorphic sex chromosomes. However there is no evidence that the divergence of the Galloanserae was accompanied by any other major chromosomal change (although isolated individual changes are apparent), due to the relatively stable chromosome number in the majority of species and the clearly established orthology of the macrochromosomes.

Chromosome number appears conserved and ranges from 2n=78 (chicken) to 2n=82 (golden pheasant, *Chrysolophus pictus* (Guttenbach *et al.,* 2003)). The majority of the changes from the ancestral form are found in chromosomes 2 and 4. Chromosome 2 is represented as two separate telocentric chromosomes (3 and 6) in the five species of pheasant, turkey (*Meleagris gallapavo*) and California quail (*Callipepla californica*). It is also represented as two telocentric chromosomes (3 and 7) in the capercaillie (*Tetrao urogallus*).

The ancestral chromosome 4 is conserved intact in all the Galliformes and indeed most birds, albeit fused to smaller chromosomes on certain occasions. In the guinea fowl (*Numidea meleagris*), a fusion has occurred between it and ancestral chromosome 9 (Shibusawa *et al.*, 2002). However the more common fusion is between ancestral chromosome 4 and ancestral chromosome 10; this is seen in chicken, the partridges; peafowl and two quail species (Blue breasted and Japanese; Shibusawa *et al.*, 2004). The pheasants, capercaillie, turkey, California quail and chachalaca (*Ortalis vetula*) all show the chicken 4p arm hybridising to ancestral chromosome 10 although it is usually described as an unassigned microchromosome. Molecular evidence (Hillier *et al.,* 2004) has suggested that ancestral chromosome 10, when it appears as chicken chromosome 4p, still retains the properties (e.g. gene density, recombination rate, CpG island distribution) of the smaller chromosome it once, was despite the fusion.

In addition to the rearrangements of chromosomes 2 and 4, there are only four other interchromosomal changes detected to date for the remainder of the karyotype. In Guinea fowl (*Numidea meleagris*), a fusion has occurred between ancestral chromosomes 6 and 7; the capercaillie shows a fusion of ancestral chromosomes 6 and 8; and the common peafowl (*Pavo cristatus*) has both a fusion of ancestral chromosomes 8 and 9 and fusion of ancestral chromosome 7 to a microchromosome.

With regard to interchromosomal rearrangements, in the Japanese quail (*Coturnix japonica*), pericentric inversions have occurred between it and the ancestral type for chromosomes 1 and 2. An apparent pericentric inversion in Red-Legged Partridge (*Alectoris rufa*) has been revealed by Kasai *et al.* (2003) using comparative BAC mapping to in fact be the repositioning of the centromere to the p terminus. The reported pericentric inversion in the Blue Breasted and Japanese quails should be viewed with caution therefore until further BAC studies are performed. One rearrangement that is probably a pericentric inversion however is the ancestral chromosome 8 which is metacentric in chicken and Chinese bamboo partridge but telocentric in other birds, confirmed by BAC mapping experiments on turkey chromosomes (Griffin *et al.,* 2008).

On a related theme, recent studies of lampbrush chromosomes suggest that, while chicken microchromosomes tend to be telocentric, Japanese quail microchromosomes are all metacentric (Galkina *et al.*, 2006). The mechanism by which this occurred (i.e. pericentric inversion, translocation or centromere relocation) remains unknown.

The Anseriformes are the nearest extant relatives to the Galliformes, diverging 90-96 million years ago, and the only other extant order from the second divergence. Among them the Greylag goose (*Anser anser*) studied by Guttenbach *et al.* (2003) shows a fusion of the ancestral 4 and 10 - an identical pattern to that seen in chicken. The swan goose (*Anser cygnoides*) studied by Jaszczak *et al.* (2002) shows evidence of rearrangements on chromosome 4, having a metacentric chromosome pair. Though painting data is not yet available to confirm that this is the ancestral 4, the accepted diploid number of 80, equal to that of the Greylag, plus the ease of hybridisation with the Greylag suggest conservation of the ancestral form as well. In the mallard duck (*Anas platyrhynchos*) cross-species chromosome painting of the macrochromosomes (Schmid *et al.,* 2000, 2005) has confirmed results of earlier G-banding studies (Denjean *et al.,* 1997; Schmid *et al.,* 2000), which suggested that there is only one interchromosomal difference between the chicken and duck karyotypes - the retention of the ancestral chromosomes 4 and 10 in duck.. Differences in chromosome morphology (Denjean *et al.,* 1997) suggested that there are also intrachromosomal rearrangements between APL5-8 and their chicken orthologues. A further FISH mapping of 57 chicken BACs revealed small intrachromosomal rearrangements in APL2, 7, 8 and Z and confirmed synteny for the orthologues of GGA9, 11, 13-15, 18 and 28 in the duck genome (Fillon *et al.,* 2007). The Muscovy duck (*Cairina* *moschata*) has not yet been painted with chicken chromosome paints, however banding studies by Denjean *et al.* (1997) showed few rearrangements in the macrochromosomes, and the diploid number is believed to be the same (2n=80).

1.2.4. The Third Divergence – The Neoaves

The final divergence is not one clear split into the "higher land" and "higher water" birds; rather, there are numerous lineages whose branching orders have yet to be fully determined. Nonetheless, phylogenomic studies have revealed a clear sister relationship between the Passeriformes, Falconiformes and Psittaciformes, and a distinction between shore birds and water birds (Hackett *et al.*, 2008). Following this event, however, many birds on all sides of the divide clearly underwent a series of microchromosomal fusions and, to a smaller extent, macrochromosomal fissions with a net result of fewer chromosomes in the karyotype. In other words, a tendency to reduce the chromosome number has been an independent, convergent event happening in several unrelated families and orders significantly after the last major divergence of the birds. As mentioned, 24% of studied birds have an average of 2n=66-74; the Laridae (gulls and terns) the Pelecaniformes (pelicans etc.) and the Psittaciformes (parrot family). Perhaps the most striking example however is seen in the Falconiformes (e.g. vultures, falcons, hawks, eagles etc.) on which the most cross-species FISH studies have been performed.

1.2.5. Karyotype Evolution Within Specific Orders

1.2.5.1. Falconiformes (and Cicconiformes)

Falconiformes examined to date have a low chromosome number (ranging from 2n=50 (American Kestrel, *Falco sparberius*) to 2n=68 (Red tailed Hawk, *Buteo jamaicensis*)) and an atypical chromosome morphology suggestive of several fissions and fusions among both the macro- and microchromosomes. The Cicconiformes (New World vultures) were formerly classed as part of the Falconiformes, but are now separated following a divergence approximately 75 million years ago (van Tuinen and Hedges 2001). They have 2n=80, and are thus closer to the ancestral avian karyotype.

Cytogenetic findings therefore along with other lines of evidence suggest that members of the Accipitridae family including old world vultures, eagles, hawks and kites are more closely related to one another than they are to the new world vultures, and that the major chromosomal changes are characteristic of the Accipitridae rather than the Falconiformes as a whole. Accipitridae that have been studied by comparative painting are the Griffon vulture (*Gyps fulvus*), Rüppell's vulture (*G. rueppellii*) and Bearded Vulture *(Gypaetus barbatus*) by de Olivera *et al.* (2005) and the Harpy Eagle (*Harpia harpyja*) by Bed'Hom *et al.* (2003). There are no large macrochromosomes in these birds, rather ~25 pairs of medium sized chromosomes and 4-6 pairs of microchromosomes suggesting frequent and whole scale microchromosomal fusion; the black-winged kite (*Elanus caeruleus)* has only a single microchromosome pair (2n=68). There are also several fissions of the larger chromosomes apparent when chicken whole chromosome paints are applied to these species; chicken chromosome 1-5 paints show extensive rearrangements; for example chicken chromosome 1 hybridises to 6 separate chromosomes ranging in size from 7 to 22 in *G. fulvus* and *G. rueppelli*; to 4 chromosomes in *G. barbatrus* ranging from 7 to 12 and 5 chromosomes ranging from 5 to 24 in the Harpy Eagle. In contrast, where data is available, chromosomes 6-10 hybridise only to a single chromosome or a larger, fused chromosome. An apparent exception is chromosome 4. The chicken chromosome 4 paint hybridises to only 2 chromosomes in all four species, a larger (~1-4) and a smaller (~13-16) suggestive of conservation of the ancestral karyotype (de Olivera *et al.*, 2005, Bed'Hom *et al.*, 2003)

Among the Ciconiiformes the best studied example is the California Condor (*Gymnogyps californianus*), which is also thought to have one of the largest wingspans of any North American bird (Raudsepp *et al.*, 2002, Stoms *et al.*, 1993). Raudsepp *et al.* (2002) found few rearrangements with chicken. A GGA2 paint hybridises to chromosome 2 of this species with weak cross hybridisation to chromosome 3. Moreover, the chicken chromosome 3 paint hybridises to California condor chromosome 3 with a weak signal on chromosome 2. Chromosome 4 has a p arm and is therefore sub-metacentric, however the chicken chromosome 4 paint detects two chromosomes (4 and 9 – presumably the ancestral 4 and 10) in the California condor suggestive of a pericentric inversion or centromere relocation on the California condor chromosome 4.

1.2.5.2. Passeriformes

The Passeriformes are the largest avian order, comprising more than half of all known bird species (IUCN Red List, 2008). The most studied is the zebra finch (*Taeniopygia guttata*), an emerging model organism for study of many issues relevant to human health and disease mainly because of its ability to communicate via complex learned vocalisations. The zebra finch has been used as a model species for sex differences in neural structure and function, influences of steroid hormones on neural networks, adult neurogenesis, steroid hormone synthesis in the brain, the neural basis for learning and complex auditory processing and auditory-motor integration. The zebra finch is also the second bird (after chicken) to have a complete genome sequencing effort. The sequence assembly is, at the time of writing, being finalised and validated via physical mapping of zebra finch BAC clones. These indicate that the assembly is largely correct, with only minor inconsistencies to the cytogenetic map (M. Völker, personal communication). Comparative genomics has also been carried out on the chaffinch, redwing and blackbird. Chicken chromosome paints 1-10 and Z reveal a few distinct rearrangements. The redwing (*Turdus iliacus*) and blackbird (*Turdus merula*) belong to the family Turdidae, and both display a fission of the ancestral chromosome 1 near or at the centromere. The chaffinch (*Fringilla coelebs*) is in the family Fringillidae; it and the zebra finch (Estrildidae) show a similar fission. Ancestral chromosome 4 is conserved in all four birds; however, due to the chromosome 1 fission it is referred to as chromosome 5 in the chaffinch and zebra finch.

1.2.5.3. Strigiformes and Columbiformes (Owls and Doves)

The Great Grey Owl (*Strix nebulosa*) and the Eagle Owl (*Bubo bubo*) from the family Strigidae were studied by Schmid *et al.* (2000) and Guttenbach *et al.* (2003) respectively. There are no interchromosomal changes from the ancestral form in the Great Grey Owl. The Eagle Owl however shows a similar fission to that seen in the Turdidae i.e. that of ancestral chromosome 1. The chicken (ancestral) chromosome 2 paint hybridises to the long arm of the largest chromosome in the Eagle Owl with the short arm of the same chromosome orthologous to ancestral chromosome 4. Finally, the ancestral 5 has undergone a fission event in Eagle Owl and split to a macro- and microchromosome.

Among the Columbiformes, the Pigeon (*Columbia livia*) retains the ancestral karyotype (Derjusheva *et al.*, 2004), but the African collared dove *(Streptopelia roseogrisea*) has a fusion of ancestral 4 and 10 (the same as chicken and goose), as well as two fusions of ancestral chromosomes 6, 7, 8 and 9 forming two larger macrochromosomes.

1.2.5.4. Psittaciformes

Like the Falconiformes, the Psittactiformes (parrots and cockatoos) contain some of the most extensive chromosomal rearrangements seen in birds. The exact divergence time of the parrot lineage is not fully understood, though mitochondrial estimates place the Galliform/Pssitaciform split at ~120Mya. Chromosome painting has been performed in three species of parrot (*Agapornis roseicollis* (peach-faced lovebird, 2n=48); *Nymphicus hollandicus* (cockatiel, 2n=72) and *Melopsittacus undulatus* (budgerigar, 2n=62)) by Nanda *et al.* (2007). They demonstrated large scale fusions and fissions in the three species, for example the apparent fission of ancestral chromosome one into two chromosomes in each of the three species. However, the size and morphology of the derived chromosomes suggests further rearrangements have occurred independently within each species lineage. An example of this can be seen in *A. roseicollis*, which has undergone a fusion of the ancestral chromosomes six and seven, and a microchromosome. Dual painting with chicken paints for six and seven reveals a "striped" pattern (Figure 1.9) which Nanda *et al.* (2007) attribute to a paracentric inversion following the fusion.

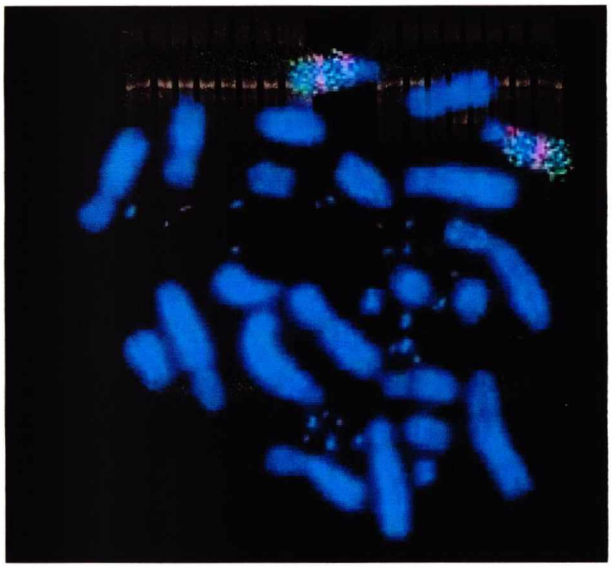


Figure 1.9: Chicken paints for GGA6 and 7 on peach-faced lovebird (*Agapornis roseicollis*) chromosomes demonstrating a paracentric inversion. From Nanda *et al.* (2007).

1.2.5.5. Charadriiformes

In a recent study, Nie *et al.* (2009) used reciprocal chromosome painting to characterise the chromosomal differences between chicken and stone curlew – one of the birds with the smallest chromosome numbers (*Burhinus oedicnemus*), 2n=42. The data suggested that the larger ancestral chromosomes (1-5) had not undergone rearrangements, the reduction in chromosome number being mediated by fusions of the smaller chromosomes - including the recurrent fusion of ancestral 4 and 10. This contrasts with the fissions of the macrochromosomes seen amongst the Falconiformes.

1.2.6. Chromosome 4

Despite the overall karyotypic conservation, apparently independent convergent changes (homoplasy) seem to have occurred during avian karyotype evolution; for instance both ancestral chromosomes 1 and 2 have displayed individual fission events around the centromere (in the Turdidae and the Eagle owl for chromosome 1 and in the California quail and the turkey/pheasant group for chromosome 2). Especially striking, however, are the multiple observed instances of fusion between the ancestral chromosomes 4 and 10. This contrasts with the conserved synteny of the ancestral chromosome 4 over 310 Myrs (Hillier *et al.*, 2004).

The question raised by these observations is whether they represent multiple independent fusions (i.e. homoplasy), a small number of fusions and multiple fissions, or another explanation, such as hemiplasy (Avise and Robinson, 2008). The evidence thus far suggests that the fusions/fissions are centric; this is certainly the case when comparing chicken and turkey, shown via the approaches of BAC mapping and by the application of turkey chromosome paints to chicken microarrays and lampbrush chromosomes (Griffin *et al.*, 2008).

A particularly fragile region of the genome (in this case probably the centromere) might explain multiple fissions; however a genomic reason as to why these two chromosomes might be prone to fusions is more complex. Similar centromeric sequences in the two chromosomes might provide one explanation, as might the proximity of the chromosomes in a germ line cell nucleus. In mammals, chromosomal rearrangements are thought to be facilitated by the presence of segmental duplications (Kehrer-Sawatzki and Cooper, 2008). Patterns of karyotype evolution can also be influenced by meiotic drive – this is thought to drive mammalian karyotype evolution through non-random segregation of chromosome fusion or fission products during female meiosis (Pardo-Manuel de Vilena and Sapienza, 2001). The preferential segregation during metaphase of higher centromere numbers to the oocyte rather than to the polar body provides a mechanism favouring chromosome fusions. Meiotic drive seems to occur in chicken, where the larger number of centromeres is preferentially segregated to the polar body (Dinkel *et al.*, 1979). This would appear to favour fissions. However, Pardo-Manuel de Vilena and Sapienza (2001) point out that spindle polarity may reverse over short time scales – for example, the change from acrocentric to metacentric karyotypes over 5-10,000 years in mice (Nachman *et al.*, 1994). Further evidence is required to determine whether such mechanisms have been a feature of avian karyotype evolution.

1.2.7. Telomeres in Avian Evolution

Nanda *et al.* (2002) used FISH to study the distribution of telomeric sequences in 16 different bird species, and showed an enrichment of telomeric DNA on microchromosomes compared with the macrochromosomes. This pattern of centric and interstitial sequence in addition to chromosome ends has been found in chicken and turkey (Galliformes), Bell's vireo (*Vireo bellii*; Passeriformes), red tailed hawk (*Buteo jamaicensis*; Falconiformes) and Inca dove *(Columbina inca*; Columbiformes . The California condor, studied by Raudsepp *et al.* (2002), in contrast, showed no interstitial hybridisation sites, similar to the house sparrow (*Passer domesticus*; Passeriformes), and lesser adjutant stork (*Leptoptilos javanicus*; Cicconiformes); signals were confined to chromosome ends. This pattern was also seen in two vultures, *Gyps fulvus* and *G. barbatrus* (Nanda *et al.*, 2006), as well as in the black-winged kite, *Elanus caeruleus* (Bed'Hom *et al.*, 2003). When compared to the macrochromosomes, telomere signals were stronger on the microchromosomes in all of the studied bird species, with the strongest signals on the smallest chromosomes. This signifies higher numbers of telomeric repeats, and it is intriguing that, although there are a low proportion of repeats in avian genomes overall, the abundance of telomeric sequences appears higher than in, for example, mammalian genomes. Despite the chicken genome being only about one-third of the size of the human genome, the proportion of telomeric sequences is much greater by about 5-10 times, i.e. 4% of the chicken genome contains telomeric sequences (Schmid *et al.*, 2000). There are three classes of telomere arrays in birds (see section 1.1.3.1; Delany, 2000), of which the third can range from hundreds of kilobases to up to 2Mb. These are the largest telomere arrays so far described for any organism (Schmid *et al.*, 2000).

One suggestion to explain patterns of telomere length is that these serve as caps to protect the gene dense microchromosomes from telomere erosion (Delany *et al.*, 2003). Additionally, birds tend to be longer lived than mammals of equivalent size (Calder, 1990), despite having 1.9-3.7 fold greater lifetime energy expenditures (Jurgens and Prothero, 1991). Haussmann *et al.* (2005) demonstrated that telomere length is correlated with survival early in life in tree swallows, and Haussmann and Mauck (2008) found that storm petrels with longer telomeres tended to live longer than those with short telomeres; and that telomere length was heritable. They also found that the longest lived storm petrels showed no sign of telomere shortening; that is, either telomeres were not being degraded (a sign of efficient protection from oxidative damage) or were being regenerated (plausible, given high levels of telomerase expression in these birds throughout their lives (Haussmann *et al.*, 2007)). Hence, a selection mechanism may exist to maintain or increase telomere length in birds.

It is still uncertain whether the interstitial telomere arrays on the larger chromosomes reflect ancient fusion points of smaller microchromosomes. Nanda *et al.* (2006) did not find any interstitial telomeres in the Old World Vultures, despite the extensive fusions, and suggested that in these birds old non-functional telomeres were not retained. Furthermore, Nanda et *al.* (2007) found few interstitial telomeres in parrots; those they saw, they suggest to be constitutive heterochromatin of coincidentally similar sequence. An alternate hypothesis that interstitial telomeres aid crossing over in meiosis has not been confirmed; indeed, there is no overlap between chicken interstitial telomeres and recombination hot-spots (Galkina *et al.*, 2005).

1.3. Nuclear Genome Organisation

While the term 'genome organisation' can be considered at a number of levels, nuclear genome organisation (or nuclear organisation) is concerned with the location of specific genes, genomic regions (or nuclear proteins) or entire chromosomes at particular nuclear positions at a set phase or time in development and with changes thereof. Although gene expression is regulated at many levels, the effect of nuclear organisation on the genome is possibly one of the least understood areas of genomics.

1.3.1. Chromosome Territories

Differences in chromatin distribution in interphase nuclei between different cell types were first noted by Flemming in 1882, and in 1885 the Austrian anatomist Carl Rabl suggested that the numbers of chromosomes were consistent within tissues through cell divisions. He proposed that in plants, chromosomes were organised such that centromeres were close to the spindle pole, while telomeres were attached to the nuclear envelope on the other side of the nucleus – in essence, a continuation of an anaphase arrangement through interphase. Though described in many cell types (Marshall *et al.,* 1997), the Rabl configuration does not appear to be common in mammals or other vertebrates.

The idea that all chromosomes occupy distinct regions within an interphase nucleus was first suggested circumstantially by experiments in the 1970s and 1980s; Stack *et al.,* (1977) describe visualising chromosomes in Chinese Hamster Ovary (CHO) cells and plants during interphase after using a modified Giemsa staining technique. Following these early experiments, research has progressed to suggesting roles for chromosome positioning in disease, development and evolution.

The nuclear location of individual chromosome territories appears to be related to their accessibility to various nuclear machineries (for example transcription factories). The subsequent effects on nuclear function has implications for the whole of developmental and cell biology (Cremer and Cremer 2001). Genome organisation is thus thought to be responsible for (or at least strongly correlated to) large-scale regulation of transcription and the mediation of normal and abnormal cellular function.

It has been shown that whole chromosomes can change their nuclear position; the first suggestion of a non-random positioning of a chromosome was the sex chromatin body (the inactive X chromosome at interphase) (Barr & Bertram 1949, Comings 1968 cited in Greaves *et al.,* 2003). The inactive X condenses and moves towards the nuclear membrane (Manuelidis 1990), ordering itself such that the expressed genes escaping inactivation are on the periphery of the territory, and the remainder are in the inactive core (Clemson *et al.,* 2006). More recently, Foster *et al.* (2005) reported the shift of sex chromosomes towards the interior of porcine spermatids in primary spermatocytes, suggesting a possible role in epigenetic control or regulation of paternal gene expression in the embryo. This repositioning of chromosomes during mammalian spermatogenesis appears to be common; an interior relocation of autosomal centromeres has been observed in human and murine spermatogenesis (Foster *et al.,* 2005; Turner *et al.,* 2006; Zalensky and Zalenskaya, 2007).

Perturbations in nuclear organisation have been associated with different cell types, states and with disease (Foster and Bridger, 2005). In some laminopathies and in progeroid cells, chromosome organisation appears compromised (Meaburn, 2007). Unsurprisingly, the position of a chromosome, and hence its neighbours, play a large role in determining the translocations that follow radiation damage (Caddle *et al.,* 2007), and probably other causes of DNA strand breaks (Gandhi *et al.,* 2008).

1.3.2. Chromosome Territory Structure

Although chromosomes, as observed in humans, occupy distinct territories, there is evidence that adjacent chromosomes may intermingle to some extent. Chromatin from adjacent chromosomes map overlap up to 46% (Branco and Pombo, 2006) and the degree of intermingling appears to correlate with the frequency of translocations (Ghandi *et al.,* 2008). Even where chromosomes do no intermingle, individual loci may come together, by chance or design, termed chromosome kissing (Cavalli, 2007). Chromosome kissing is thought to have implications for both chromosomal rearrangements and for gene regulation, as specific parts of those chromosomes can interact to form functional domains (Cockell, 1999, Croft *et al.*, 1999).

One model for how chromosome territories may be constructed suggests that there may be an interchromatin compartment in which the chromosome territory surface contains invaginations permitting the penetration of transcription factors and other gene expression machinery (Cremer *et al.,* 2006). Another, the lattice model, suggests more extensive intermingling of peripheral chromatin fibres from adjacent territories (Branco and Pombo, 2007). In a broad sense, the interchromatin model postulates bringing the transcription factors to the genes, while the lattice model suggests bringing the genes to the transcription factors (Heard and Bickmore, 2007).

Interphase chromosomes are secured in their respective positions by nuclear tethering. There is general agreement that these attachments are to the nuclear lamina and nuclear matrix, although a minority opinion is that they involve some ill-defined internal nuclear structure (Pederson 2004).

1.3.3. Models for Nuclear Organisation

Of the several models for how chromosomes can be organised in the nucleus, two appear to predominate, at least in vertebrates. In the first, a gene-density based model, chromosome position is determined by relative gene density (with the gene dense chromosomes towards the centre of the nucleus). Human lymphocytes for example follow a gene density related organisational pattern (Croft *et al.*, 1999). In the second model, a chromosome size based distribution, the larger chromosomes are found towards the nuclear periphery and the smaller chromosomes towards the nuclear centre. Such organisation has been reported for quiescent human fibroblasts (Bolzer *et al.*, 2005, Bridger and Bickmore, 1998, Sun *et al.,* 2000). It is thought that cells with elliptical nuclei may be more prone to a chromosome size related organisation, while cells with spherical nuclei may be more prone to gene-density based organisation (Bolzer *et al.,* 2005).

There are also several examples of chromosome position changing during cell differentiation and/or disease. For instance, repositioning of the X chromosome has been seen in neurons in epilepsy sufferers (Borden and Manuelidis, 1988), the repositioning of the sex chromosomes and the centromeres of the autosomes towards the nuclear centre has been reported in mammalian spermatogenesis (Foster *et al.*, 2005; Turner *et al.*, 2006; Zalensky and Zalenskaya, 2007). An extreme rearrangement has been reported in rod cells of nocturnal mammals, in which heterochromatin localises to the nuclear centre and euchromatin to the nuclear periphery. It is thought that this configuration acts as a lens, channelling light towards the light-sensing segments more efficiently than the conventional pattern found in diurnal mammals (Solovei *et al.*, 2009).

1.3.4. Nuclear Organisation in Birds

Studies of nuclear organisation in birds are extremely limited, in part due to the comparatively recent development of chromosome specific probes and elucidation of even one full bird karyotype (Masabanda *et al.,* 2004). Nonetheless, since the development of these resources, some insight has been given into avian nuclear organisation.

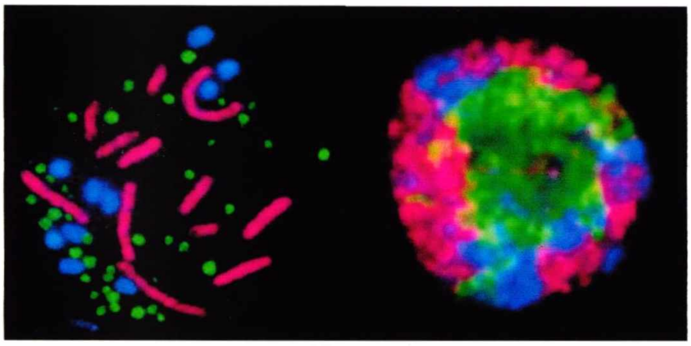


Figure 1.10: Chromosome paints for larger (red) and smaller (blue) macrochromosomes and for microchromosomes (green) on metaphase (left) and interphase (right). From Habermann *et al.* (2001).

Habermann *et al.* (2001) conducted the first detailed two dimensional study and three dimensional reconstruction of chromosome territories in chicken fibroblasts and neurons. They used whole chromosome paints for GGA1-10 and Z, and 19 pairs of microchromosomes (from 14Mb to 4Mb). In both cell types, the largest chromosomes GGA1-5 and Z, plus the smaller chromosomes GGA6-10 were predominantly found at the periphery of the nucleus, while the microchromosomes formed clusters, mainly towards the centre of the nucleus (though some microchromosomes appeared towards the periphery of the nucleus, between the macrochromosome territories). Of course, given that the smaller chromosomes are also more gene rich, this arrangement potentially fits both the size-related and the gene density related models. In total 21 neuronal nuclei and 28 fibroblast nuclei were analysed. In neurons, chromosomes 1-5 and Z were peripheral, while 6-10 shifted slightly towards the centre. The microchromosome territories were central in both cell types, though were more peripheral in the neurons than in the fibroblasts. From this they suggested that this radial arrangement may be a common motif in all chicken cell types.

Federico *et al.* (2005) used GC rich and GC poor isochores from chicken to probe Falconiformes, which have a very different karyotype structure (e.g. de Olivera *et al.*, 2005, Nanda *et al.*, 2006). They found that the GC rich isochores were internal at interphase and that the GC poor isochores were more peripheral in both chicken and falcons. In this group, it appears that a gene density based organisation may be more dominant, though no individual chromosome position information is available.

There is evidence that chromosome position is cell type specific. Studies of chicken sperm heads indicate that most chromosomes do not appear to adopt a non-random position as they do in somatic cells (Solovei *et al.*, 1998; Tsend-Ayush *et al.*, 2008). This is in stark contrast with the situation in the sperm of monotremes, marsupials and placental mammals, where nuclear organisation seems to be more ordered (e.g. Greaves *et al.*, 2001, 2003; Zalensky and Zalenskaya, 2004).

These studies so far suggest that there is some evolutionary conservation of nuclear organisation between birds and mammals; there is certainly evidence that such conservation exists across the 90 million years between human and pig (*Sus scrofa*). Observations have been made suggesting the gene-dense regions of pig cell nuclei correspond to a more internal position (Federico *et al.*, 2004). The argument can be made that if conservation is seen in mammals across this timescale, then it should also be seen in birds, where genome conservation seems to be generally higher. Chicken is commonly described in the literature, however, as an example of a species fitting both the chromosome size and gene density models (e.g. Foster and Bridger, 2005, Hepperger *et al.*, 2008). Further information is therefore needed at a higher resolution in order to determine whether one pattern or the other is dominant.

1.3.5. Positioning of individual loci

Substantial research has shown that individual gene loci alter their nuclear position based on transcriptional status. For example, a study in mouse embryonic stem cells demonstrated movement of the developmentally activated gene *Mash1* from the nuclear periphery or from pericentromeric heterochromatin towards the interior of the nucleus (Williams *et al.*, 2006). Genes for immunoglobulins IgH and IgK, during murine lymphocyte development, also reposition to the interior of the nucleus on activation (Kosak, 2002). The unrelated genes *CFTR*, *GASZ* and *CORTBP2* associate with heterochromatin and the nuclear periphery in a number of different human cell types when repressed, and with euchromatin at the nuclear interior when activated (Zink *et al.*, 2004). Silenced genes have been demonstrated to migrate to centromeric clusters during shutdown, while active genes appear to migrate to "transcription factories" (identified by RNA polymerase II) and dynamically co-localise during transcription (Osborne *et al.*, 2004).

Similarly, other loci have been seen to move to the nuclear periphery upon transcriptional silencing. As an experimental test of this, whole chromosome territories were repositioned to the nuclear lamina in human HT1080 cells (Finlan *et al.*, 2008). Reduced expression was seen for some, though not all, genes on the repositioned chromosomes. This indicates that there is not a direct causal link between nuclear position and transcriptional status of any given gene; Kozubek *et al.*, (2002) highlighted that some chromosomes have a more internal location than others but this was not necessarily related to transcription within these cells. More recently, Morey *et al.* (2009) induced activation of Hox loci, which then loop out from their respective chromosome territories. Flanking genes, also relocalised, did not show changes in expression levels. However, inactive Hox alleles tended to remain within the chromosome territory. These data support the emerging view that locus position is a consequence, not a cause, of gene expression levels.

Indeed, in some cases, association with the nuclear periphery appears to favour gene activation – for example, in fission yeast, *Schizosaccharomyces pombe*, the *INO1* locus relocates to the nuclear periphery, where an integral membrane protein is required for gene activation (Brickner *et al.*, 2004). In budding yeast, *Saccharomyces pombe*, the *HKX1* locus associates with nuclear pore complexes upon activation (Taddei *et al.*, 2006). During erythroid differentiation in mouse, the β-globin locus is activated at a low level at the nuclear periphery, then re-localises to the interior for full activation (Ragoczy, 2006). In mouse T-helper (Th1) cells, the IFN-γ gene is constitutively associated with the nuclear periphery, irrespective of expression state; it is thought that, in this case, transcriptional regulators, as opposed to the actual genes, may be undergoing relocation (Hewitt *et al.*, 2004). Other genes are unaffected by radial position within the nucleus – for example, the *PLP* gene in oligodendrocyte differentiation (Nielson et al., 2002). Therefore, it appears that while the nuclear periphery is not necessarily inhibitory to transcription, it can be significantly involved in, and even required for, repressing certain genes (Ruault *et al.*, 2008, Deniaud and Bickmore, 2008).

Further evidence suggests that a more direct functional interaction exists between locus position and constitutive or facultative heterochromatin. A study in mouse B lymphocytes found transcriptionally inactive genes, but not transcriptionally active genes associated with Ikaros-heterochromatin foci (Brown, 1997). Ikaros is a transcription activator and repressor involved in the development of a number of hematopoetic cell types (Georgeopolous, 2002). It is found associated with heterochromatin in interphase nuclei (Brown *et al.*, 1997). Further studies in mouse suggested that genes reposition as they pass through stages of the cell cycle (Brown *et al.*, 1999). Transcriptionally inactive genes were recruited to centromeric heterochromatin in cycling primary B lymphocytes as the cells prepared for division; this behaviour was not observed in quiescent lymphocytes (Brown *et al.*, 1997). Other cytokine genes are positioned away from heterochromatin in resting murine T cell nuclei (Grogan *et al.*, 2001). Similarly, the mediator of a family of mammalian transcriptional repressors, KAP1, has been seen to colocalise with repressed genes and pericentric heterochromatin (Briers *et al.*, 2009).

Most recently, *in vivo* imaging technologies have allowed the tracking of chromatin domains surrounding induced gene loci. These have shown the unfolding of chromatin fibres following gene activation (Hu *et al.*, 2009). Such technological advances will undoubtedly assist in answering questions in this field that remain outstanding – for example, the relationship between chromosomal positioning and locus positioning. Little data is available in birds, and that only for loci on macrochromosomes (Stadler *et al.*, 2004), yet the size of the microchromosomes might imply that entire chromosome territories become involved in changes of locus position.

1.4. Chicken Genomics

Many mammalian genomes are either completely sequenced or are in the advanced stages of being mapped (137 mammalian genome projects listed on the Genomes Online (GOLD) database as of January 2009; Liolios *et al.*, 2008), one of the most recently completed being the duck-billed platypus, *Ornithorhynchus anatinus* (Warren *et al.*, 2008). Among avian species however, the chicken is so far the only one to be completely sequenced (though zebra finch (*Taeniopygia guttata*) is in the process of being sequenced and the raw sequence data is available for download and analysis (http://genome.wustl.edu/genome.cgi?GENOME=Taeniopygia%20guttata). The chicken provides a bridge across the evolutionary gap between mammals and other vertebrates and thus serves as the principal model for the approximately 10,000 avian species (Burt 2005). Birds share a common ancestor with theropod dinosaurs; hence chicken was the first non-mammalian amniote to have its genome sequenced. The reasons for its choice lies in the pivotal role it plays as a model species for human disease, in developmental biology, in the study of genome function and as an important agricultural animal. The chicken has been, and is, used as a model for many human diseases; for example musculoskeletal diseases such as muscular dystrophy (Yoshizawa *et al.*, 2004), infectious diseases such as salmonellosis (Shah *et al.*, 2005), cardiovascular diseases, autoimmunity, different types of cancer, as well as nervous system disorders such as retinal degeneration (Hirst *et al.*, 2001).

1.4.1. Genetic Mapping

A genetic (linkage) map is based on the segregation of markers in meiotic products, with distances in centiMorgans (cM). 1cM represents a recombination frequency of 1%, and can in principle rise to 50%, representing markers on separate chromosomes. The frequency with which two markers are transmitted together allows an estimation of the distance between them, and hence the construction of linkage maps.

In chicken, three populations have been used to construct genetic linkage maps. The East Lansing population (Crittenden *et al.*, 1993) consists of 52 BC1 animals derived from a backcross between a partially inbred JungleFowl line and a highly inbred White Leghorn line. The Compton population (Bumstead and Palyga, 1992) consists of 56 BC1 animals, derived from a backcross of two inbred White Leghorn lines that differed in their resistance to salmonella. The Wageningen population consists of 456 F2 animals from a cross between two broiler dam lines originating from the White Plymouth Rock breed (Groenen *et al.,* 1998). The number of informative meioses for the two backcross populations varies from 20-56 with an average mapping resolution of 5-7cM. In the Wageningen population the number of informative meioses varies from 15-886 resulting in a mapping resolution of 1cM (Groenen *et al.,* 1998). The three chicken linkage maps have since been integrated into one consensus linkage map (Groenen *et al.*, 2000).

Linkage mapping in other birds is less advanced, even in agriculturally important species. Huang *et al.* (2005) developed a linkage map in the duck based on 138 microsatellite markers, repeated motifs with high polymorphism found in all prokaryotes and eukaryotes, with some results repeated in Huang *et al. (*2006). This builds on the small amount of previous work in duck genomics; Maak *et al. (*2000) developed seven microsatellite markers in the Pekin, while Stai & Hughes (2003) characterised microsatellite loci in the domestic and wild Muscovy. What these mainly highlight is the paucity of information in comparison with the chicken.

Some comparative evidence from genetic mapping in the zebra finch and the great reed warbler suggests the chicken genetic map is unusually long in comparison to passerine birds; the zebra finch genetic map is only 60% that of the chicken (Hale *et al.,* 2008), and the reed warbler map is only 6-13% that of the chicken (Dawson *et al.,* 2007). However, the reasons for this are as yet unclear. Comparisons with more recent genetic maps e.g. in the ostrich (Huang *et al.,* 2008) and collared flycatcher (Backström *et al.,* 2008) may help to resolve this question.

An important utility of linkage maps lies in the identification of quantitative trait loci (QTL) (Burt *et al.,* 2005). QTL are traits of agricultural interest, determined by the different alleles carried by animal that affect phenotype – such as the behaviour of feather pecking in chickens (Keeling *et al.,* 2004), that affect the welfare of the birds, as well as more directly physical attributes. Research is being carried out into more than 200 QTLs in areas of disease susceptibility and resistance plus egg production and leanness (Masabanda *et al.*, 2004). QTL mapping benefits especially from comparative physical mapping, since it requires the integration of genetic and physical maps.

1.4.2. Physical Mapping of the Chicken Genome

A number of chicken BAC libraries have been constructed (Lee *et al.*, 2003, Liu *et al.*, 2003, Crooijmans *et al.*, 2000). A total of 57,091 clones, combined into 2331 contigs, from these libraries have been used for the construction of a physical map of the chicken genome (Ren *et al.*, 2003). Using the Wageningen BAC library, at least one BAC clone has been isolated for markers that have been mapped at 10cM intervals on the chicken linkage map (Crooijmans *et al.*, 2000). In order for the linkage map and cytogenetic maps to be integrated, BAC clones have been isolated with markers from almost every linkage group of the consensus linkage map. In total, more than 1500 BAC clones from the Wageningen BAC library have been isolated and assigned to a linkage group, which represents genome coverage of 5% (Crooijmans *et al.*, 2000).

All libraries that were ultimately used for the genome sequence assembly were prepared from DNA of a single female Red Junglefowl (RJF #256, last reported living in retirement on the campus of Michigan State University). The bird came from an inbred line (UCD 001) to minimize heterozygosity and to provide sequence coverage for both Z and W sex chromosomes. The chicken BAC-based physical map was developed in parallel with the sequence assembly.

1.4.3. Chicken Genome Sequencing

The first draft of the chicken genome was assembled using whole-genome sequencing strategies, including BAC, fosmid and plasmid paired-end reads (Hillier *et al.,* 2004). This approach was used in conjunction with the "whole genome shotgun sequencing" approach. The shotgun technique breaks the DNA into fragments ranging from 2kb to 150kb in length. This DNA library was read in 800bp lengths from either end of each fragment using an automated DNA sequencer, and assembled into contiguous sequences (contigs) *in silico*. The BAC-based physical map (20-fold clone coverage) enabled the genome sequence assembly to be knitted together. The BAC-map along with the genetic map provided the main scaffolding for the assembly into larger ordered and oriented groupings (ultracontigs) as the mechanism for chromosomal assignment (Burt, 2005).

This approach produced an assembly of approximately 1.05 gigabases, about one third the size of a typical mammalian genome. A 6.6x coverage draft sequence was achieved resulting in the following main observations: a nearly threefold difference in genome size between the chicken and mammalian genomes, which reflects lower interspersed repeat content, fewer pseudogenes and segmental duplications, and reduced intergenic distances within the chicken genome. It was shown that intrachromosomal rearrangements such as inversions are more common than translocations when long blocks of syntenic regions of the chicken-human genomic sequences are aligned. Alignment of the chicken and human genomes identifies at least 70 megabases (Mb) of sequence that is highly likely to be functional in both species. However, many of the chicken-human aligned non-coding sequences occur far from genes, in clusters that seem be selected for functions not yet fully understood (Hillier *et al.,* 2004).

The sex chromosomes were poorly represented in the final assembly. Unlike the rest of the genome, the W chromosome has a high repeat content, resulting in little sequence data; the current assembly (release 2.1) has only ~260kb assigned to a chromosome cytogenetically similar in size to GGA8 (assigned 30.7Mb). Targeted sequencing will be necessary to complete the assemblies of the sex chromosomes (Burt, 2005).

The autosomes' sequence coverage was 98%, based on overlaps with an independent set of BAC clones. Overlaps with cDNA clones suggested 5%-10% of genes were missing from the final assembly; the problem may have been due to gene duplications and GC-rich sequences. For example, the MHC region on chromosome 16, a rich source of duplicated genes, was inadequately represented. Further work to complete the chicken genome sequence to a high quality for comparative genomics and gene discovery is required (Burt, 2005).

The latest release of the chicken genome assembly has now anchored approximately 95% of the 1.05Gb genome to chromosomes 1-28, 32, Z, W and two additional linkage groups that have not been cytogenetically assigned. The data is available through the Ensembl database (www.ensembl.org/Gallus_gallus) (Hubbard *et al.,* 2009). Automated and curated genome annotation has been used for the identification and prediction of gene sequences. This has enabled many of the genes conserved between birds and mammals to be identified (Hillier *et al.,* 2004).

Comparison between the chicken genome and other vertebrate genomes can shed light on questions regarding gene gains/losses (Hillier *et al.,* 2004) – for example, whether a human gene with no homologue in chicken represents a gain in the human lineage or a loss in the chicken lineage. Comparisons carried out between human, chicken and Fugu (*Takifugu rubripes*) suggest that at least one third of genes are conserved in all vertebrates. Other evidence suggests that rates of gene loss were higher in the avian lineage and that there have been fewer gene duplications in birds (Burt 2005). Certain genes appear to have been lost from the chicken lineage, including vomeronasal receptors, caseins, and some genes of the immune system. On the other hand, birds have more keratins specific to feathers, and mammals have lost the avidin egg proteins (Burt 2005).

1.4.4. Microarray Technologies

The message that appears from comparative painting and mapping studies in birds is that the karyotypes of species studied to date are mainly highly conserved; despite this, there are difficulties in hybridising chromosome paints, and particularly smaller probes (e.g. BACs, YACs, cosmids), as the evolutionary distance increases. Consequently, attention is now focussing on higher resolution microarray based approaches. The different approaches available with microarray technology allow for broad investigations into differences in gene regulation within or between species, or indeed for differences in whole genome structure.

1.4.4.1. Expressed sequence tag (EST) and cDNA microarrays

A number of EST and cDNA arrays have been produced for chicken. Burnside *et al.* (2005) generated an array based on 11,447 cDNAs from immune tissue and a DT40 cell line ESTs. More global gene expression arrays have been used to investigate differences between different chicken lines (for example, fat versus lean, or fast-growing versus slow-growing) (Cogburn *et al.*, 2003). Another immune based array, containing known chicken ESTs as well as novel chicken immune related genes has been recently developed (Smith *et al.*, 2006).

1.4.4.2. Single nucleotide polymorphisms (SNP) microarrays

SNP arrays are now being widely using in human genome wide association studies, to identify SNPs associated with disease or phenotypic variation. Their use is being extended to other animals; for example, cows and pigs (Kamiński *et al.*, 2008). These arrays are also now being used for comparative genomic studies. Flynn & Carr (2007) interrogated a human SNP array with chimpanzee, gorilla and codfish mitochondrial DNA, recovering about 88% of the gorilla sequence, but only 4% of the codfish sequence – representing short conserved regions between primates and fishes. To date, in birds, a chicken 20K SNP array has been developed for the investigation of differences in chicken lines, with interest in producing arrays for other agriculturally important birds, such as turkey and duck (Crooijmans, 2008).

1.4.4.3. Oligonucleotide microarrays

To determine large scale alterations in genome structure (e.g. deletions, duplications) it is possible (and increasingly becoming more informative) to use SNP arrays for array-based comparative genomic hybridisation (aCGH). However, to date, especially in birds, the most informative available arrays are oligonucleotide based. Affymetrix produces a 70mer chicken array with 21,120 oligonucleotides, while Roche NimbleGen produces a 385,000 oligonucleotide 50mer chicken tiling path array. The utility of these arrays for comparative genomics comes in part in the investigation of copy number variation (e.g. Fadista *et al.*, 2008). The Nimblegen array is the platform that was used for the study of copy number variation presented in this thesis (chapter 6).

1.5. Copy Number Variation

One of the most exciting recent advances in genomics has been the realisation that copy number variants (CNVs) contribute substantially to normal and disease related phenotypic variation. CNVs are defined as a polymorphism in the number of copies of a DNA fragments 1kb or larger (Feuk *et al.,* 2006), excepting insertions or deletions of transposable elements (Freeman *et al.,* 2006). Recent evidence has shown that CNVs play a significant role in normal and disease-related genetic and phenotypic variation in humans and other primates (Emanuel and Saita 2007; Freeman *et al.,* 2006). For example, a number of CNVs have been linked in humans with schizophrenia, autism and other psychiatric disorders (St Clair, 2008, Stefansson *et al.,* 2008). There have also been associations of HIV-AIDS susceptibility with CNVs at the *CCL3L* locus in humans, chimpanzees and macaques (Gornalusse *et al.,* 2009, Degenhardt *et al.,* 2009, Shostakovitch-Koretsyaya *et al.,* 2009). Analysis of copy number variation in humans, via array-comparative genomic hybridisation (aCGH) using a combination of oligonucleotide tiling-path microarrays, and single nucleotide polymorphism (SNP) microarrays has suggested that copy number variable regions (CNVRs) comprise about 12% of the genome (Redon *et al.,* 2006). To date, detailed studies of CNVs in vertebrates have been performed only in mammals – for example, mice (Cutler *et al.,* 2007, Liang *et al.,* 2008, Snijders *et al.,* 2005), pigs (Fadista *et al.,* 2008) and primates (Perry *et al.,* 2006, 2008, Dumas *et al.,* 2007). Information is therefore lacking on patterns of copy number variation in other vertebrate groups.

CNVs are known to be associated with segmental duplications (large, >1kb, regions, with low copy number and >90% sequence identity (Lander *et al.,* 2001)). Segmental duplications are thought to facilitate non-allelic homologous recombination (Lupski 1998; Stankiewicz and Lupski 2002). A correlation has been seen in primates between the locations of segmental duplications and breakpoints of chromosomal rearrangements (Kehrer-Sawatzki and Cooper, 2008), as well as between CNVs and breakpoints for common chromosomal rearrangements (Sebat *et al.,* 2004), and it has been suggested that chromosomal fusions also may be facilitated by the presence of segmental duplications (Emanuel and Saitta, 2007). CNV generation is therefore thought to be driven, at least in part, through non-allelic homologous recombination following ancestral segmental duplications (Perry *et al.,* 2006, Repping *et al.,* 2006).

Cross-species CNV studies, and studies of the evolutionary significance of CNVs have largely focused on primates and revealed numerous lineage-specific gene gains and losses and CNVs (e.g. Bailey and Eichler 2006; Dumas *et al.,* 2007; Fortna *et al.,* 2004; Locke *et al.,* 2003; Newman *et al.,* 2005; Samonte and Eichler 2002). These support the hypothesis that gene duplication is a major factor in evolutionary change (Ohno, 1970); human specific duplications of the AQP7 gene are thought to lie behind key physiological adaptations in thermoregulation and energy utilisation permitting endurance running (Dumas *et al.,* 2007). Other CNVs detected at the AMY1 (salivary amylase) locus appear to have been under selection during human evolution (Perry *et al.,* 2007), and it seems likely that selection for or against CNVs at certain loci will prove common in human and other genomes (Gresham *et al.,* 2008).

1.5.1. CNVs in Birds

The only published cross-species study of CNVs in birds comes from an analysis of CNVs in chicken and turkey (Griffin *et al.,* 2008). Chicken and turkey DNA were hybridised to the Roche NimbleGen whole-genome tiling-path array and 16 CNVs were identified; chicken and turkey diverged approximately 35Mya. In comparison, a lower resolution BAC-array targeted at only segmental duplications detected 58 CNVs between chimpanzee and human (Perry *et al.,* 2006) which diverged 6 Mya. These findings suggest that the low karyotypic variability between these birds is mirrored by a low level of CNVs. Both phenomena may be due to the low level of segmental duplications in avian genomes. Almost 50% of chicken CNVs mapped to the same genomic regions as the CNVs mapped from a comparison of chicken and turkey genomes, which parallels findings in primates (Perry *et al.,* 2006) and suggests that there may be hotspots for CNVs similar to those for chromosomal rearrangements. The CNVs between chicken and turkey were mostly associated with genes and the CNV regions were more highly conserved in avian, amphibian and fish lineages, suggesting a link with egg-laying species. This, however, is only very preliminary data, and much more information is required in order to understand patterns of copy number variation in birds. Such work is therefore one of the specific aims of this thesis, through the analysis of CNVs in a range of bird species.

1.6. The Evolution of Avian Genome Structure

Individual features of avian genomes (for example, the presence of microchromosomes, or a high chromosome number) can be seen in other vertebrate orders; however, the "so many, so small" pattern is unique to birds. This has lead to considerable speculation into the processes of genome evolution in birds, and the suggestion of a model by which the karyotype could have evolved, outlined by Burt (2002). An understanding of the evolution of the avian karyotype requires an understanding of the evolution of vertebrate genomes.

1.6.1. Vertebrate Genome Evolution

It is thought that the ancestral vertebrate genome, approximately 450Mya, contained 10-13 pairs of chromosomes (Nakatani *et al.*, 2007; Kohn *et al.*, 2006). Two rounds of whole genome duplication followed, resulting in around 40 chromosome pairs in the gnathostome (jawed vertebrate) ancestor (Nakatani *et al.*, 2007; Putnam *et al.*, 2008). Some fusions are thought to have reduced chromosome number in the osteichthyan karyotype (n=31) and further in the ancestral amniote karyotype (n=26), approximately 310 Mya. Previous attempts at reconstructing vertebrate genome evolution have suggested an ancestral amniote chromosome number of 18 pairs (Kohn *et al.*, 2006; Jaillon *et al.*, 2004); in either model, the ancestral amniote karyotype is hypothesised to be similar to the current chicken karyotype. Microchromosomes may have first formed around 310Mya, at the time of the ancestral amniote, or perhaps earlier, up to 440Mya, at the time of the osteichthyan ancestor. Following the divergence at 310Mya, the mammalian lineage underwent successive fusions and rearrangements, while some repeat expansions were seen in the lineages leading to snakes, turtles and crocodiles (Shedlock *et al.*, 2007). Organ *et al.*, (2007) inferred the genome sizes of non-avian dinosaurs through measurements of cell sizes from fossils. They found that measured theropod dinosaur genomes averaged about 1.78pg compared to ornithischian dinosaur genomes of about 2.49pg (Figure 1.11). From this, it appears that genome sizes began to decrease between 250-230 Mya (Ellegren, 2007).

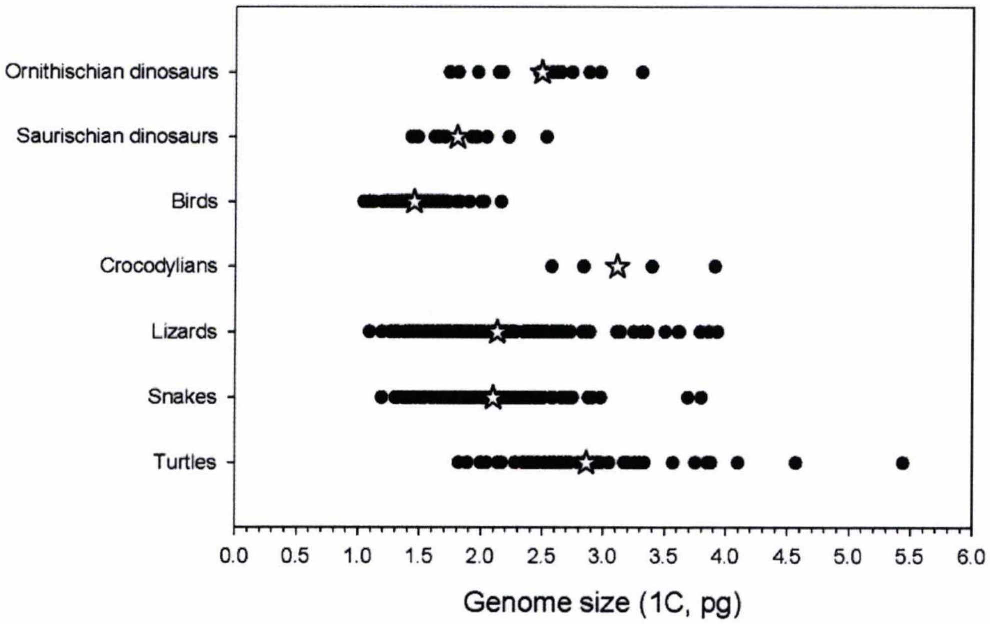


Figure 1.11: Genome sizes in birds and other non-avian reptiles. Genome sizes in the dinosaurs show evidence for size constraint, a pressure maintained in birds. From Gregory (2008).

This size reduction appears to have continued through the evolution of birds – notably, the bird groups which have been studied to date generally have the smallest and most constrained genome sizes of any vertebrates (Figure 1.12).

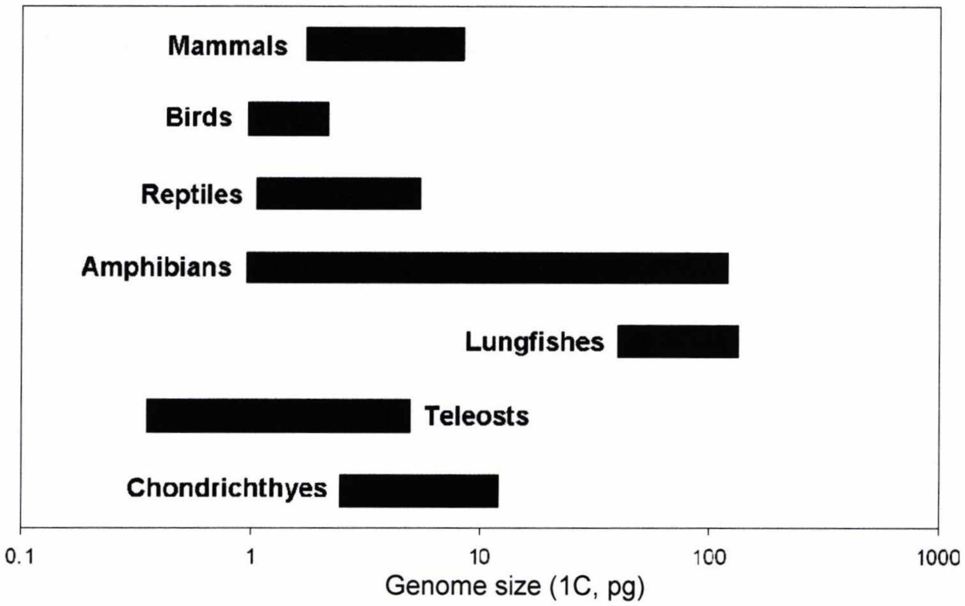


Figure 1.12: Ranges of genome sizes (in picograms) of various vertebrate taxa (complied from www.genomesize.com)

1.6.2. Isochores

Another feature distinguishing the chicken genome (and potentially other avian genomes), shared with mammals, is the isochore structure. Isochores are long (>300kb) regions found within some vertebrate genomes characterised by relative homogeneity in base composition (Bernadi, 2000); that is, they are GC-rich or GC-poor compared with the surrounding regions. It should be noted that there are no sharp boundaries between different isochores, rather GC content changes continuously below the 300kb resolution. In essence, they are the genomic correlates to chromosome bands (Saccone *et al.*, 1993). They have been found in all amniotes so far studied, and thus it has been proposed that they evolved in the amniote ancestor, ~360-310Mya (Chojnowski *et al.*, 2007). There have been two different opinions over the evolution of isochores – a disagreement between neutralist and selectionist viewpoints. Bernadi (2000) maintains a selectionist view, that isochores evolved in response to homeothermy. The argument is that GC rich sequences are more stable at higher temperatures, thus selected for in homeothermic organisms. However, isochores have been found in snakes (Hamada *et al.*, 2003), turtles, crocodiles (Hughes *et al.*, 1999) and alligators (Chojnowski *et al.*, 2007), all poikilothermic ("cold-blooded"). A neutralist viewpoint, arguing for variable mutation processes in different genomic regions, has yet to be demonstrated. However, another selective hypothesis for increasing GC content is biased gene conversion (BGC) (Galtier *et al.*, 2001).

1.6.3. Biased Gene Conversion

There is evidence that the genomes of amniotes have a higher level of CpG methylation than those of amphibians or teleost fishes (Belle *et al.*, 2004). It has been shown that in mammalian cells, methylated cytosine will spontaneously deaminate to thymine; additionally, cytosines within CpG islands in humans appear to have a mutation rate 10 times higher than other nucleotides (Gianelli *et al.*, 1999). Hence, DNA mismatches will be generated, and there will be a selective pressure to repair them in favour of guanine. DNA mismatch repair enzymes preferentially repair in favour of guanine 90% of the time (Brown and Jiricny, 1987). That is, a TG mismatch can be repaired to CG or to TA, and the CG repair is more likely to occur.

This provides a mechanism for GC-rich isochores to evolve in the amniote ancestor (Duret *et al.*, 2006). Alleles can have a heterozygous site, one allele having an AT, the other having a GC. Following recombination, there will be a mismatch, which must be repaired. As seen, there is selection pressure for mismatches to be repaired in favour of GC content (Figure 1.13). Hence, the gametes will be biased towards GC content in areas of high recombination. In both mammals and birds, chromosomes with high recombination rates tend to have a higher GC content (Jensen-Seaman *et al.*, 2004; Hillier *et al.*, 2004).

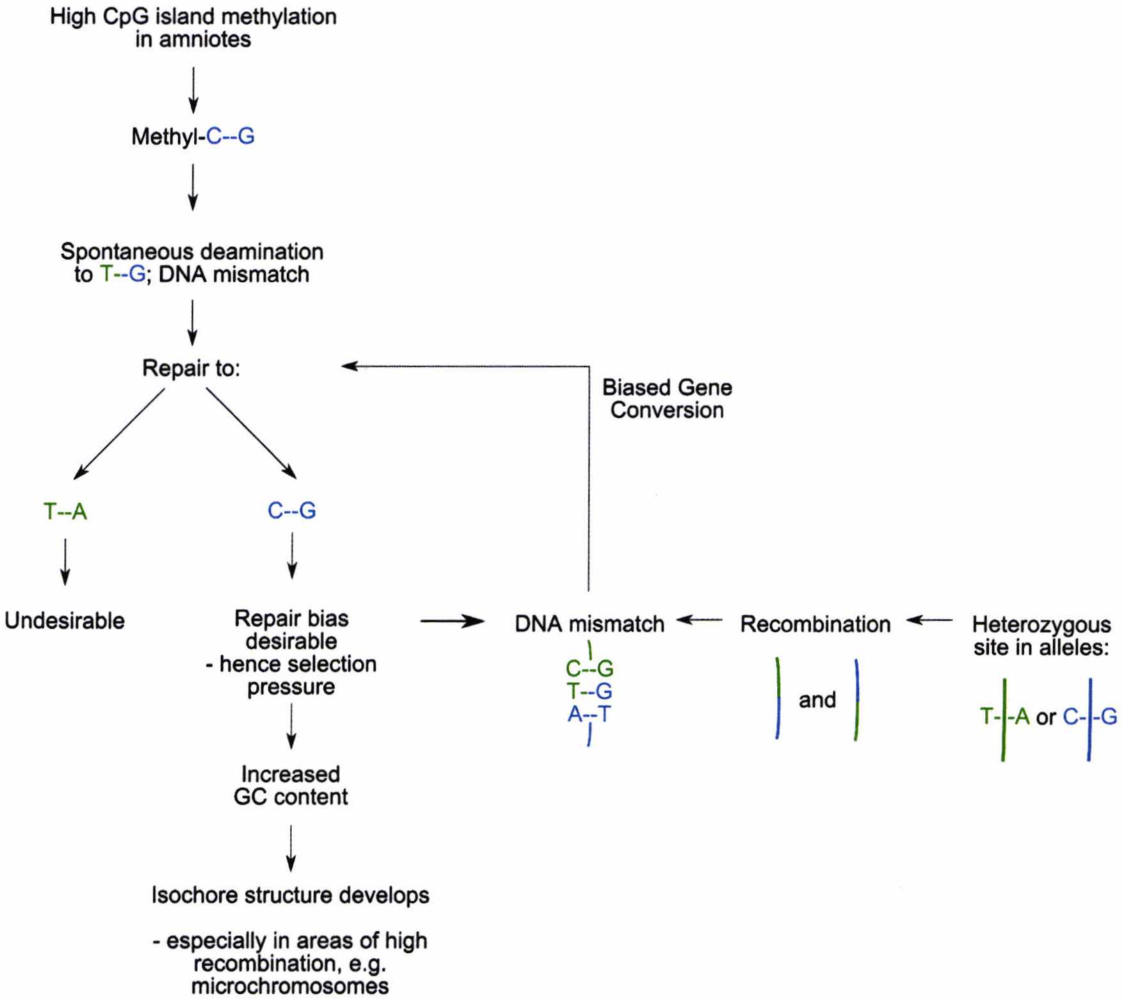


Figure 1.13: The process and development of biased gene conversion.

In mammals, it appears that isochores are being lost (Duret *et al.,* 2002), and that homogenisation of GC content is taking place. It is theorised that where BGC is not active (where recombination is not high enough) an AT mutation bias may dominate (Duret *et al.,* 2006); the numerous chromosomal rearrangements in mammals will have reduced recombination rates, thereby explaining why mammals are losing their isochore structure.

1.6.4. Evolution of the bird lineage

Although this may appear to indicate bird genome size is a relic of processes in theropod dinosaurs, it has been shown that genome size continued to fall in at least some bird lineages to the current average of 1.45pg (range 0.97-2.16pg; www.genomesize.com). This, it seems, may be in part due to a reduction in repetitive content. The most common repeat found in the chicken genome, the CR1 long interspersed nuclear element (LINE), is mainly distributed on the macrochromosomes, and is found conserved across many bird species (Coullin *et al.,* 2005; see also section 1.1.3). CR1-like elements have also been found in the genomes of reptiles (Vandergon & Reitman 1994), mammals (Jurka 2000), amphibians (Kajikawa *et al.,* 1997), and invertebrates (Drew & Brindley 1997). Due to their prevalence, they have been implicated in a structural or functional role in the genome, possibly through the regulation of gene expression (Sanzo *et al.,* 1984), although most of the CR1 elements in the chicken genome today appear to be inactive (Hillier *et al.,* 2004). Recent studies have also indicated that over the last 207 Myrs, the proportion of CR1 elements in the chicken lineage has decreased 6 fold – while undergoing a 10 fold increase in the alligator lineage and a 3 fold increase in the turtle lineage (Shedlock *et al.,* 2008). The features of vertebrate genomes outlined above are summarised in Figure 1.14.

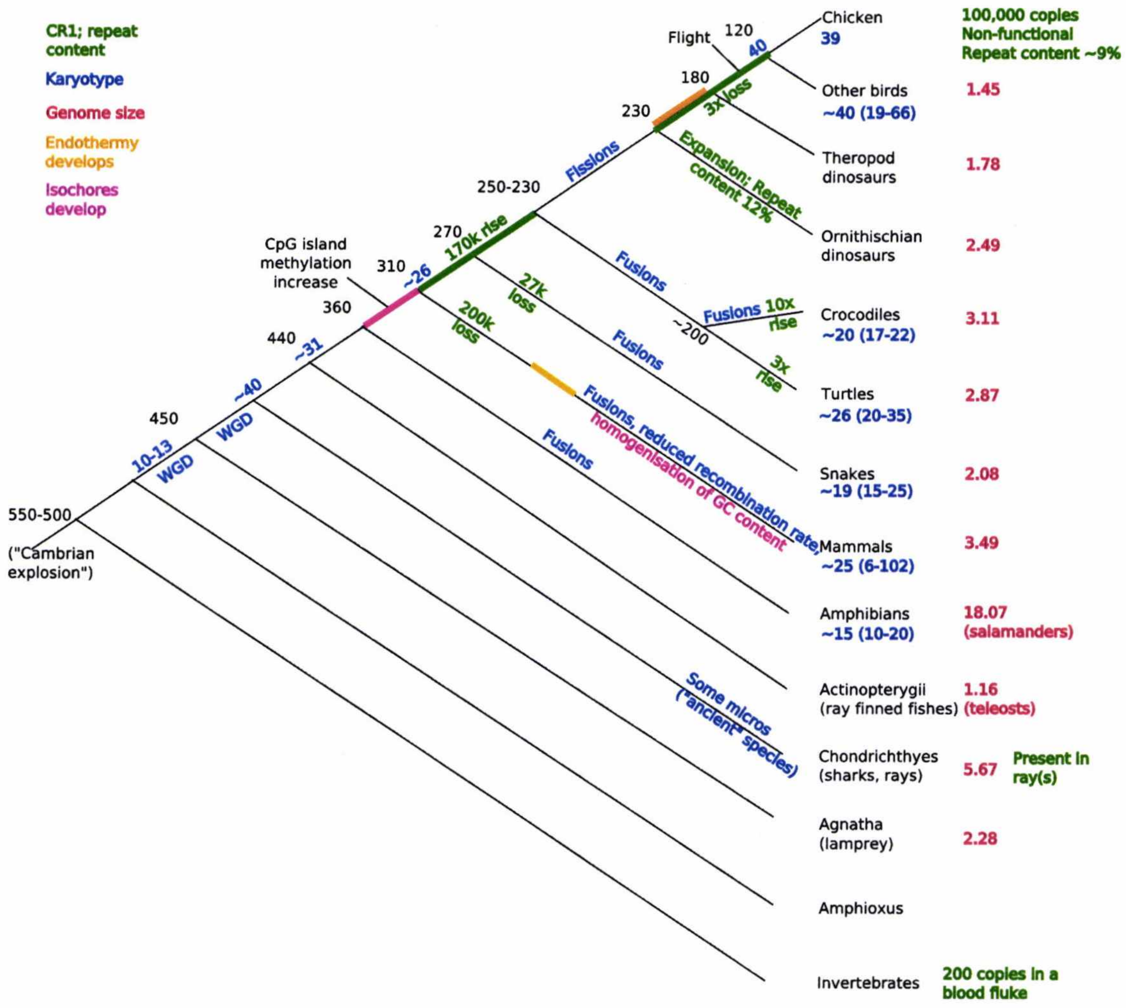


Figure 1.14: Elements of vertebrate genome evolution. A rough phylogeny is shown of major divergences leading to modern day birds. Green annotations show CR1 repeat content in each lineage, showing it to be an ancient repeat that has decreased in number in birds. Blue shows estimated haploid chromosome numbers along the backbone of the phylogeny, and changes affecting karyotype (WGD = Whole Genome Duplication). Red shows average genome size in picograms of orders where available. Orange bars outline when homeothermy developed in mammals and birds. The purple bar shows the development of the isochore structure of the amniote genome, and the current loss of isochores in mammals.

The idea of genome evolution that is suggested from the above is one of multiple sequential reductions in repeat diversity in many lineages of birds and non-avian reptiles. There are also no known instances of natural polyploidy in birds, though this has been seen in reptiles and has been noted in some mammals (Gregory, 2005). Hence, there still appears to be a selective pressure for a small genome in at least some birds. One hypothesis to explain this is the energetic requirement of flight, which is thought to have evolved around 150 Mya (Feduccia, 1995). Genome size correlates with cell size; a small genome permits a small cell. A smaller cell has a greater surface area:volume ratio than a larger cell, thus can sustain a higher metabolic rate. The original genome size reduction in the dinosaurs is suggested to be for a similar reason, though the metabolic requirement was likely to meet the demands of homeothermy, rather than flight (Organ *et al.*, 2007). It can also be noted that the other class of flying vertebrates, the bats, have a smaller genome size than the mammalian mean (Burton *et al.*,1989, Smith and Gregory, 2009), and appear to have considerably fewer microsatellites than other mammalian species (Van Den Bussche *et al.*, 1995). An additional, compatible, hypothesis has been put forward by Gregory (2002). This begins with the observation that genome size correlates with neuron size and brain complexity inversely correlates with neuron size in certain amphibians (Roth *et al.*, 1994). Birds with complex, metabolically active brains and a high relative brain size may have an additional selection pressure for a small genome (Andrews and Gregory, 2009).

The hypothetical picture thus far of the ancestral vertebrate genome leading to the bird lineage, is of a small genome size, with a low repeat content, and perhaps 20 microchromosome pairs in the karyotype (Burt, 2002), plus a selection pressure to maintain a small genome. Chromosomal evolution (driven by, for example, fissions, fusions, deletions, insertions, inversions, translocations) is dependent on the rate of mutation and the rate of fixation (Burt *et al.*, 1999). The mutation rate of all rearrangements except fissions is dependent on the frequency of homologous sites within the genome. One major contributor of homologous sites are repeats, most of which are derived from transposable elements (Kidwell and Holyoake, 2001). The birds studied to date have on average a lower repeat content than mammals, amphibians or reptiles; they therefore also have had less opportunity for chromosomal rearrangements other than random fissions. Assuming random fixation of fissions, and an even size distribution of chromosomes in the ancestral karyotype, the expected size distribution would be of many small chromosomes, and a few macrochromosomes, with no abrupt size division between them – the karyotype found in the majority of birds that have been studied (Burt *et al.*, 2002). Under this model, the karyotype structure (i.e. the presence of microchromosomes) is a consequence of selection pressure to maintain a small genome, and not a direct adaptation in its own right.

This model also provides an explanation for the other characteristics of chicken (and potentially other avian) microchromosomes, for example the high GC content, and low number of repeats. Due to obligate crossing over during meiosis, microchromosomes have a higher recombination rate than macrochromosomes (for the chicken, this is estimated as one crossover per 30Mb for the macrochromosomes and one per 12Mb for the microchromosomes (Hillier *et al.*, 2004), though this may be high compared to other birds; the zebra finch genetic map is only a quarter of the size of the chicken genetic map (Stapely *et al.*, 2008)). A high recombination rate allows for the efficient removal of repeats via non-allelic homologous recombination (Abrusán *et al.*, 2008) – the same mechanism that is thought to drive CNV and segmental duplication generation, without a genome size constraint.

Once class of repetitive sequence that does not appear to have been reduced during bird evolution is the telomeric repeat, $(TTAGGG)_n$. Chicken genomes contain a greater proportion of telomeric sequence than human genomes (Schmid *et al.*, 2000), and telomere length has been linked to both maintenance of the microchromosomes and to longevity (see section 1.2.7). The potential adaptive benefits to long telomeres may outweigh pressures to reduce genome size.

1.7. General Significance of Avian Genomics

Chicken research has had a significant impact on fundamental biology and the chicken has been a popular model organism for over 100 years. The chicken is also ideal for studying vertebrate development due to the ease of access to and manipulation of the chicken embryo using incubated eggs (Stern 2005). Chicken erythrocytes are nucleated, unlike those of mammals, which makes them a valuable model for studies of chromatin structure. As such, most avian genomic research has been carried out on the chicken. The sequencing of the genome also took place when the most closely related species to mammals with a sequenced genome was the puffer fish, which had diverged from the common ancestor 100 million years before birds and mammals split. From this perspective, the chicken is an important resource for studying genome evolution (Burt and Pourquie, 2003).

New tools such as the electroporation of chicken embryos and the use of RNAi to knock down gene expression are likely to make the chicken embryo an even more powerful model for the molecular study of development in vertebrates in the future (Stern, 2005; Burt, 2005). The immortalised DT40 cell line is derived from chicken, and has shed much light on research in human cancers (Chang and Delany, 2004).

Modern selective breeding has made considerable progress in the last 90 years in both egg and meat production traits. World egg production increased to 62.6 million tonnes per year in 2007 (faostat.fao.org) and broiler meat production increased to 7.4 million tons per year (USDA foreign agricultural service, www.fas.usda.gov). However, with the success has come the development of a number of undesirable traits. For example, in broilers chickens there has been an increase in congenital disorders, such as ascites and lameness, reduced fertility, and reduced resistance to infections (Burt, 2005). In the egg-layers there has been an increase in the incidence of osteoporosis linked to an increase in the production of eggs. Given that the genetic progress in egg and meat production is predicted to reach its limit within 20 years (Burt, 2002), the poultry industries have incentives to reduce costs as well as develop new methods of delivering high quality products to the consumers. Increased requirements for food safety, and increased customer demand for 'organically' reared birds mean there is a need to reduce the use of chemicals and antibiotics, as well as to increase genetic resistance to pathogens. It would be difficult to acquire these new traits by conventional genetic selection, as it is difficult and costly. However, the development in poultry genomics over the last few years suggests new solutions to these problems (Burt, 2005).

Much of the above is applicable to other birds; the most obvious avian targets for comparative genomics include the zebra finch because of its importance as a neurological model; the turkey for agricultural reasons (and its relatedness to chicken); and the duck, again for agricultural reasons and as a target for immunological studies because of their resistance to avian influenza (Munster *et al.*, 2006). The precise basis of duck influenza resistance is still unknown, although evidence has suggested the involvement of the Toll-like receptors (MacDonald *et al.*, 2007) and there is considerable interest in avian immunological genomics (Keeler *et al.*, 2007). Its agricultural importance alone makes the Pekin duck a target for genomic studies with worldwide duck consumption being between 4 and 5% of the total poultry market (Food and Agriculture Organization of the United Nations; http://faostat.fao.org). Given that the zebra finch has a significant sequencing and genome mapping effort associated with it, the most relevant and informative targets for comparative genomics in birds are the turkey and duck. Of these, the duck genome has been least well characterised. Therefore, an important next step in avian genomics, for both agricultural and evolutionary standpoints is to enrich the understanding of genome organisation in both species, but especially in the duck.

1.8. Rationale for this thesis

It is clear from the existing literature that there are specific features characterising the (still somewhat limited) number of avian genomes studied to date (e.g. patterns of karyotype evolution), and there are some general hypotheses for how such features may have evolved. There are also detailed comparative genomic approaches used to investigate avian (and other) genomes that point to a number of open questions remaining in avian genomics.

To date, only one detailed comparative cytogenetic map between bird genomes has been published, between chicken and turkey (Griffin *et al.*, 2008). These birds are closely related, and as such the small number of inter- and intra-chromosomal rearrangements seen (particularly among the microchromosomes) may not be reflected across wider evolutionary distances. Thus, a greater number of comparative maps are needed. A comparative map between chicken and duck is the obvious next step, from both agricultural and evolutionary perspectives, as outlined in section 1.7. A practical concern associated with this is the ability of investigators to easily identify avian microchromosomes.

The previous data on nuclear organisation in chicken (e.g. Habermann *et al.*, 2001) has demonstrated a general trend, but leaves open the question of whether chromosomes follow preferentially a chromosome size or gene density based organisation (see section 1.3.4). Given that chicken is often held as an example of a species in which both patterns of organisation are followed, it is important to resolve this question for at least one cell type as a precursor to further nuclear organisation studies in birds. Nothing at all is known about patterns of nuclear organisation in other birds; testing the hypothesis that these patterns are conserved in birds as they are in mammals is also of interest.

Increasingly, it is becoming clear that CNVs are involved in phenotypic variation, both normal and disease related. However, almost nothing is known about patterns of CNVs in birds. While detailed investigations of CNVs within species are certainly needed, there is also a need for a more general overview of copy number variation between bird species. For instance, how CNVs relate to the specific features of avian genomes outlined, and how these patterns compare and contrast with other vertebrate (specifically mammalian) genomes.

1.9. Specific Aims

Given the importance of avian genomics in general and duck as a primary model species, the overall objective of this thesis was to perform in-depth investigations into genome organisation in duck with specific reference to comparison with chicken.

The specific aims of this thesis were therefore as follows

1. To establish the relative physical sizes of chicken, turkey and duck chromosomes (compared to published Ensembl estimates) and thereby devise a means through which anonymous clones can be assigned to avian chromosomes within a minimum number of steps.

2. To perform comparative physical mapping (by FISH) between chicken and duck with a view to the generation of a cytogenetic genome map in duck and the establishment of inter- and intra- chromosomal rearrangements between the two species.

3. To provide a detailed appraisal of nuclear organisation in chicken embryonic fibroblasts, and perform comparative genomic experiments in turkey and duck. In particular to establish whether size-related or gene-density-related models best fit avian embryonic fibroblast nuclei. Further, to test the hypothesis that avian chromosome territories dynamically alter in their nuclear position in response to changed gene expression in avian (chicken and duck) macrophages.

4. To test the hypothesis that inter-specific copy number variation (CNV) can be established between a range of avian species, to devise a means of expressing such data, and to speculate on the possible ramifications for genome evolution.

2. Materials and Methods

2.1. Preparation of cell suspensions

2.1.1. Fibroblast culturing

2.1.1.1. Preparation of media

The media used in culturing was Dulbecco's Modified Eagle's Media (DMEM) with LG and 1000mg/ml glucose. Media was prepared beforehand and stored at 4°C. When required, 1L of media was warmed to 37°C together with 100ml Chick Serum (Gibco), 20ml Penicillin/Streptamycin (Gibco) and 10ml glutamine (200mM 100x, Gibco). The latter three solutions were kept at -20°C until required. The complete media was made up in a class II hood. A small quantity of media was poured away, sufficient to allow the extra 130ml to fit in the bottle. This measurement was not precise. The chick serum, pen/strep and glutamine were poured into the media, which was then dated and labelled as complete. It was either used at once, or returned to 4°C for storage. Due to the approximately 40 day half-life of glutamine, complete media cannot be left for long periods of time.

2.1.1.2. Primary culture preparation

Eggs were opened in a class II hood. The hood was sterilised with UV for a minimum of 10 minutes before opening. Once open, it was sprayed with ethanol. The egg shell was cut with a scalpel making a small hole into which forceps could fit. The forceps were then used to enlarge the hole until it was big enough to remove the embryo through. The embryo was grasped by the neck using the forceps and pulled out, or the entire contents of the egg were tipped into a 10cm diameter plastic petri dish. Once acquired, the embryo was placed in a petri dish containing approximately 2ml of 1xPBS. The head and limbs were cut off, and an anterior-posterior cut was made along the ventral surface to allow the internal organs to be scraped out.

A 70μm filter (VWR) was placed in a petri dish containing 5ml complete DMEM. The remains of the embryo were put in the filter, and macerated with the plunger of a 10ml syringe until it was judged that no more fibroblasts could be obtained. The fibroblast-containing media was pipetted into a 175ml culture flask (vented cap) [company] containing 21ml complete DMEM. Since 1ml of media was absorbed by the filter, the final volume in the flask was 25ml. If the embryo was small, a 75ml flask was used, containing 11ml DMEM for a final volume of 15ml. The flasks were incubated at 37°C with 5% CO_2.

2.1.1.3. Refreshing

Cells were refreshed when they were insufficiently confluent to warrant splitting or harvesting. They were also refreshed if the culture was left for three days since the nutrient level in the media would have declined. Non-adherent cells were removed during refreshing. Thus it was useful to refresh new primary cultures and provide less competition for nutrients. The media in the flasks was tipped out and new media pipetted in. 25ml of complete DMEM was used for a 175ml flask and 15ml for a 75ml flask.

2.1.1.4. Splitting

Flasks were split when the cells appeared confluent but did not show sufficient dividing cells to warrant harvesting, or when it was desired to keep a culture for a longer period of time to allow more flasks to be harvested. If the cells appeared extremely confluent and had been growing quickly, it was decided to split the flask 1 in 3 rather than the standard 1 in 2 to allow more time before they were split again or harvested.

To split cells, for example 1 in 2 in 175cm^3 culture flask, the media was removed and 8ml of verscene (0.197g EDTA/1 litre 1xPBS (Sigma), pH7) was added to wash the flask, then removed. 2ml of trypsin (0.25% (Gibco)) and 4ml of verscene was added to the flasks, (the trypsin was added to the flasks to detach the adherent fibroblast cells from the flask wall). The sides of the flasks were tapped to ensure the fibroblast cells had detached from the flask; this was checked under a microscope. 3ml of each cell suspension was put into two new flasks with a further 22ml of fresh media and incubated.

2.1.2. Fibroblast harvesting

Cultures were selected for harvesting when confluent with a high number of dividing cells visible. Demecolceine solution (10µg/ml; Sigma) was added (200µl/T175; 150µl/T75) and the cultures incubated for 40 minutes at 37°C. The cells were trypsinised to remove them from the flask (see section 2.1.2).

After trypsinising the cells, 6ml was added to 8ml of complete media in a 15ml falcon and was spun down for 5 minutes at 400g. The supernatant was removed to approximately 0.5ml and the pellet was resuspended, to ensure that the pellet would not clump on the addition of KCl. Approximately 1.5ml of 0.075M KCl was added in a drop-wise fashion with a pasteur pipette with constant gentle agitation, so as not to burst cells. A further 5ml of KCl (to hypotonically swell the cells) was added. The tube was inverted to mix and incubated at 37°C for 20mins. The hypotonic treatment was stopped with 10 drops of fixative (3:1 methanol: acetic acid), again inverted to mix and spun down at 1500 rpm for 5 minutes.

The waste was poured off and pellet flicked up before the addition of about 1.5ml of freshly prepared fixative dropwise with constant gentle agitation as above. Further fixative was added to 5ml, inverted to mix and spun down at 400g for 5 minutes.
This step was repeated four more times, to remove any debris and to ensure a clean sample. The sample was stored at -20^{0}C.

2.1.3. Blood (Lymphocyte) Culturing

Whole blood was collected from adult birds into standard heparined vacutainers. 3ml room temperature Histopaque-1077 (Sigma) was added to a 15ml falcon tube. 3ml whole blood was added carefully to form a layer above the histopaque. The tube was centrifuged at 400g for 30 minutes, and the upper (plasma) layer was aspirated and discarded. The second (lymphocyte) layer was transferred to a new 15ml Falcon tube and 10ml RT PBS was added and gently mixed. Tubes were centrifuged at 250g for 10 minutes and the supernatant discarded. The pellet was resuspended in 5 ml sterile PBS and centrifuged at 250 x g for 10 minutes. The supernatant, save 0.5ml was discarded and the pellet resuspended. Cells were transferred to a T25 culture flask containing 10ml RPMI with 10% foetal chick serum, penicillin/streptomycin, L-glutamine and concanavalin A (100μg/ml) and incubated at 39.5°C, 5% CO2 for 72 hours. Demecolcine solution (0.05 μg/ml) was added to the culture and incubated for one hour. Cells and medium were transferred to a 15ml falcon tube and centrifuged at 400g for 5 minutes. After this, hypotonic treatment and fixation proceeded as described in section 2.1.2, save that the KCl was at 39.5°C. Fixed samples were stored at -20°C

2.1.4. Macrophage culture

Macrophages were prepared from blood cultures from chicken and duck. Whole blood cultures were incubated as above at 41°C with 5% CO_2 for 48 hours, after which the media was refreshed, removing non-adherent cells. Following a further 48 hour incubation, all cells types save macrophages were lost. 500ng/μl lipopolysaccharide was added to half of the cultures, and allowed to incubate for 24 hours. The cultures were harvested for RNA extraction (2.5.2.1) or for fixation and FISH.

2.2. BAC DNA Preparation

2.2.1. Isolation of chicken BAC clones

The isolation of BAC clones covering the complete chicken genome has been described in detail previously (Aerts *et al.,* 2003). The BAC clones were derived from the Wageningen chicken BAC library, constructed from the DNA of a White Leghorn chicken (Crooijmans *et al.,* 2000). Screening of the library was performed by a two-dimensional screening procedure (Crooijmans *et al.,* 2000). In the first step, the plate pools were screened to identify the plates that were positive for the marker. In the second step, the row- and column-pools of these plates were screened to find the coordinates of the positive BAC clone.

The selection of the markers used to isolate the BACs was based on the position of the markers on the chicken consensus linkage map (Groenen *et al.*, 2000). Markers were chosen at regular intervals across the different linkage group/chromosomes.

2.2.2. LB Agar preparation

32.0g LB agar (Invitrogen) was added to 1 litre of ddH_2O and sterilised at 120°C for 15mins, then left to cool to 50°C, whereupon 600µl of the antibiotic chloramphenicol (25mg/ml) (Fluka Biochemika) was added to give a final concentration of 15µl/ml of chloramphenicol. For preparation of agar plates; cooled agar was poured into sterile plastic Petri dishes, to prevent any unwanted contamination, approximately 30ml per plate and allowed to set before being stored at 4°C.

2.2.3. Terrific broth preparation

16g of terrific broth (Invitrogen) was added to 1 litre of ddH_2O, autoclaved, and cooled to 50°C, 600µl of 25mg/ml chloramphenicol stock was added to give a final concentration of 15µl/ml. The broth was used to inoculate the starter culture for the midi-prep (Qiagen).

2.2.4. Plating and inoculation of BACs

A disposable sterile loop was inserted into the glycerol stock of the BAC clone then streaked onto an agar plate to obtain single colonies. The plate was turned upside down and then incubated at 37°C overnight. A colony was picked from the plate using a pipette tip and mixed into terrific broth. A colony was added to two tubes containing 25ml of terrific broth and incubated in a 37°C shaker overnight, to ensure growth of the cultures.

2.2.5. Qiagen Plasmid Midi kit

The midi prep was carried out following the manufacturer's instructions. A starter culture of 25ml LB medium containing chloramphenicol was inoculated using a single colony and incubated at 37°C overnight with vigorous shaking (300rpm). The bacterial cells were then harvested by centrifugation at 6000g for 15mins at 4°C. The bacterial pellet was resuspended in 4ml of buffer P1 (resuspension buffer). 4ml of buffer P2 was added (cell lysis buffer), which was mixed gently and thoroughly by inverting 4-6 times. The cells were incubated at room temperature for 5mins. 4ml of chilled buffer P3 (neutralisation buffer) was added and then mixed immediately but gently by inverting 4-6 times. The cells were incubated on ice for 15mins, then centrifuged at 15,000g for 1 hour at 4°C. The supernatant containing plasmid DNA was removed promptly and centrifuged again for 30mins. Meanwhile a QIAGEN-tip 100 was equilibrated by applying 4ml of buffer QBT (equilibration buffer to equilibrate the tip). The supernatant was applied to the QIAGEN-tip 100 and entered the resin by gravity flow. The QIAGEN-tip 100 was washed twice with 10ml of buffer QC (wash buffer). To elute the DNA, 5ml of buffer QE (wash buffer) was added to the column. The precipitation of the DNA was carried out by adding 3.5ml of RT isopropanol to the eluted DNA. This was mixed and centrifuged immediately at 15,000g for 30mins at 4°C. The supernatant was discarded. The DNA pellet was washed with 2ml of room temperature 70% ethanol and centrifuged at 15,000g for 10mins. The supernatant was discarded carefully so as to not disturb the pellet. The pellet was air dried for 5-10min and redissolved in 50μl TE buffer. The redissolved DNA was then stored at -20°C for labelling via nick translation.

2.2.6. Nick Translation

The following reagents were prepared per sample:

Reagent	Concentration	Amount (μl)
NT Buffer (50mM Tris-HCl, pH 8.0, 5mM $MgCl_2$, 50μg/ml BSA)		5
β-mercaptoethanol	0.1M	5
dNTPs (0.5mM A,C,G; 0.12mM T)		2.5
Biotin / digoxigenin	1mM	2.5
E. coli polymerase I	10U/ul	1
DNase (1:100)	10μg/ml	8
Total		24

24μl of the master mix was pipetted into a microcentrifuge tube, and 26μl of DNA to be labelled was added. DNA was acquired from a midi prep in the case of BACs or DOP-PCR in the case of chromosome paints. Samples were vortexed and spun briefly to mix, then incubated at 16°C for 2 hours. Following incubation, the samples were placed on ice to pause the reaction, and 3μl was run on a 2% agarose gel. If the sample showed a smear of less than 500kb, the reaction was stopped by the addition of 5μl 0.5M EDTA. If the fragments were still too large, the reaction was continued for an appropriate time and a new gel run.

2.2.7. DNA precipitation

50μl 5M ammonium acetate and 250μl 100% ice cold ethanol were added to each sample. These were vortexed, and left at -80°C for 2 hours or overnight. The samples were centrifuged at 13,000rpm at 4°C for 25 minutes. The supernatant was discarded, and 200μl 70% ice cold ethanol was added. The samples were again spun at 13,000rpm at 4°C for 25 minutes. The supernatant was discarded and the samples allowed to air dry at room temperature. 10μl hybridisation mixture was added, and the samples vortexed. They were aged at room temperature for 24 hours or at 55°C for 1.5 hours before being stored at -20°C.

2.3. Fluorescent in-situ hybridisation (FISH)

2.3.1. Slide preparation

Cell samples were spun at 1500rpm for 5 minutes. Fixative (3:1 methanol:acetic acid) was prepared and kept on ice. Slides (Superfrost, VWR) were rinsed with fix and air dried. The supernatant was removed from the cell samples and the pellets resuspended. Using a pasteur pipette, a drop of cell suspension was dropped from a height onto the slide. 5-6 drops of fixative was added and gently agitated for 10 seconds. The slide was rinsed in fix and allowed to air dry. Slides were aged before use to harden the chromosomes; this was for 24 hours at room temperature; overnight at 37°C; or for 1 hour at 70°C.

2.3.2. Same species FISH

Slides were aged, either for 24 hours at room temperature, at 37°C overnight or at 70°C for 1 hour. The aged slides were put through an ethanol series (70, 80, 100%) for 5 minutes each, then air dried. 100μl RNase A solution (Promega) was added to each slide, which were then covered with a 22x50mm coverslip and incubated in moist chambers at 37°C for 1 hour. During the RNase treatment the probes were prepared. 2μl probe was added to 6μl hybridisation mixture and 1μl herring sperm DNA (Sigma). At 40 minutes into the RNase treatment the probes were denatured at 75°C for 5 minutes, then put at 37°C to pre-anneal. When the RNase treatment finished, the slides were washed in 2xSSC for 10 minutes, then put through an ethanol series (70, 80, 100%) and air dried. They were denatured in 70% formamide/2xSSC for 1 minute 30 seconds, put through an ethanol series (70% ice cold, 80, 100% room temperature) and air dried. The probes were added to the slides, which were covered with 18x18mm coverslips and sealed with rubber cement. The slides were incubated in moist chambers at 37°C for 24 hours.

Following incubation, the slides were washed in 2xSSC, and the coverslips removed. The slides were put into 50% formamide/2xSSC for 20 minutes at 37°C, then into 2xSSC, 0.1% Ipegal for 1 minute. They were then put into storage buffer 4xSSC, 0.05% Ipegal for at least 15 minutes (maximum time 3 days). The slides were blocked in 4xSSC, 0.05% Ipegal, 3% BSA for 25 minutes, after which 100µl detection mix was added to each slide. The detection consisted of 4xSSC, 0.05% Ipegal, 1.5% BSA plus 1:200 of Cy3-streptavidin (Amersham) or FITC-anti-digoxigenin (Roche). A 22x50mm coverslip was placed on each slide, and they were incubated at 37°C in moist chambers for 35 minutes. The slides were washed in storage buffer for 10 minutes, then rinsed in distilled water and air dried before being mounted in Vectashield anti-fade with DAPI (Vector).

2.3.3. Cross-species-FISH

This was performed as per same species FISH, with changes to the probe volume and post-hybridisation washes. The amount of probe was doubled (i.e. 2µl was used) and the amount of hybridisation mixture was reduced to compensate. Slides were incubated for at least 48 hours in the case of chicken probes on turkey chromosomes, and at least 72 hours in the case of chicken probes on duck chromosomes. The post-hybridisation washes had a lower stringency; 40% formamide in 2xSSC at 30°C for 20 mins.

2.3.4. Dual colour FISH

For dual colour FISH, the hybridisation mixture was fully replaced by the second probe. The hybridisation and post-hybridisation washes were carried out as standard, save that both fluorochromes were included in the detection mix at appropriate concentrations.

2.4. DNA Amplification by PCR

2.4.1. Primary DOP-PCR (paint)

The following reagent mix was prepared per reaction and added to a tube containing microdissected or flow-sorted chromosomes:

Reagent	**Amount (μl)**
5x Buffer D (Invitrogen)	3
6MW Primer (50uM) CCGACTCGAGNNNNNNATGTGG	0.6
dNTP mix (2.5mM) (Invitrogen)	1.2
SuperTaq (10U/ml)	0.06
PCR water	10.14
Total	15

The samples were run on a thermocycler under the following profile:

Step	**Cycles**	**Temperature (^{o}C)**	**Time (m:s)**
1	1	94	9:00
2	8	94	1:00
		30	1:30
		72	3:00 (Ramp to 72^{o}C at 0.3^{o}C/second)
3	26	94	1:00
		62	1:00
		72	1:30
4	1	72	10:00
Hold at 4^{o}C			

3μl of each sample was run on a 2% agarose gel; the remainder was stored at -20°C.

2.4.2. Secondary DOP-PCR (paint)

The following reagent mix was prepared per reaction:

Reagent	**Amount (μl)**
5x Buffer D (Invitrogen)	20
6MW Primer (50uM)	4
dNTP mix (2.5mM) (Invitrogen)	8
SuperTaq (10U/ml)	0.2
ddH_2O	65.8
1^0 PCR products	2
Total	100

The samples were run on a thermocycler on the following profile:

Step	**Cycles**	**Temperature (°C)**	**Time (m:s)**
1	1	94	3:00
3	25	94	1:00
		62	1:00
		72	1:30
4	1	72	8:00
Hold at 4°C			

3μl of each sample was run on a 2% agarose gel; the samples were stored at -20°C for use in labelling PCR or nick translation.

2.4.3. Labeling DOP-PCR (paint)

The following master mix was prepared:

Reagent	**Amount (µl)**
5x Buffer D (Invitrogen)	20
DOP Primer (50uM)	4
dACG mix (2.5mM) (Invitrogen)	8
dTTP (1mM)	16
Biotin / digoxigenin (1mM)	4
SuperTaq (15U/ml)	0.2
PCR water	55.8
2^0 PCR products	2
Total	100

The reactions were run following the same profile as a secondary amplification (section 2.4.2). Following the reaction, the samples were precipitated as described in section 2.2.7 and stored at -20°C.

2.5. Microarray based studies

2.5.1. Array - Comparative Genomic Hybridisation (array-CGH)

2.5.1.1. Extraction of genomic DNA

Whole blood was used for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit according to the manufacturers' instructions. Briefly, 10μl whole blood was added to 20μl Proteinase K and 180μl 1xPBS and mixed. 20μl 20mg/ml RNase A was added and incubated at room temperature for 2 minutes. 200μl Buffer AL was added, mixed by vortexing and incubated at 56°C for 10 minutes. 200μl ethanol was added and mixed by vortexing. The mixture was pipetted into a DNeasy Mini spin column and spun at 6000g for 1 minute. 500μl of buffer AW1 was added and the column was spun at 6000g for 1 minute. 500μl of buffer AW2 was added and spun at 20,000g for 3 minutes. 100μl buffer AE was added, incubated for 1 minute at room temperature, then spun at 6000g for 1 minute to elute the DNA. The elution step was repeated using the same collection tube. Samples were run on a 2% agarose gel to check fragment size, and DNA concentration was measured using a Qu-bit fluorometer (Invitrogen). Multiple samples were pooled and ethanol precipitated to an appropriate concentration (250ng/μl).

2.5.1.2. Array - Comparative Genomic Hybridisation (array-CGH)

Chicken and duck genomic DNA was diluted to 250ng/μl, and 1.5μl was checked on a Nanodrop spectrophotometer for purity. Absorbance ratios of A260/280 greater than 1.8 and A260/230 greater than 1.9 were required. The NimbleGen chicken whole-genome tiling array (Catalogue Number/Design Name B3791001-00-01, galGal3 WG CGH – Roche NimbleGen, Milton Keynes, UK) was used for the array CGH experiments. It contains 385,000 50-mer oligonucleotides with an average spacing of 2,586 base pairs (source – UCSC, build – galGal3). Test DNA was extracted from blood samples and feather pulp from 8 species (see section 2.5.1.1). The reference (Red Jungle Fowl) DNA, from the same animal used in the chicken genome sequencing project, was kindly provided by Dr Hans Cheng (Michigan State University). Labelling of genomic DNA and hybridization to the NimbleGen array were performed by the company (NimbleGen) and used random priming to incorporate modified nucleotides by either amino-allyl or direct linkage to either of the two dyes used (Cy3 and Cy5). All of the hybridizations in this experiment used two dyes per slide (Cy3 and Cy5). Red Jungle Fowl reference DNA (Cy5) was co-hybridized with test DNA (Cy3).

CGH analysis proceeded in three stages, normalization, window averaging and segmentation. After combining the signal intensity and genomic coordinate information, the Cy3 and Cy5 signal intensities were normalized to one another using Qspline normalization (Workman *et al.,* 2002). Qspline is a robust non-linear method for normalization using array signal distribution analysis and cubic splines. Once normalized, the data was prepared for DNA segmentation analysis. This included a window averaging step, where the probes that fall into a defined base pair window size are averaged, using the Tukey's biweight mean (Tukey, 1960). The Tukey's biweight method yields a robust weighted mean that is relatively insensitive to outliers, even when extreme. A new position was assigned to this average, which is the midpoint of the window. A window size of 25kb was used. The circular binary segmentation algorithm (Olshen *et al.,* 2004) was used to segment the averaged $\log_2$ ratio data. DNA segments were called by attempting to break the segments into sub-segments by looking at the t-statistic of the means. Permutations ($n = 1000$) were used to provide the reference distribution. If the resulting p-value was below the threshold (default of $p = 0.01$), then a breakpoint was called. A pruning step was used to remove spurious segments, rejecting segments where the standard deviation of the means was not sufficiently different. By default, a cut off of 1.5 standard deviations was used.

The preceding analysis steps were performed by Roche Nimblegen. The data provided was in the form of CSV (comma separated variable) files containing the location (e.g. chromosome, start position, stop position) of each segment detected by their algorithm. A template was designed in Microsoft Excel to take this data, from all species analysed, and further interrogate it. Following the approach of Redon *et al.* (2006), only CNVs containing more than 4 probes were considered for analysis.

The template scanned the input data for overlaps in CNV position. Overlapping CNVs were assigned to a CNVR. The complete list of CNVRs was output, with their start and stop positions, and the number of species sharing them. The $\log_2$ ratio indicated whether the CNV was a gain or loss relative to the reference sample; from this, each CNVR was designated as containing a gain, a loss, or both. Summary information was calculated on all this data (e.g. maximum, minimum, mean, median CNV and CNVR sizes). The list of CNVRs was then used to draw a graph, in which the CNVRs were listed along the x axis, and each species was listed on the y axis. Where a species has a CNVR, a dot was drawn. The dot was either blue (if the CNVR was unique to that species) or black (if the CNVR was shared). The current version of the template is designed to accept up to 5000 CNVs, from up to 13 species.

2.5.1.3. Assessment data spread in aCGH data

The spread of the data in $\log_2$ plots was assessed as a measure of noise. In order to compare the levels of noise between the species tested, standard deviations of log2 ratios were taken for every 10 probes across the unaveraged dataset (384,000 probes) – i.e. across 38,400 overlapping approximately 25kb windows. The resulting standard deviations were grouped into 0.01 size bins from 0 to 2 and plotted as a frequency distribution, with the interpretation that higher standard deviations would imply higher noise. The differences between distributions were tested using Mann-Whitney U tests, which tests the null hypothesis that independent samples (not assumed to be normally distributed) come from the same underlying distribution.

2.5.1.4. Analysis of segmental duplication data in chicken

The segmental duplication data published for chicken genome galGal2 (2004) release (Hillier *et al.,* 2004, Chung *et al.,* 2003) (accessible in Excel format from http://eichlerlab.gs.washington.edu/help/eray/CHICKEN/chicken.html) was converted to match the galGal3 (2006) release from which the microarrays were designed, using the

LiftOver tool in the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgLiftOver) with default settings. Converted segmental duplications were compared with CNVR data.

2.5.2. Nuclear organisation in avian macrophages

2.5.2.1. Extraction of RNA from macrophages

RNA was extracted from pre- and post-stimulated chicken macrophages using a Qiagen RNeasy mini kit (#74104) according to the manufacturer's instructions. Briefly, cell cultures in T75 flasks were trypsinised as per section 2.1.2, the cells added to 8ml complete RPMI and spun at 300g for 5 minutes. The supernatant was removed and the pellet resuspended in 600μl lysis buffer RLT. The cell suspension was pipetted into a QIAShredder column (Qiagen) and spun at 8000g for 2 minutes. 600μl of 70% ethanol was added and mixed by pipetting. The mixture was loaded into a QiaSpin column and spun at 8000g for 1 minute. The flow-through was discarded and 700μl buffer RW1 was added. The column was spun at 8000g for 1 min. 500μl of wash buffer RPE was added, and spun at 8000g for 15 seconds. Another 500μl of buffer RPE was added, and spun at 8000g for 2 minutes to dry the spin column. The RNA was eluted from the column in 30μl RNase free water by spinning at 8000g for 2 minutes. RNA concentration and purity were assessed on a Nanodrop spectrophotometer.

2.5.2.2. Hybridisation to Ark-Genomics 5K Chicken Immune Array

The ARK-Genomics chicken 5K immune cDNA microarray v3.1 was chosen for macrophage transcriptional analysis. The array is constructed from 2 new immune-related cDNA libraries and contains 5190 elements (in duplicate). Clones on the array originate from tissues known to contain high levels of cells related to the immune system, namely Bursa, Peyers patch, thymus and spleen. Represented on the array are genes that are known to cluster with existing chicken ESTs as well as unique genes, some of which have no known homologies and represent novel genes in the chicken collection. A series of reference genes (i.e. genes of known immune function) are also present on the array (Smith *et al.*, 2006).

Two technical replicates were performed for each sample. The RNA amplification, labelling. hybridisation and visualization was performed by ARK-Genomics according to their standard protocols (http://www.ark-genomics.org/protocols/). The mean was taken of the technical replicates. The ratio of signal in the LPS stimulated samples against the unstimulated samples was calculated. Genes were considered as being highly up- or down-regulated if the ratios were greater than 1.25 or less than 0.75 respectively.

2.6. Microscopy

FISH slides were analysed on an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD digital camera using appropriate filters. Images were captured using SmartCapture software (Digital Scientific UK) prior to being exported as TIFFs for analysis.

2.7. Image analysis

2.7.1. FLpter analysis

Analysis was performed using ImageJ (Abramoff *et al.*, 2004), following the protocol described by Lichter *et al.* (1990). The chromosome lengths were measured using the measure tool. The total length of the chromosome with the BAC signal was measured, then the length of the signal to the p terminus was measured. These were expressed as a ratio, with p terminus being 0.0 and the q terminus being 1.0.

2.7.2. Chromosome size measurements

Chromosome areas were measured on metaphase spreads using the freeform selection tool. The relative sizes of the chromosomes were calculated by comparing the labelled chromosome of interest to an easily identified small 'marker' chromosome. These marker chromosomes were GGA8, APL5 and MGA7. Each image was split into individual RBG channels. Using the blue (DAPI) channel the area of both GGA8 and the chromosome with the BAC signals were measured using the freehand selection tool and the analysis function.

Where chromosomes have undergone an evolutionary fission event (as in the case of ancestral chromosome 2 in the turkey), the areas of the derived chromosomes were summed and considered orthologous to the intact chromosome in chicken. Likewise, in the case of fusions (as in ancestral chromosomes 4 and 10 in chicken), the sum of the separate chromosomes was considered orthologous to the derived fused form. Hence the areas of MGA3+6 are orthologues to GGA2, and the areas of MGA4+9 and APL4+10 are orthologues to GGA4.

2.7.3. Determination of Nuclear Position of Chromosome Territories

The position of chromosomes within the nucleus was measured using an automated method based on Croft *et al.* (1999). A macro written for ImageJ (Michael Ellis, Digital Scientific UK) split each image of a nucleus to separate RGB planes and then converted the blue image (representing the DAPI counterstain) to a binary mask from which concentric regions of interest (rings) of equal area were created. The proportion of signal in each channel within each ring was measured relative to the total signal for that channel within the area covered by the binary mask. Results were logged and output to a spreadsheet for analysis. At least 50 nuclei were analysed for each BAC, providing 100 signals per BAC. All interphase nuclei examined for each species were derived from the same embryo.

The proportion of signal within each shell was normalised against the DAPI intensity with the same shell to compensate for flattening of the nucleus (Boyle *et al.*, 2001). The overall percentage of normalised signal within each shell was then calculated and a χ^2 test was performed to test for a significant difference to a random distribution. A random distribution was assumed to be an equal proportion of normalised signal in each shell, i.e. 20% in each shell. The percentage signal within each shell was used to calculate an 'overall' position for the signal in each nucleus image. The median value of the overall positions for all nuclei with a specific probe was taken as the overall position for the probe. Median values were used given the non-normal distribution of the data; similarly, interquartile ranges were calculated instead of standard errors of the mean. Spearman's rank (non-parametric) correlation coefficients were calculated to test for relationships between chromosome sizes, chromosome positions and gene densities (available for chicken only).

3. Specific aim 1: To establish the relative physical sizes of chicken, turkey and duck chromosomes (compared to published Ensembl estimates) and thereby devise a means through which anonymous clones can be assigned to avian chromosomes within a minimum number of steps.

3.1.Background

Cytogenetic mapping involving FISH experiments on metaphase chromosomes (or interphase nuclei) is the lowest resolution of the techniques used for gene mapping. It has thus been a common feature of all genome projects to date (e.g. Schmid *et al.,* 2000; 2005) and can be important for karyotype definition. The process of genome sequencing usually involves the development of a library of clones from which a physical map can be assembled and to which the developing genome sequence can be assigned (see section 1.4.2). Although the method of isolation theoretically ensures that clones are linked to a particular locus in the developing sequence assembly (and therefore already assigned to a particular chromosome region), BACs can often be clearly shown by FISH to map to a different chromosome than expected from *in silico* approaches (Aerts *et al.,* 2005). This is often due to repetitive sequences, which can cause inaccuracies in *in silico* assignments. Furthermore, clones are frequently generated that are not assigned to the sequence assembly (termed anonymous clones). These can however be assigned using FISH (Aerts *et al.,* 2005).

Essential for cytogenetic mapping is the ability to identify unequivocally the chromosome on which the FISH signal appears. Cytogenetic experience of human karyotyping is relatively common; however, for other animals there is little or no cytogenetic expertise. In these cases, labs may rely on multicolour FISH (m-FISH) experiments, which can be time consuming, expensive and prone to technical difficulties (Jentsch *et al.,* 2001). Furthermore, although for many mammals, karyotyping is no more difficult than in humans (e.g. chimpanzee, pig), for other animals (e.g. mice, dogs) karyotyping is much more difficult as the chromosomes are more numerous, and banding patterns are less distinct (Macgregor, 1993). This is especially true of birds because karyotyping by standard means is literally impossible (Griffin *et al.,* 1999; Masabanda *et al.,* 2004).

As the genomes of an increasing number of avian species begin to have mapping and sequencing efforts associated with them, the robust and reproducible cytogenetic identification of chromosomes in a range of birds will become more important. The upcoming publication of the zebra finch genome sequence will require confirmation of microchromosomal syntenies and of *in silico* comparisons with the chicken genome. A high throughput strategy therefore requires a simple and expedient way of identifying avian chromosomes on which an anonymous clone may lie.

Furthermore, studies of nuclear organisation (see also chapter 5), recognised as important for understanding both normal and disease related cellular phenotypes, are also dependent on accurate measurements of chromosome sizes, and on the reliability of reported genome assemblies. A first step therefore towards a detailed analysis of nuclear organisation in birds is to establish chromosome sizes in chicken and other birds of interest for future genome sequencing efforts. From the perspective of nuclear organisation, it is also of interest to assess whether the chromosome size estimates reported in Ensembl are reflective of physically measured chromosome sizes.

3.2. Specific Aims

Considering the above, the detailed specific aims of this chapter were as follows:

Specific aim 1a: To measure chromosome sizes of chicken, duck and turkey by comparison to the smallest chromosome recognisable by morphology.

Specific aim 1b: To test the hypothesis that Ensembl estimates of chromosome length are accurate representations of physical chromosome size as measured down the microscope.

Specific aim 1c: To combine FISH and image analysis approaches to devise a means through which anonymous (e.g. BAC) clones can be assigned to avian chromosomes within two easy steps.

3.3. Results

3.3.1. Determination of chromosome sizes in chicken, turkey and duck

BAC clones with known position for chromosomes GGA1-24, 26-28 were hybridised to chicken, turkey and duck metaphase spreads. Chromosome sizes were measured relative to easily identifiable 'marker' chromosomes – chosen on the basis of being both small and easily identifiable based simply on their morphology and/or DAPI banding pattern. The marker chromosomes chosen were GGA8, MGA7 and APL5. GGA8 is metacentric; MGA7 is sub-metacentric; APL5 is telocentric with a distinctive central DAPI negative band. Measurements of chromosome sizes were performed in ImageJ (Figure 3.1, Figure 3.2).

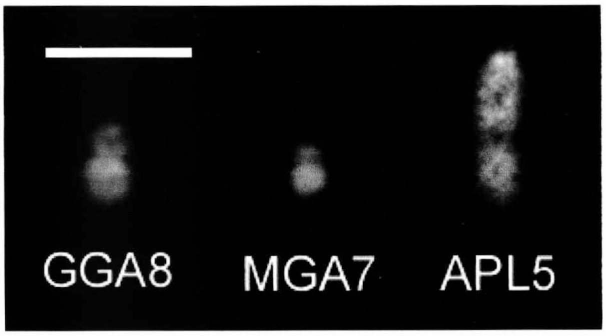
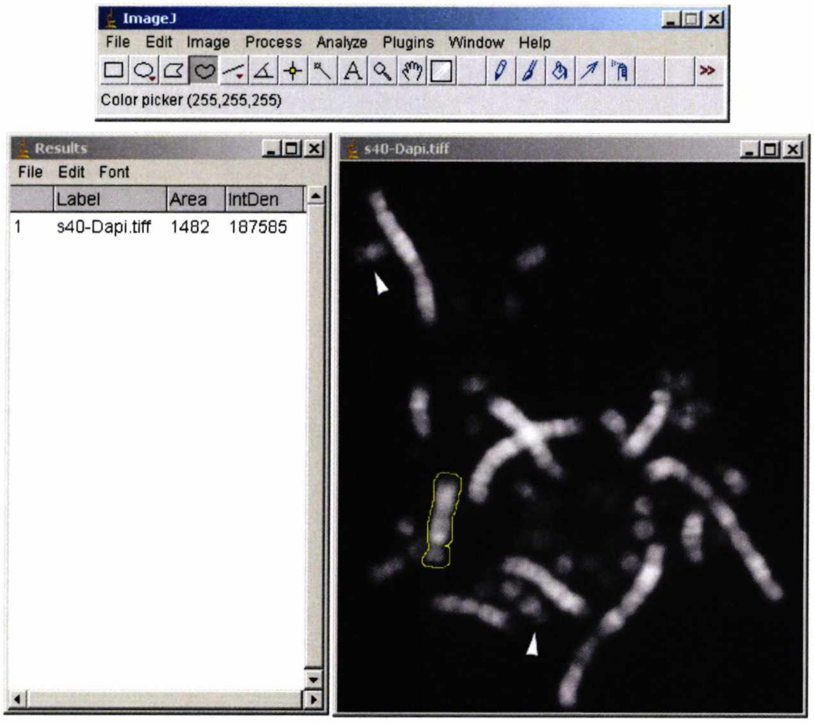


Figure 3.1: Marker chromosomes used for chromosome size measurements; all are readily distinguishable in a metaphase spread. Scale bar represents 5μm.



Color picker (255,255,255)

	Label	Area	IntDen
1	s40-Dapi.tiff	1482	187585

Figure 3.2: ImageJ screenshot showing measurement of GGA4 area. Arrows indicate GGA8.

Two chromosomal rearrangements distinguish chicken and turkey, with known orthologies. The ancestral chromosomes 4 and 10 (GGA4q, 4p) are conserved in duck as APL4 and a microchromosome. The duck chromosome orthologous to GGA4p was identified using BACs WAG112C24 (Figure 3.3) and WAG23I06 (see also chapter 4).

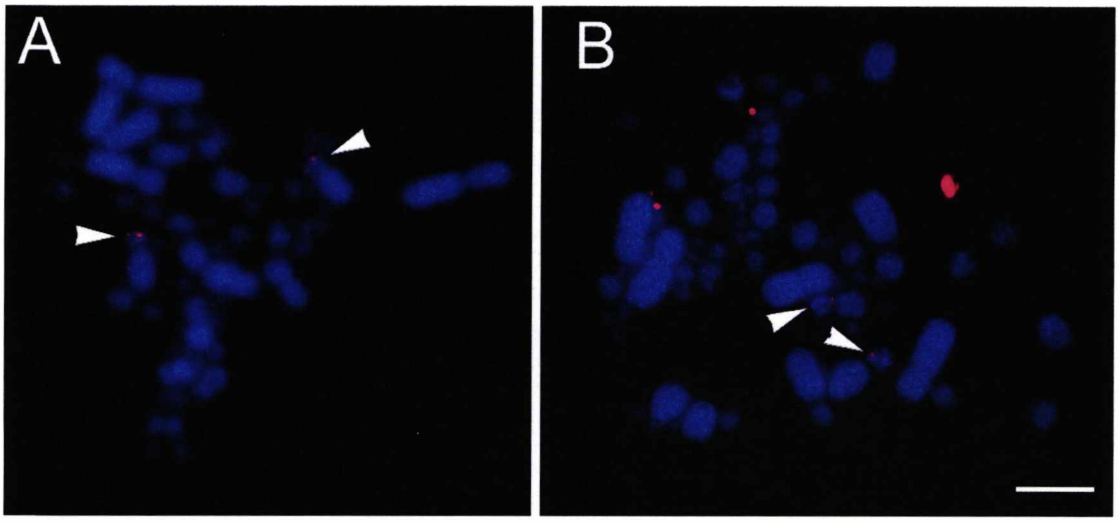


Figure 3.3: BAC WAG112C24 hybridised to A) chicken, GGA4p; B) duck, APL10 (arrowed). Scale bar represents 5µm.

The area of the GGA4p orthologue chromosome was compared with the areas of other duck chromosomes (Figure 3.4). It was determined that it is the tenth largest chromosome, i.e. APL10.

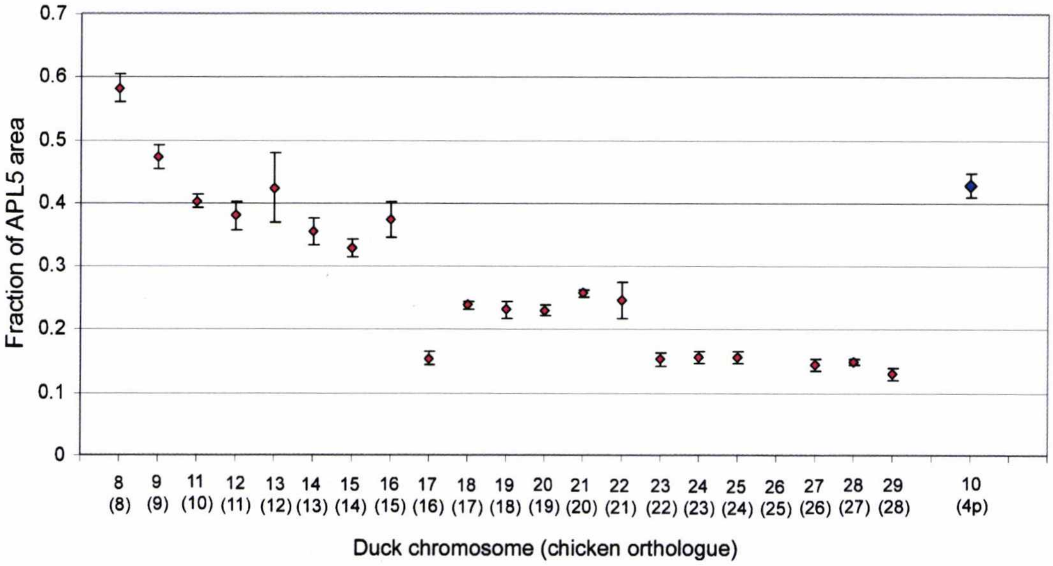


Figure 3.4: Identification of the duck microchromosome orthologous to GGA4p. Measured areas of duck microchromosomes are shown as a fraction of APL5 area. The orthologue of GGA4p (blue, right) is seen to be APL10 by size. Error bars represent standard error of the mean.

The relative chromosome sizes as measured in chicken, turkey and duck are presented in Table 3.1. With a measure of the sizes of chromosomes in each of the three species, a comparison was made between orthologous chromosome sizes. The data show a strong correlation between chicken and turkey orthologous chromosome sizes (Spearman's rank, $\rho=0.97$, $n=25$, $p<0.0001$), and between chicken and duck orthologous chromosome sizes (Spearman's rank, $\rho=0.96$, $n=29$, $p<0.0001$), indicating few differences in chromosome size order in these species. For the purposes of ranking the data, where the ancestral 2 has undergone a fission in the turkey lineage, the areas of MGA3+6 have been summed and considered orthologous to GGA2. Likewise, in the case of the fusion of ancestral 4 and 10 in chicken, the sum of the separate chromosomes in turkey and duck is considered orthologous to the derived fused form. The combined areas of MGA3+6 were found to be greater than the area of GGA2; similarly, the areas of MGA4+9 and APL4+10 were found to be greater than the area of GGA4.

Chicken					Turkey			Duck		
GGA	Area c.f. GGA8	SE	Base pairs (Ensembl)	Ratio c.f. GGA8	MGA	Area c.f. MGA7	SE	APL	Area c.f. APL5	SE
1	4.66	0.01	200,994,015	6.55	1	4.05	0.01	1	2.78	0.05
2	3.04	0.01	154,873,767	5.05	6	1.25	0.01	2	2.14	0.04
					3	1.90	0.01			
3	2.35	0.02	113,657,789	3.71	2	2.45	0.01	3	1.59	0.03
4	1.84	0.02	94,230,402	3.07	9	0.60	0.01	10	0.43	0.02
					4	1.70	0.01	4	1.08	0.02
5	1.71	0.01	62,238,931	2.03	5	1.44	0.01	5	1.00	-
6	1.25	0.01	37,400,442	1.22	8	0.92	0.01	6	0.59	0.03
7	1.14	0.01	38,384,769	1.25	7	1.00	-	7	0.61	0.01
8	1.00	-	30,024,636	1.00	10	0.80	0.01	8	0.58	0.02
9	0.80	0.01	25,554,352	0.78	11	0.70	0.01	9	0.47	0.02
10	0.72	0.01	20,909,726	0.70	12	0.53	0.01	11	0.40	0.01
11	0.58	0.01	19,020,054	0.63	13	0.43	0.02	12	0.38	0.02
12	0.57	0.03	19,821,895	0.66	14	0.46	0.01	13	0.42	0.05
13	0.62	0.02	17,279,963	0.58	15	0.27	0.01	14	0.35	0.02
14	0.53	0.02	20,603,938	0.69	16	0.42	0.01	15	0.33	0.01
15	0.55	0.02	12,438,626	0.41	17	0.41	0.02	16	0.37	0.03
16	0.31	0.03	239,457	0.01	18	0.16	0.02	17	0.15	0.01
17	0.47	0.02	10,632,206	0.35	19	0.30	0.02	18	0.24	0.01
18	0.38	0.02	8,919,268	0.30	20	-	-	19	0.23	0.01
19	0.38	0.02	9,463,882	0.32	21	0.31	0.01	20	0.23	0.01
20	0.43	0.02	13,506,680	0.45	22	0.42	0.02	21	0.26	0.01
21	0.22	0.01	6,202,554	0.21	23	0.21	0.02	22	0.25	0.03
22	0.34	0.02	2,228,820	0.07	24	0.16	0.02	23	0.15	0.01
23	0.23	0.04	5,666,127	0.19	25	0.25	0.01	24	0.16	0.01
24	0.30	0.03	5,910,111	0.20	26	0.18	0.02	25	0.16	0.01
26	0.24	0.03	4,255,270	0.14	28	-	-	27	0.14	0.01
27	0.22	0.01	4,841,970	0.09	29	0.13	0.03	28	0.15	0.01
28	0.22	0.02	4,512,026	0.16	30	0.13	0.02	29	0.13	0.01
Z	2.17	0.01	74,602,320	2.43	Z	2.06	0.01	Z	1.20	0.05
W	1.12	0.01	259,642	0.0.1	W	1.28	0.01	W	0.53	0.04

Table 3.1: The areas of measured chromosomes in chicken (GGA), turkey (MGA) and duck (APL). Orthologous chromosomes are aligned. GGA2 corresponds to MGA3 and MGA6. GGA4 corresponds to MGA4 and MGA9, and to APL4 and APL10. SE represents standard error of the mean. Chromosome base pair lengths and ratios to GGA8 are based on data from Ensembl.

3.3.2. Comparison of chicken Ensembl base pair estimates with measured areas

The measured areas for chicken were compared with the published base-pair estimates from Ensembl (www.ensembl.org/Gallus_gallus; Release 52, Hubbard *et al.,* 2009) (Table 3.1). A correlation was seen between the two data sets (Spearman's rank, $\rho=0.86$, $n=29$, $p<0.0001$). Plotting the chicken area data versus the Ensembl data revealed that the measured areas were smaller than might be expected for the larger chromosomes (Figure 3.5A), but much closer to a 1:1 ratio for the microchromosomes (Figure 3.5B). Some chromosomes did not fit the trend; the Ensembl estimates were smaller than the measured size of the chromosome. These were chromosomes GGA16, 22 and W.

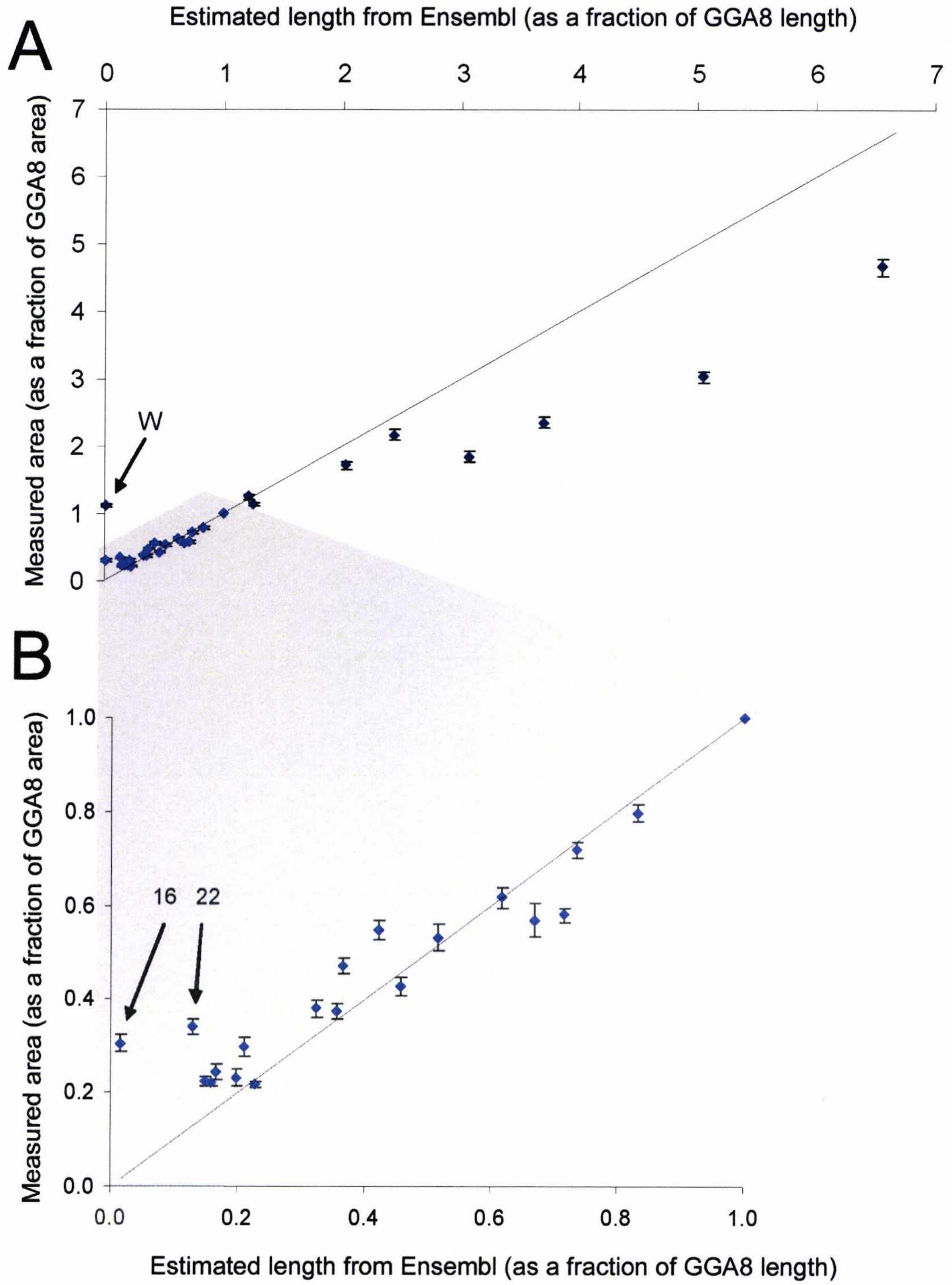


Figure 3.5 Comparison of chicken chromosome lengths from Ensembl with measured areas. (A) shows all chromosomes. The smaller chromosomes GGA8-28 are highlighted in grey. This region is expanded in (B). Lines show 1:1 ratios. Most microchromosomes fit closely to Ensembl predictions; macrochromosomes tend to be smaller than expected. Three major outliers, GGA16, 22 and W are indicated. Error bars represent standard error of the mean.

3.3.3. Assignment of BACs using a two step FISH approach

Based on the measured chromosome areas, a two step FISH approach was devised for the assignment of anonymous clones to avian chromosomes. In the first step, the clone was hybridised, and the area of its chromosome was measured relative to the marker chromosome. The two chromosomes with reference ratio measurements immediately greater than the derived value, and the two immediately less than the derived value were selected. Occasionally it was necessary, for the smaller chromosomes, to take three values above and three below to narrow down the candidates to six. This then prompted four (or six) parallel dual colour experiments with previously assigned BACs to facilitate a final assignment.

This method was tested in chicken; 28 BACs were mapped using the approach described; the identities of these BACs were obscured until after assignments had been made. Details of the BACs used for mapping are given in Table 3.2.

Marker	Ratio to GGA8	GGA	Assignment "short list"	Assignment correct?
WAG12F03	0.71	11	9, 10, 13, 11	Yes
WAG129C21	0.72	15	9, 10, 13, 11	Yes
WAG18H2	0.65	13	9, 10, 13, 11	Yes
WAG47D10	0.67	12	9, 10, 13, 11	within 6
WAG93I01	0.55	15	11, 12, 15, 14	Yes
WAG117D7	0.50	17	15, 14, 17, 20	Yes
WAG28L18	0.41	23	17, 20, 19, 18	No
WAG14G12	0.87	13	9, 10, 13, 11	Yes
WAG41P17	0.52	20	15, 14, 17, 20	Yes
WAG13J20	0.37	24	19, 18, 22, 16	within 6
WAG13I13	0.52	17	15, 14, 17, 20	Yes
WAG92I14	0.83	10	9, 10, 13, 11	Yes
WAG8K20	0.72	10	9, 10, 13, 11	Yes
WAG15I8	0.63	10	9, 10, 13, 11	Yes
WAG8G10	0.71	10	9, 10, 13, 11	Yes
WAG40G23	0.44	17	14, 17, 20, 19	Yes
WAG119F5	0.37	18	19, 18, 22, 16	Yes
WAG99H21	0.32	24	18, 22, 16, 24	Yes
CTG1704	0.49	19	15, 14, 17, 20	within 6
WAG122G20	0.35	18	19, 18, 22, 16	Yes
WAG20E8	0.35	24	19, 18, 22, 16	within 6
WAG19B13	0.38	18	17, 20, 19, 18	Yes
WAG67B16	0.58	12	13, 11, 12, 15	Yes
WAG1D2	0.43	18	14, 17, 20, 19	within 6
WAG40F11	0.34	26	18, 22, 16, 24	within 6
WAG14H23	0.41	18	17, 20, 19, 18	Yes
WAG55M22	0.50	19	15, 14, 17, 20	within 6
CTG0107	0.52	19	15, 14, 17, 20	within 6
Number successful (within 6):				19 (27) / 28

Table 3.2: Assignment of clones to chromosomes based on size measurements. Short lists for dual colour FISH experiments were drawn up from physical area measurements. Only one BAC was not correctly assigned within 6 chromosomes.

The ratios derived proved to be reliable in narrowing down the ratio to, in most cases, four chromosomes. According to the results, ratio measurements could narrow down the chromosome of interest to four possible candidates for 19 of the BACs (68%). If six possible candidates were considered then all but one were narrowed down (96%). It was noted that estimates did become less reliable with decreasing chromosome size.

3.4. Discussion

3.4.1. Chromosome sizes in chicken, turkey and duck

This study has demonstrated that across these three species the overall karyotypic conservation is mirrored by the relative sizes of orthologous chromosomes, indicating that there has been very little expansion or reduction in chromosome size – and hence genome size – over 90 million years (the divergence between chicken and duck; van Tuinen *et al.,* 2001). This highlights the conservation among birds in general, and among the Galloanserae (land and water fowl) in particular. The combined areas of turkey chromosomes 3 and 6 were larger than the area of GGA2, and the areas of the two chromosomes corresponding to ancestral chromosomes 4 and 10 in turkey and duck were larger than the area of GGA4. This suggests that the fission of ancestral chromosome 2 was accompanied by an expansion of genetic material, most likely centromeric repeats as the new centromeres were established. Conversely, the fusion of the ancestral chromosomes in the GGA4 lineage was accompanied by a reduction in size, again consistent with centromeric heterochromatin loss, and the loss of centromeric telomere sites (Nanda *et al.,* 2002). A loss of repetitive material is also consistent with theories of genome size constraint in birds (Gregory, 2002, Burt *et al.,* 1999).

3.4.2. Comparison of Ensembl estimates with measured areas of chicken chromosomes

The pattern of measured chromosome sizes in chicken broadly fits that from the Ensembl data, with a few obvious exceptions. These are GGAW, 16 and 22. GGAW is slightly larger than GGA8, as seen down the microscope; the assigned sequence however only covers ~260kb. This is due to the high heterochromatic repetitive content of the W chromosome (Burt, 2005, Hillier *et al.,* 2004), evidenced by its bright DAPI staining. The sequence data for GGA16 also under represents the true size of the chromosomes, likely due to repetitive content; GGA16 contains the chicken MHC region, and nucleolar organiser region (NOR) not yet represented in Ensembl, thus a large number of genes are missing from the sequence assembly (see also chapter 7). While GGA22 appears larger than the sequence data would predict, it is unclear whether this represents un-accounted for gene content, or heterochromatin.

Notably, the measured sizes of the larger macrochromosomes are smaller than expected from the sequence data. This may be explicable by the higher amounts of heterochromatin on the macrochromosomes as compared with the microchromosomes (Burt 2002, Hillier *et al.,* 2004). Although it might be expected that this would cause the measured chromosome sizes to be even larger than the Ensembl estimates suggest, these regions could undergo increased compaction and condensation. By this hypothesis, microchromosomes should be less affected, according with the better agreement seen between the measured sizes and the Ensembl data.

3.4.3. Development of a two step FISH mapping approach

Although m-FISH experiments can theoretically be used to resolve complex karyotypes and allow BAC mapping in a single step, in practice such methods can be precluded by cost, time and resources. The approach developed here is, potentially, transferable to all birds. It should be noted, however, that the chosen marker chromosome needs to be of a sufficiently small size to allow reliably distinguishable chromosome size ratios to be derived. For example, the use of APL5 as a marker chromosome might make the measured differences between chromosomes too small for a reliable short-list of only 4-6 chromosomes to be drawn up. Therefore in some other avian species it might be appropriate to perform a dual colour rather than a single colour first experiment, using marker chromosome identifier probe(s) to narrow down the assignment. Obviously, difficulty of assignment is inversely proportional to chromosome size and therefore this approach becomes less accurate for the smallest chromosomes. The only probes thus far assigned to the chicken genome for GGA29 onwards are single cosmids for GGA29, 30 and 31 and chromosome paints for GGA33-38 (Masabanda *et al.,* 2004). Thus cytogenetic mapping is unlikely to become commonplace to chromosomes of this size in other avian species.

The accuracy of the measurements must also be taken into consideration. Although a simple technique, the user must be precise in measuring chromosome area, and able to reliably identify the marker chromosome. An example of this would be the tendency for those new to the chicken karyotype to confuse chromosomes 8 and W (similar in size and, to some degree, morphology).

A second source of potential error is biological; it has been mentioned that the number of tandem repeats of rRNA genes on GGA16 can vary dependent on chicken breed, with a variation of up to 2Mb in repeat content between breeds (Delany and Krupkin, 1999). It is possible that other elements affecting chromosome size are polymorphic within populations. In birds especially, and indeed any organism with microchromosomes, the effects of relatively minor differences could have a significant effect. Quantitatively, the smallest chicken chromosomes are estimated to contain approximately 4Mb (Schmid *et al.*, 2000, 2005). A gain or loss of 0.5Mb – which has been observed as a copy number variant between chicken and turkey, albeit on a larger chromosome (Griffin *et al.*, 2008) – could appear as a 12.5% change in the size of the chromosome. Again, this tends towards the increased reliability of the technique among the larger microchromosomes.

Thirdly, the observed size of chromosomes is dependent upon their degree of condensation. Metaphase preparations from unsynchronised cultures will contain metaphase spreads at a number of different stages of condensation; at each stage in the condensation process, the ratio of a given chromosome's size to that of another chromosome will vary to some degree. Those using the technique should ensure, as far as possible, that their cell cultures are synchronised.

3.5. Conclusion

The approach presented here represents a method which can be used to map BACs, cosmids, YACs and PACs in species with differential chromosome sizes beyond the reach of conventional karyotyping. The caveat of course is that as chromosome sizes become smaller and less distinguishable, the importance of user accuracy increases. The information produced here lays the groundwork for more detailed work in comparative genomic investigations into physical mapping (for example, see chapter 4) and nuclear organisation (see chapter 5).

4. Specific aim 2: To perform comparative physical mapping (by FISH) between chicken and duck with a view to the generation of a cytogenetic genome map in duck and the establishment of inter- and intra- chromosomal rearrangements between the two species

4.1. Background

The duck (*Anas platyrhynchos,* APL) is an obvious avian target for comparative genomics, for agricultural reasons and also for immunological studies because of their resistance to avian influenza (see section 1.7). Previous chromosome painting and cytogenetic mapping data in duck is described in detail in section 1.2.3. However, to date, duck genomics is limited to 240 genetic markers and 85 physical chromosomal markers. A crucial next step is to enrich the physical map as a forerunner for sequencing efforts that are currently underway in China (Huang *et al.*, 2008).

Traditionally, the presence of microchromosomes has caused problems in defining avian karyotypes which has only been circumvented recently in chicken, using molecular cytogenetic techniques (Masabanda *et al.*, 2004); similar studies on microchromosomes remain limited (e.g. Fillon *et al.*, 2007, Kayang *et al.*, 2006, Griffin *et al.*, 2008).

Therefore, the purpose of this chapter was to develop a detailed cytogenetic map for the duck based on comparative FISH mapping of a large number of chicken BACs and thus provide markers for duck chromosomes. This will facilitate physical gene mapping and will permit an improved integration of the existing cytogenetic and genetic mapping information (Huang *et al.*, 2006). Such a map will reveal whether there are hitherto undiscovered chromosomal inversions that are of interest from an evolutionary perspective (that is, it has been suggested that species specific differences will accumulate in these regions, thereby giving insights into the genetic mechanisms underlying speciation (Price, 2008; Kirkpatrick and Barton, 2006)). Finally it will allow for a better definition of the duck karyotype, in particular through identification of the duck microchromosomes.

4.2.Specific Aims

Given the above rationale, the specific aims of this chapter were as follows:

Specific aim 2a: To map 155 representative chicken BAC clones to duck metaphases; thereby to generate a comparative molecular cytogenetic genome map of the duck.

Specific aim 2b: To establish the existence of intra-chromosomal rearrangements between the two species.

Specific aim 2c: To test the hypothesis that inter-chromosomal rearrangements among the microchromosomes exist between chicken and duck.

Specific aim 2d: To develop a set of FISH marker clones for duck microchromosomes; thereby to define the duck karyotype.

4.3. Results

4.3.1. A comparative molecular cytogenetic genome map of the duck

Of 400 BACs that successfully hybridized to chicken metaphases, 155 (39%) could be visualized with confidence on duck chromosomes (for example, Figure 4.1). These covered the majority of the karyotype i.e. APL1-29 (except 26). Figure 4.2 shows the G-banded ideograms for GGA and APL1-8, with the positions of the BACs mapped to these chromosomes (full data in Table 4.1 (overleaf)). Only one interchromosomal difference was detected among the macrochromosomes, the retention of the ancestral chromosomes 4 and 10 in duck.

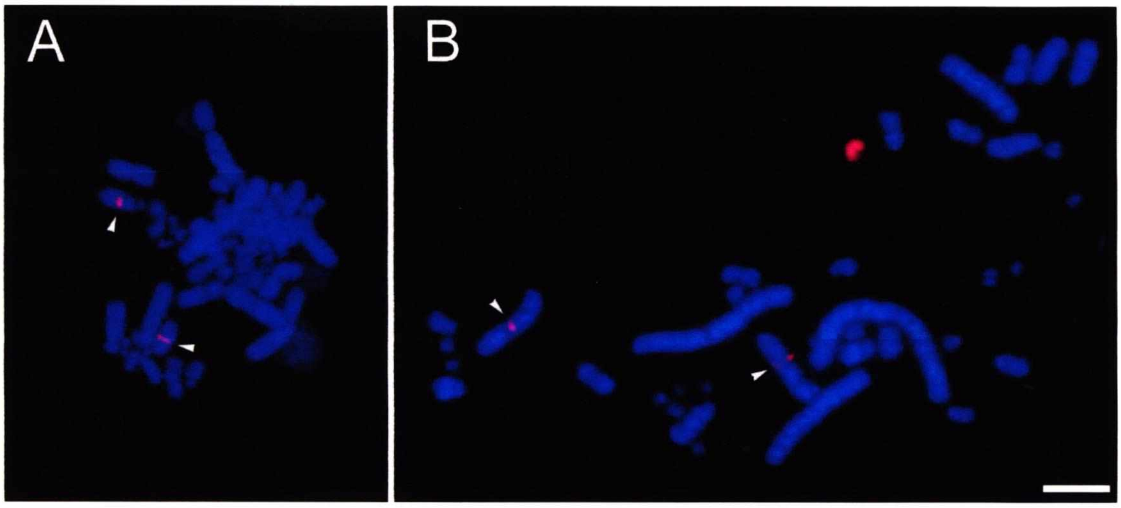


Figure 4.1: Example FISH image of BAC WAG27H3 mapping to A) GGA5 and B) APL5. APL5 can be easily recognised, as described in the previous chapter. Scale bar represents 5µm.

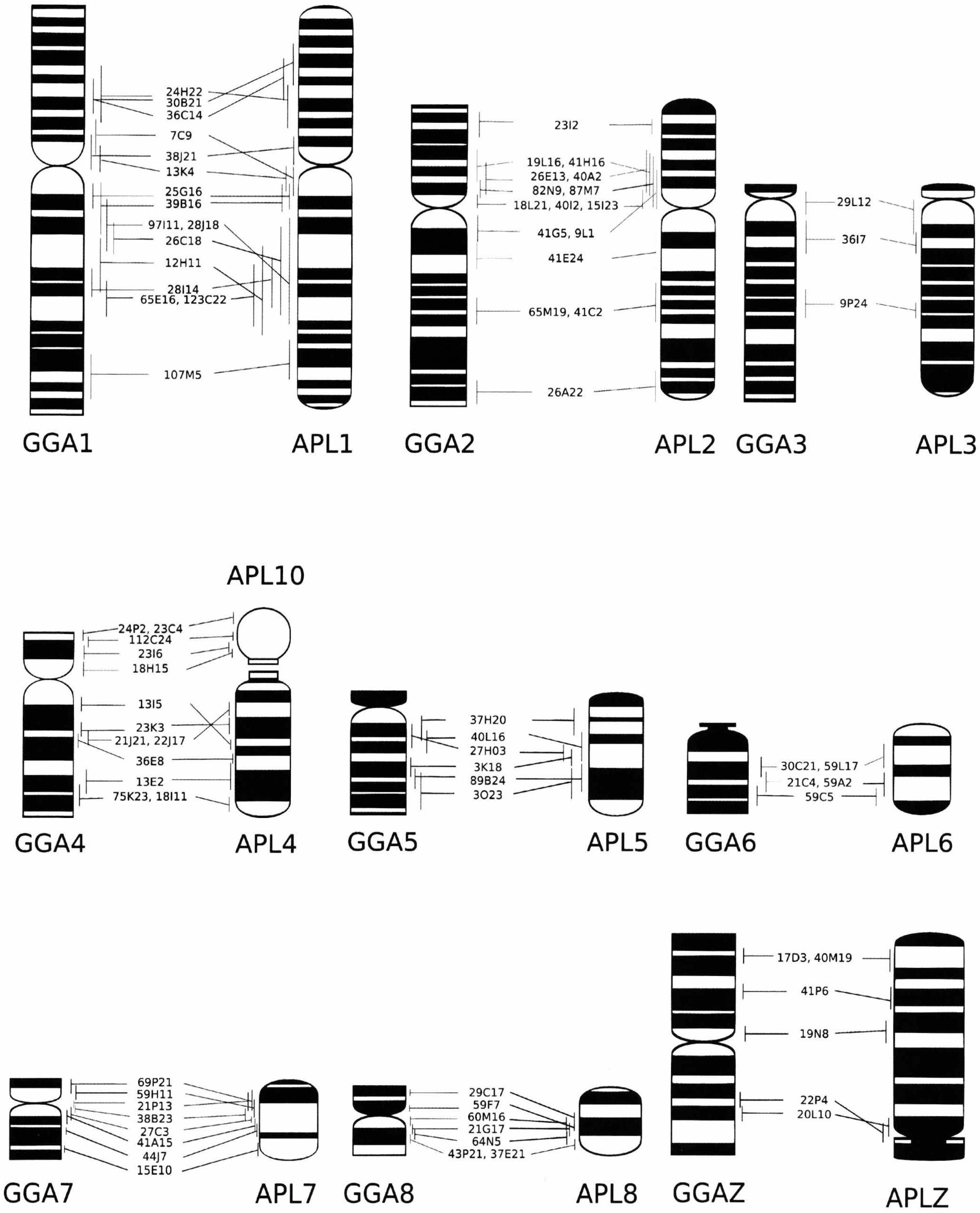


Figure 4.2: The G-banded ideograms of chicken and duck chromosomes one to eight and Z are shown with the positions of all BACs successfully hybridised to both species as determined by FLpter measurements. Intrachromosomal rearrangements can be seen on GGA and APL1, 2, 4, 7, 8 and Z. GGA4p corresponds to APL10. Note the orientation of APLZ. Ideograms were prepared from Ladjali-Mohammedi *et al.* (1999) and Fillon *et al.* (2007). Error bars represent one standard deviation. Details are presented on the following page.

GGA BAC clone	GGA chromosome	GGA Marker	GGA FLpter	GGA FLpter SD	GGA metaphases	APL chromosome	APL FLpter	APL FLpter SD	APL metaphases
WAG24H22	GGA1p	LEI0068	0.23	0.03	9	APL1p	0.24	0.02	8
WAG30B21	GGA1p	LEI0194	0.24	0.03	8	APL1p	0.14	0.03	10
WAG36C14	GGA1p		0.24	0.03	6	APL1p	0.19	0.02	4
WAG7C9	GGA1p		0.32	0.03	6	APL1q	0.40	0.02	9
WAG38J21	GGA1p		0.38	0.03	7	APL1p	0.36	0.06	7
WAG13K4	GGA1p		0.42	0.03	9	APL1q	0.42	0.03	6
WAG25G16	GGA1q	LEI0101	0.47	0.03	4	APL1q	0.47	0.03	8
WAG39B16	GGA1q	MCW0068	0.48	0.03	10	APL1q	0.48	0.03	3
WAG26C18	GGA1q	MCW0200	0.58	0.02	9	APL1q	0.64	0.08	6
WAG12H11	GGA1q	LEI0091	0.63	0.04	8	APL1q	0.75	0.09	5
WAG28I14	GGA1q	LEI0169	0.69	0.02	6	APL1q	0.68	0.03	7
WAG65E16	GGA1q	LEI0107	0.73	0.02	8	APL1q	0.73	0.05	5
WAG107M5	GGA1q	ADL0101	0.90	0.04	8	APL1q	0.89	0.03	7
WAG23I2	GGA2p	MCW0082	0.06	0.02	8	APL2p	0.09	0.02	8
WAG19L16	GGA2p	ADL0309	0.21	0.03	3	APL2p	0.27	0.06	5
WAG40A2	GGA2p	ADL0176	0.25	0.03	6	APL2p	0.23	0.04	4
WAG41H16	GGA2p	MCW0239	0.25	0.04	7	APL2p	0.24	0.04	11
WAG26E13	GGA2p	MCW0131	0.25	0.03	5	APL2p	0.25	0.04	9
WAG85M7	GGA2p	ADL0120	0.27	0.02	3	APL2p	0.27	0.06	7
WAG82N9	GGA2p	MCW0274	0.29	0.03	3	APL2p	0.29	0.04	3
WAG15I23	GGA2p		0.31	0.04	11	APL2p	0.41	0.04	9
WAG40I2	GGA2p		0.34	0.03	10	APL2p	0.39	0.06	7
WAG18L21	GGA2q		0.36	0.03	10	APL2p	0.37	0.03	9
WAG9L1	GGA2q	LEI0129	0.42	0.03	8	APL2p	0.31	0.03	6
WAG41G5	GGA2q		0.42	0.03	11	APL2p	0.33	0.07	8
WAG41E24	GGA2q		0.51	0.04	10	APL2q	0.55	0.04	11
WAG41C2	GGA2q	LEI0147	0.67	0.03	2	APL2q	0.69	0.04	8
WAG65M19	GGA2q	ABR0008	0.68	0.02	7	APL2q	0.67	0.05	9
WAG26A22	GGA2q	MCW0157	0.96	0.02	7	APL2q	0.96	0.02	7
WAG29L12	GGA3	MCW0261	0.09	0.04	7	APL3	0.12	0.04	8
WAG36I7	GGA3	ADL0370	0.24	0.05	6	APL3	0.28	0.03	5
WAG9P24	GGA3	MCW0127	0.55	0.04	4	APL3	0.60	0.04	2
WAG112C24	GGA4p	ADL0317	0.05	0.02	8	APL10	0.45	0.05	6
WAG24P2	GGA4p		0.06	0.02	9	APL10	0.80	0.09	8
WAG23C4	GGA4p		0.06	0.03	9	APL10	0.90	0.06	5
WAG23I6	GGA4p	ADL0203	0.12	0.02	6	APL10	0.34	0.06	4
WAG18H15	GGA4p		0.15	0.04	10	APL10	0.24	0.08	6
WAG13I5	GGA4q		0.40	0.06	12	APL4	0.52	0.07	9
WAG22J17	GGA4q		0.47	0.05	15	APL4	0.17	0.07	8
WAG23K3	GGA4q		0.53	0.04	9	APL4	0.34	0.05	7
WAG36E8	GGA4q		0.56	0.03	7	APL4	0.61	0.07	10
WAG21J21	GGA4q		0.58	0.03	7	APL4	0.25	0.08	10
WAG75K23	GGA4q		0.88	0.06	12	APL4	0.86	0.05	8
WAG13E2	GGA4q	LEI0063	0.89	0.04	6	APL4	0.92	0.04	5
WAG18I11	GGA4q		0.93	0.03	9	APL4	0.92	0.03	10
WAG37H20	GGA5	MCW0263	0.24	0.06	4	APL5	0.22	0.07	4
WAG40L16	GGA5	MCW0193	0.38	0.06	3	APL5	0.46	0.08	5
WAG27H3	GGA5	ROS0013	0.42	0.06	8	APL5	0.51	0.06	11
WAG03K18	GGA5	MCW0210	0.62	0.05	7	APL5	0.54	0.03	4
WAG89B24	GGA5	MCW0113	0.70	0.06	9	APL5	0.71	0.07	6
WAG03O23	GGA5	ADL0166	0.81	0.08	5	APL5	0.72	0.08	5
WAG59L17	GGA6		0.45	0.11	11	APL6	0.41	0.13	4
WAG30C21	GGA6	ADL0040	0.49	0.07	4	APL6	0.36	0.10	4
WAG21C4	GGA6		0.62	0.12	10	APL6	0.65	0.10	5
WAG59C5	GGA6		0.80	0.07	10	APL6	0.73	0.11	10
WAG69P21	GGA7	LEI0064	0.08	0.05	6	APL7	0.26	0.07	11
WAG59H11	GGA7		0.17	0.07	12	APL7	0.33	0.11	8
WAG38B23	GGA7		0.40	0.13	10	APL7	0.38	0.11	7
WAG27C3	GGA7	MCW0201	0.44	0.07	3	APL7	0.46	0.03	2
WAG41A15	GGA7		0.45	0.11	11	APL7	0.54	0.09	10
WAG44J7	GGA7		0.64	0.06	4	APL7	0.59	0.08	4
WAG15E10	GGA7		0.86	0.05	8	APL1q	0.84	0.07	9
WAG29C17	GGA8	MCW0275	0.14	0.03	6	APL8	0.45	0.04	3
WAG59F7	GGA8		0.29	0.09	11	APL8	0.50	0.14	8
WAG60M16	GGA8	ADL0302	0.45	0.08	3	APL8	0.54	0.05	10
WAG21G17	GGA8		0.61	0.10	9	APL8	0.63	0.10	6
WAG64N5	GGA8		0.72	0.14	9	APL8	0.74	0.09	11
WAG43P21	GGA8	ADL0301	0.75	0.05	3	APL8	0.87	0.07	4
WAG37E21	GGA8		0.80	0.11	9	APL8	0.87	0.06	10
WAG40M19	GGAZ		0.08	0.04	14	APLZ	0.91	0.04	8
WAG17D3	GGAZ		0.11	0.04	19	APLZ	0.91	0.04	8
WAG41P6	GGAZ		0.27	0.04	10	APLZ	0.69	0.08	10
WAG19N8	GGAZ		0.48	0.05	10	APLZ	0.56	0.06	10
WAG20L10	GGAZ		0.74	0.05	10	APLZ	0.11	0.06	11
WAG22P4	GGAZ		0.80	0.06	8	APLZ	0.16	0.06	9

Table 4.1: Chicken BACs successfully hybridised to duck macrochromosomes. FLpter represents Fractional Length from the p terminus (Lichter *et al.*, 1990); SD represents standard deviation.

4.3.2. Intra-chromosomal rearrangements between chicken and duck

FISH mapping suggested intrachromosomal rearrangements between GGA and APL1, 2, 4, 7, 8 and Z. BACs WAG24H22, WAG30B21 and WAG36C14 clearly evidenced a rearrangement on GGA1p and APL1p (Figure 4.2; Table 4.1). The order of BACs was not completely inverted, suggesting that the underlying rearrangement may be a translocation rather than a paracentric inversion. BAC WAG7C9 mapped to GGA1p and APL1q, indicating a small pericentric inversion. Some BACs mapping to GGA1q and APL1q suggested possible rearrangements on these chromosome arms; however, substantial variation in the FLpters determined for these BACs in duck made it difficult to distinguish artefacts from real changes in marker order.

BACs WAG42G5 and WAG9L1 both mapped to GGA2q and APL2p, evidencing a pericentric inversion (Figure 4.3). However, BAC WAG18G1, which mapped close to the centromere on GGA2p, also hybridised close to the centromere in APL2p. This demonstrated that the inversion involves only a small fraction of 2p.

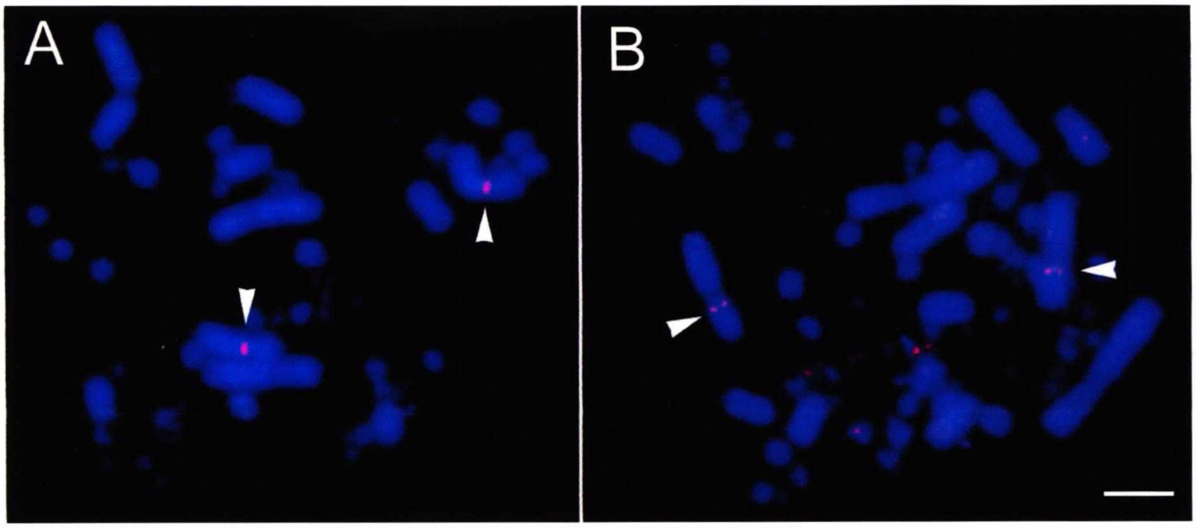


Figure 4.3: WAG41G5 mapping to A) GGA2q and B) APL2p evidencing a pericentric inversion. Scale bar represents 5µm.

BACs WAG13I5 (Figure 4.4), WAG23K3, WAG21J21 AND WAG22J17 clearly demonstrated a paracentric inversion on GGA and APL4.

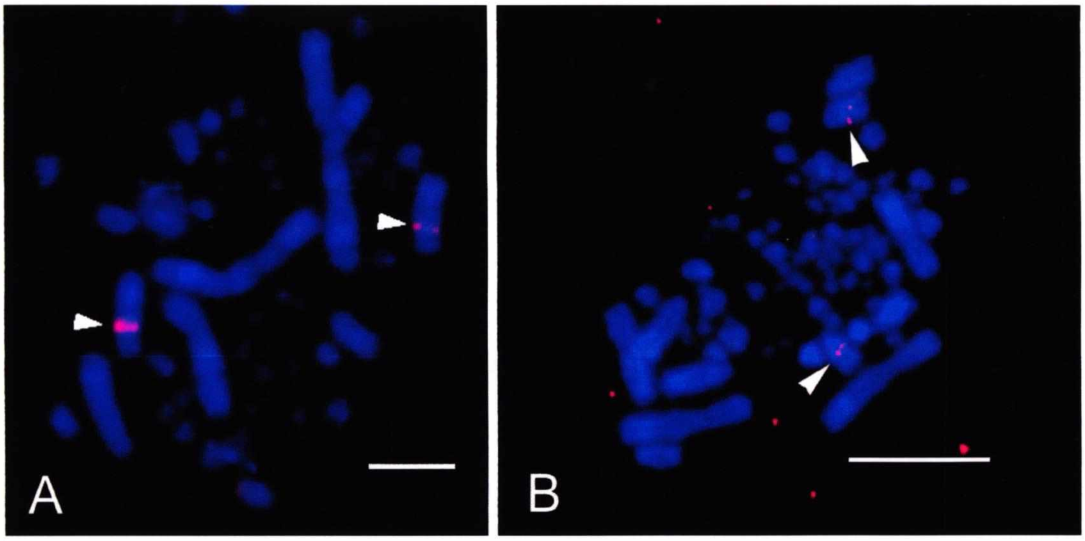


Figure 4.4: BAC WAG13I5 on A) GGA4q (FLpter 0.40) and B) APL4 (FLpter 0.52) demonstrating part of the paracentric inversion. Scale bars represent 10μm.

The morphological differences between GGA and APL7 were reflected in a change in marker order involving BACs WAG69P21 (Figure 4.5), WAG59H11 and WAG21P13. However, like in the rearrangement on GGA and APL1p, marker order was not completely inverted, indicating that this rearrangement may be more complex than a simple pericentric inversion. Similarly, FISH mapping results did not provide clear evidence for a pericentric inversion causing the morphological differences between GGA and APL8.

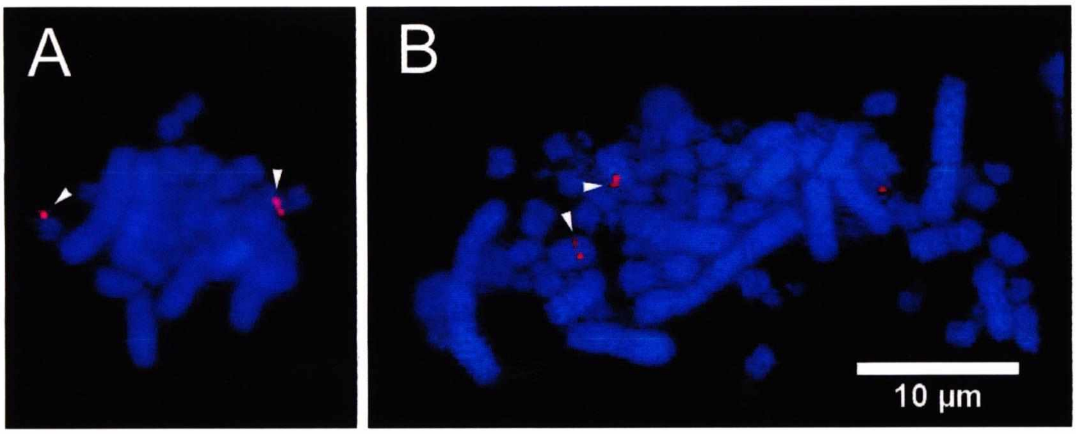


Figure 4.5: BAC WAG69P21 mapping to A) GGA7p (FLpter 0.08) and B) APL7 (FLpter 0.26).

Marker order on GGAZ and APLZ chromosome was largely conserved, with the possible exception of a small inversion involving BACs WAG22P4 and WAG20L10. Thus, it seems that the morphological differences between the metacentric GGAZ and the subtelocentric APLZ are due to the formation of a neocentromere rather than a pericentric inversion.

4.3.3. Inter-chromosomal rearrangements among the microchromosomes

The dual-colour FISH experiments demonstrated synteny among the microchromosomes (APL9, 11, 14-16, 19, 21, 27-29; Figure 4.6). The BACs successfully hybridised to the microchromosomes are shown in Table 4.2 (overleaf).

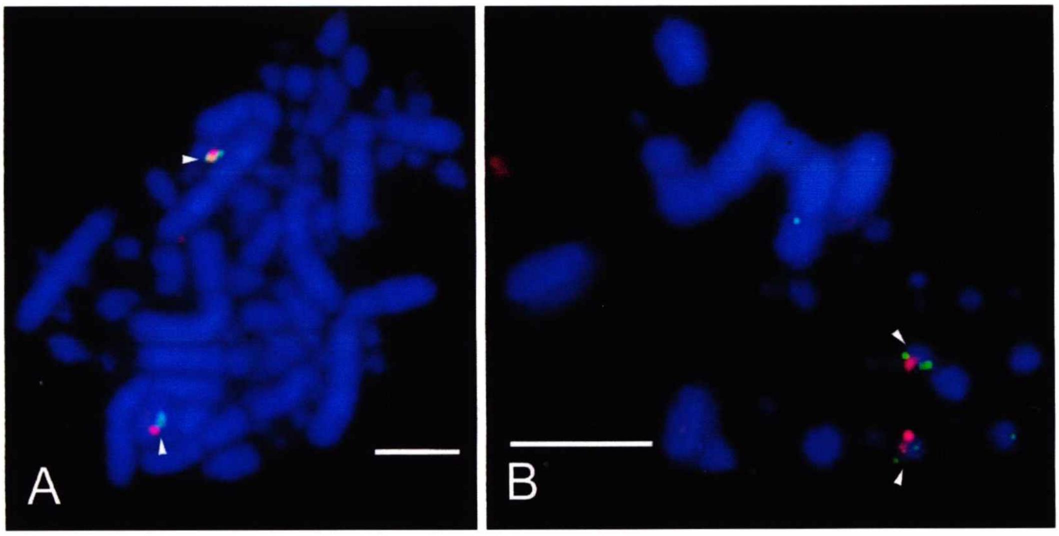


Figure 4.6: Synteny among the microchromosomes was tested by dual colour FISH. An example is shown using BACs with markers CRYBA4sts1 (red) and LEI0083 (green) on A) GGA15 and B) APL16. Scale bars represent 5μm.

Chicken chromosome	Marker associated with BAC	Position on chicken genetic map (cM)	Duck chromosome (APL)
9	ROS0078	0	9
9	ADL0191	44	9
9	MCW0134	132	9
10	B2Msts1	0	11
10	ADL0272	44	11
10	CYP19	60	11
10	MCW0035	82	11
10	MCW0132	92	11
10	MCW0003	105	11
11	ADL0308	69	12
12	SCW0019	26	13
12	LEI0099	63	13
13	MCW0244	-1	14
13	MCW0213	22	14
13	MSX2sts1	41	14
13	CAMLGsts1	70	14
13	POU4F3sts1	71	14
14	MCW0296	0	15
14	GCT0908	0	15
14	ADL0200	16	15
15	LEI0083	0	16
15	MCW0031	7	16
15	LEI0120	13	16
15	SFPQ	25	16
15	ABR0070	35	16
15	MCW0211	49	16
15	MCW0080	49	16
15	CRYBA4sts1	54	16
16	MCW0371	-	17
16	LEI0258	0	17
17	ADL0149	32	18
17	MCW0151	57	18
18	ADL0184	-	19
18	MCW0045	0	19
18	HUJ0010	0	19
18	ROS0022	24	19
18	ADL0290	35	19
18	MCW0219	47	19
18	ROS0027	48	19
19	CTG1704	-	20
19	CTG7040	-	20
19	MCW0256	13	20
19	SCW0024	24	20
20	GTC20050	-	21
20	MCW0119	0	21
20	ADL0193	16	21
20	ADL0034	26	21
20	FZFsts1	62	21
21	AGRIN	24	22
21	PLOD	64	22
22	ROS0073	0	23
22	TVSB3sts1	21	23
23	CTG1080	-	24
23	CTG1100	-	24
23	ADL0262	0	24
23	LEI0102	0	24
23	MCW0165	1	24
23	ADL0289	7	24
23	LEI0339	8	24
23	LEI0090	10	24
24	ROS0113A	20	25
24	ROS0123	30	25
24	APOA1sts	53	25
24	LEI0069	58	25
26	CTG1120	-	27
26	ABR0330	26	27
26	MCW0286	33	27
26	MCW0069	47	27
26	LEI0074	67	27
27	MCW0146	32	28
27	MCW0328	47	28
27	GCT0022	50	28
28	CTG0070	-	29
28	LEI0135	0	29
28	ABR0341	24	29
28	ABR0032	30	29
28	ABR0054	31	29
28	GCT0902	60	29
28	ADL0299	60	29
28	LEI0067A	64	29

Table 4.2: Markers on duck microchromosomes. BACs with these markers successfully hybridized to duck chromosomes. The position of the marker on the chicken genetic map is given; for dual colour FISH experiments, markers were chosen as far apart as possible on the same chromosome.

4.3.4. Definition of the duck karyotype

The duck chromosome orthologous to GGA4p was identified using BACs WAG112C24 and WAG23I06, and determined to be APL10 (section 4.1.1). There were no major differences in chromosome size between the remaining microchromosomes and their chicken orthologues. Given that no other rearrangements have been detected among the microchromosomes, chromosome numbering in duck is proposed to follow chicken for APL1-9; GGA4p corresponds to APL10; thereafter APL = GGA+1.

4.4. Discussion

4.4.1. The cytogenetic map of the duck

The duck physical map presented here is one of the most detailed physical maps obtained by comparative FISH mapping for any bird species to date. Comparative cytogenetic maps allow the transfer of physical and genetic information from one species to another, expediting the process of moving from a mapped quantitative trait locus (QTL) to functional gene. A physical map reveals the fine details of chromosomal rearrangements, and is essential for identifying candidate genes (Ruyter-Spira *et al.,* 1996). The data are also valuable in a broader evolutionary context. Birds are characterized by their relatively stable genomes; having comparative maps available simplifies further comparative evolutionary studies. With only Galliformes previously studied in any detail, this map of an Anseriform can pave the way for future studies giving greater insight into the mechanisms of genome evolution in birds.

4.4.2. Interchromosomal rearrangements between duck and chicken

The conservation of ancestral chromosomes 4 and 10, as APL4 and APL10 are consistent with both the previous studies on duck and with broader patterns of avian karyotype evolution (Fillon *et al.,* 2007, Griffin *et al.,* 2007). These chromosomes appear intact in almost all birds, and ancestral 4 is seen intact in human chromosome 4 as well (Chowdhary and Raudsepp, 2000, Hillier *et al.,* 2004).

The present study extended the data previously available for synteny among the microchromosomes. Fillon et al (2007) showed synteny for 7 microchromosome pairs (APL9, 12, 14-16, 19, 29); here synteny was demonstrated for 10 pairs (APL9, 11, 14-16, 19, 21, 27-29). The lack of detected rearrangements makes it reasonable to suggest that synteny is likely to be conserved among the remaining microchromosomes – including the as-yet unexamined microchromosomes for which no markers exist. Indeed, no sequence data from the chicken genome has yet been assigned to these smallest chromosomes, and it is still unclear why, although there is a suggestion that there may be a cloning or sequencing bias against microchromosomal sequences (Douaud *et al.,* 2008). Data on microchromosomal synteny in other bird species are restricted to the Japanese quail (*Coturnix japonica*; Kayang *et al.,* 2006) and the turkey (Griffin *et al.,* 2008). Despite the paucity of data, the emerging picture is one of remarkable conservation among avian species, with the exception of a few groups where large-scale interchromosomal rearrangements are common (such as the Falconiformes or Psittaciformes (e.g. Nanda *et al.,* 2007, de Olivera *et al.,* 2005).

4.4.3. Intrachromosomal rearrangements between chicken and duck

The BAC mapping data are consistent with intrachromosomal rearrangements distinguishing chromosomes GGA and APL1, 2, 4, 7, 8 and Z, which confirms and expands on previous findings (Fillon *et al.,* 2007). The detection of additional rearrangements on GGA1 and APL1 and GGA4q and APL4 was due to the much higher number of BACs hybridised in this study compared to previous studies. Likewise, higher-resolution mapping demonstrated that the morphological differences between GGAZ and APLZ are probably due to the formation of a neocentromere rather than a pericentric inversion. This type of chromosomal rearrangement was previously reported in birds only for the red-legged partridge (Kasai *et al.,* 2003) and the Japanese quail (Galkina *et al.,* 2005). However, despite the good coverage of the duck cytogenetic map presented here, it was not possible to determine unequivocally the nature of all chromosomal rearrangements observed between chicken and duck (for example, those between GGA7 and 8 and APL7 and 8). It seems likely however that, in addition to peri- and paracentric inversions and neocentromere formation, translocations contributed to avian genome evolution. This conclusion is based on the order of BACs associated with rearrangements on GGA and APL1, 7 and 8, which is not entirely consistent with the order expected if the rearrangements were inversions.

4.4.4. Evolutionary implications

Based on the results presented here, it appears that while the available data from comparative FISH mapping suggest a relatively low frequency of intrachromosomal rearrangements in the evolution of bird genomes, the underlying processes may be more diverse than previously appreciated. Initial *in-silico* comparisons of the draft zebra finch genome with the chicken genome also indicate translocations may be more common than initially thought (Völker, M, Skinner, BM, Griffin, DK, unpublished data). Undoubtedly, the higher resolution afforded by genome sequencing projects such as that of the zebra finch and that of the duck will help to resolve this question.

Reconstruction of avian genome evolution has been impeded by a lack of cytogenetic data. Comparison with the turkey cytogenetic map (Griffin *et al.,* 2008) suggested that APL8 likely represents the ancestral state; the order of BACs on turkey chromosome 10 (ancestral chromosome 8) and the morphology of this chromosome is the same as in duck, indicating that the rearrangement has occurred in the chicken lineage. However, it was not possible with the current data set to decide whether the metacentric Z chromosome in chicken or the subtelocentric Z chromosome in duck is more likely to represent the ancestral state, as the ancestral karyotype of the Galloanserae is not known. For GGA1, 2, 4q and 7, the chicken and turkey maps show no rearrangements; hence it was not possible to determine which changes occurred in the duck lineage, and which occurred in the Galliform lineage.

Among the rearrangements that were detected in the present study, the inversion observed in GGA4q and APL4 is of particular interest. Morphological differences in GGA4 have been described between different chicken breeds (Musa *et al.,* 2005), and the ancestral bird chromosome 4 (corresponding to GGA4q and APL4) is also one of the chromosomes most prone to convergent independent fusions in birds (with ancestral chromosome 10) (Griffin *et al.,* 2007). This contrasts with the conserved synteny of the ancestral bird chromosome 4 in humans (Chowdhary and Raudsepp, 2000, Hillier et al., 2004). Together, the data suggest that rearrangements in chromosome 4 may be more common than has been suspected from previous comparative genomic studies, and analyzing them will prove valuable for understanding avian and other vertebrate genome evolution.

From an evolutionary perspective, the detection of inversions is potentially important in understanding the process of speciation; it has been suggested that inversions facilitate genic divergence through the suppression of recombination, thus promoting speciation (Kirkpatrick and Barton, 2006, Navarro and Barton, 2003, Noor *et al.*, 2001, Rieseberg, 2001). The available data are limited but seem to indicate that inversions, including inversion polymorphisms within populations, are not uncommon in birds, which highlights the importance of further studies concerning their potential contribution to speciation in birds (Price, 2008). In this context, the inversions detected in this and previous studies highlight areas of the genome that are of particular interest for studies of the genetics of speciation.

4.4.5. Technical considerations

The efficiency of hybridization of chicken BAC clones to duck chromosomes (approximately 40%) contrasts with the findings of Yuan et al (2005), who only achieved successful hybridisation for two out of 18 BAC clones, and concluded low sequence conservation between chicken and duck. The data here is more consistent with Fillon et al (2007), who used longer hybridisation times and lower stringency hybridization buffer. There are some chromosomes covered here (for example GGA16) to which clones were not successfully hybridised in previous studies. This is likely attributable to the longer hybridisation in this case (72h as opposed to 48h).

4.4.6. Definition of the duck karyotype

Well-defined karyotypes are essential components of comparative genomic studies, required for the transfer of genetic information from a model species to less well-characterised one. Chromosome banding and macrochromosome painting studies had previously shown orthology of APL1-8 and Z to GGA1-3, 4q, 5-8 and Z. However, it was not known which duck chromosome corresponded to chicken chromosome 4p; Fillon et al (2007) suggested that this was approximately APL10-13. This study has used a combination of BAC mapping and area measurements to determine that it is APL10. Moreover, it provides the first convincing evidence that rearrangements among the microchromosomes are rare or non-existent, at least between chicken and duck. Taken together, these results enabled the unequivocal definition of APL1-29. Attempts to establish synteny among the smallest duck microchromosomes by using chromosome paints for GGA29-39 have been unsuccessful as these paints failed to hybridise across species. However, given the lack of detected rearrangements in the microchromosomes that have been investigated in this study and others (Fillon *et al.*, 2003, 2007), it seems not unreasonable to assume high levels of conserved synteny among these chromosomes as well.

Accordingly, it is suggested that duck chromosomes be numbered as per chicken for 1-9; APL10 corresponds to GGA4p; then GGA10 onwards correspond to APL11 onwards. Only one chromosome measured was smaller than numbering would suggest – that is APL17, the orthologue of GGA16, the chicken NOR chromosome. This is due to the size of GGA16 originally being overestimated (Masabanda *et al.*, 2004); however, Masabanda et al (2004), in their molecular cytogenetic definition of the chicken karyotype, suggested to retain the numbering to avoid confusion. Likewise, APL17 was so named for consistency in this study. As seen from the chromosome size measurements (section 3.3.1), the size differences between the smaller chromosomes are slight; therefore it seems unnecessary to renumber the remaining chromosomes. The successful hybridization of at least one BAC from GGA1-28 (except 25) thus means markers are now available for APL1-29 (except 26).

4.5. Conclusions

The comparative cytogenetic map of the duck presented here provides an improved definition of the duck karyotype, which highlights the conservation seen among the genomes of many bird species, and how little structural genetic variation is readily apparent. The combination of area measurements and FISH mapping of chicken BACs allowed the identification of markers for chromosomes APL1-24 and 26-29 which will facilitate future mapping studies in the duck and assist the transfer of further genetic information directly from chicken to duck. While overall the evolutionary conservation of bird karyotypes has been demonstrated, the intrachromosomal differences found highlight areas of future interest for evolutionary and functional studies.

5. Specific aim 3: To provide a detailed appraisal of nuclear organisation in chicken embryonic fibroblasts and perform comparative genomic experiments in turkey and duck.

5.1. Background

As reviewed in section 1.3, based on evidence from mammals it seems reasonable to suggest that changes in nuclear organisation might correlate with specific developmental stages, and that this may reflect a general relationship between nuclear organisation and embryology. The well-described embryonic phenotypes of chicken make it ideal to study this phenomenon. In order for such work to be instigated, however, a baseline understanding of nuclear organisation is needed in at least one cell type with which other studies can then be compared. It is unclear whether the positions of microchromosomes correlate with their individual sizes, or whether they exist in a more flexible 'pool' towards the centre of the nucleus. It is also not known whether nuclear position correlates better with gene density or chromosome size. From the perspective of evolutionary conservation of nuclear organisation, nothing at all is known in avian species other than chicken, making it impossible to decide whether the patterns of nuclear organisation observed in chicken hold true for avian genomes in general as they do in primates. Obvious targets for such studies are the turkey and the duck. This is because detailed comparative cytogenetic maps are available for both species, and the chromosomal rearrangements distinguishing them from chicken have been described in detail (Griffin *et al.*, 2008, Fillon *et al.*, 2007, chapter 4 of this thesis). Therefore, in this study, chicken BAC clones were hybridised to chicken, turkey and duck fibroblast nuclei, and the relative nuclear positions of chromosome territories in each species were assessed.

Two major models have been proposed to describe how chromosomes are organised in the interphase nucleus. First, in a "gene density based" organisation, the gene poor chromosomes are found towards the periphery and the gene rich chromosomes are more interior. In a second model, a chromosome size based distribution, the largest chromosomes are found toward the periphery of the nucleus and the smaller chromosomes are more interior.

To date, most studies of nuclear organisation in vertebrates have focused on humans and other mammals. Our knowledge of nuclear organisation in birds is limited to a small number of studies in the chicken, and knowledge of individual chromosome positions in somatic cells in chicken is restricted to GGA1-5 and Z. There is therefore a need for more detailed information on nuclear organisation in chicken.

It is also known that individual gene loci alter their nuclear position based on transcriptional status. As detailed in section 1.3.5, some loci have been seen to move toward the interior of the nucleus upon activation (e.g. Williams *et al.,* 2006, Zink *et al.,* 2004), with others moving to the nuclear periphery upon transcriptional silencing (Finlan *et al.,* 2008). Given the size of avian microchromosomes, it is possible that entire chromosome territories would alter position upon transcriptional regulation of genes within them; this has not previously been investigated. Avian macrophages represent a useful cell type for such work; they are readily isolated from blood samples, and can be easily stimulated by use of LPS (Qureshi *et al.,* 2000), which mimics the acute phase response of bacterial infection (Xie *et al.,* 2000, Cheng *et al.,* 2004). Furthermore, the effects of LPS on gene regulation in chicken macrophages have been described in a preliminary study (Bliss *et al.,* 2005), and a number of genes are known to be induced or repressed. Therefore, as an extension to the fibroblast nuclear organisation study, chromosome position was examined in chicken and duck macrophages with and without stimulation by lipopolysaccharide (LPS). RNA was isolated from chicken macrophages with and without LPS stimulation, and was hybridised to a chicken immune cDNA microarray. Chromosomes containing significantly up- or down-regulated genes upon LPS stimulation were targeted for nuclear organisation studies in chicken and duck macrophages.

5.2. Specific Aims

Given the above, the detailed specific aims of this chapter were as follows:

Specific aim 3a: To use gene density estimates calculated from Ensembl and the chromosome size information provided in chapter 3 to identify chicken chromosomes that do not follow the chromosome size - gene density correlation; thereby to identify possible discriminators for distinguishing between a chromosome size or gene density based nuclear organisation.

Specific aim 3b: To establish a means of determining nuclear organisation (chromosome territory position) for the majority of the karyotype in three avian species (chicken, turkey and duck).

Specific aim 3c: To provide a detailed, per-chromosome, appraisal of nuclear organisation in chicken embryonic fibroblasts, and to establish a "baseline" for future studies on avian nuclear organisation.

Specific aim 3d: To repeat the above on turkey and duck nuclei for the purposes of comparative genomics; thereby to test the hypothesis that the nuclear position of orthologous chromosomal segments is conserved between the three species.

Specific aim 3e: To provide preliminary evidence supporting either the "size-related" or "gene-density-related" models of nuclear organisation in avian embryonic fibroblasts.

Specific aim 3f: To isolate RNA from chicken and duck blood with and without activation by lipopolysacharide (LPS) and interrogate a 5k immuno-specific microarray (chicken) to identify chromosomes containing significantly up- and down-regulated genes.

Specific aim 3g: To apply FISH probes for the chromosomes of interest identified above on the macrophage nuclei from chicken and duck and establish the nuclear positions of these chromosomes in LPS-stimulated and un-stimulated macrophages, testing the hypothesis that nuclear organisation alters depending on transcriptional state.

5.3. Results

5.3.1. Appraisal of gene density of chicken chromosomes and correlation with chromosome size

The relative areas of chicken chromosomes (see section 3.3.1) were plotted against their gene density as calculated from estimated gene numbers from the Ensembl database (Figure 5.1). This analysis confirmed the expected negative correlation between chromosome size and gene density, i.e. smaller chromosomes tended to have higher gene densities than bigger chromosomes (Spearman's rank, $\rho= -0.63$, $n=25$, $p=0.0006$). Two outlying chromosomes were identified, GGA16 and 22 (circled in Figure 5.1); the gene densities for these two chromosomes are much lower than the gene densities for other chromosomes of similar size. The removal of these chromosomes from the analysis strengthened the correlation ($\rho= -0.88$, $n=23$, $p<0.0001$).

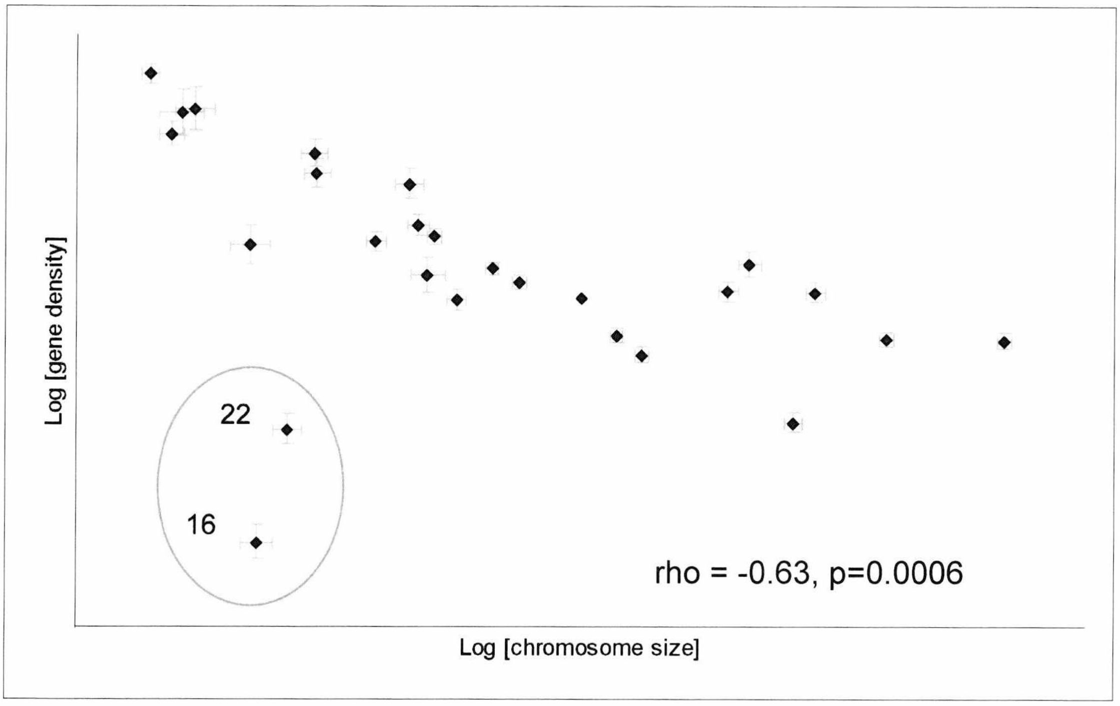


Figure 5.1: Chromosome size plotted against gene density in chicken. Outliers GGA16 and 22 are circled. Error bars represent standard error of the mean. Log values for size and density are shown to improve clarity with the smaller chromosomes; correlations are unaffected.

5.3.2. Nuclear location of chromosome territories in chicken

5.3.2.1. Identification of chromosome territory position

Position analysis of FISH images was carried out in ImageJ using a custom macro (provided by Michael Ellis, Digital Scientific UK). Each nucleus was divided into 5 concentric rings of equal area, and the percentage of signal within each ring was calculated. The data were output to a spreadsheet for analysis (details on the analysis method are described in methods section 2.7.3).

Preliminary analysis using this macro revealed that using chromosome paints to assess chromosome territory position yielded inaccurate results. Many of the chromosome territories for the larger chromosomes, while clearly located on the nuclear periphery, often appeared predominantly in more interior shells after analysis, simply due to the chromosome territory being large enough to overlap two or more shells. Pooling results from several evenly spaced BACs along each chromosome however provided more reliable and reproducible results.

An image of a nucleus showing a signal for the GGA1 BAC containing marker LEI0194, with the five ring template overlaid is shown in Figure 5.2. Example graphs of the normalised data from the ring analysis are shown in Figure 5.3, showing BACs that are predominantly peripheral (A), medial (B) and internal (C).

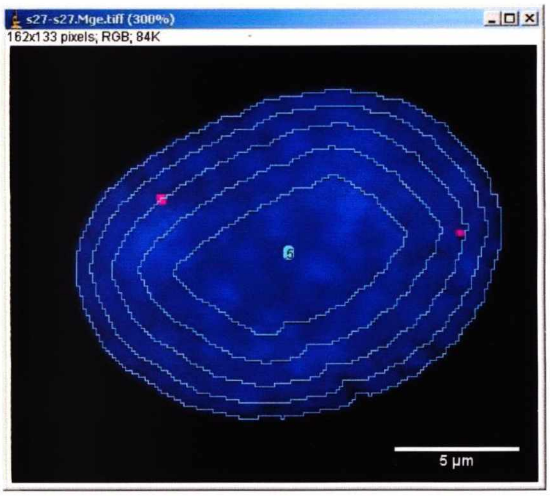


Figure 5.2: Screenshot of chicken fibroblast nucleus with BAC containing marker LEI0194 and ImageJ macro applied

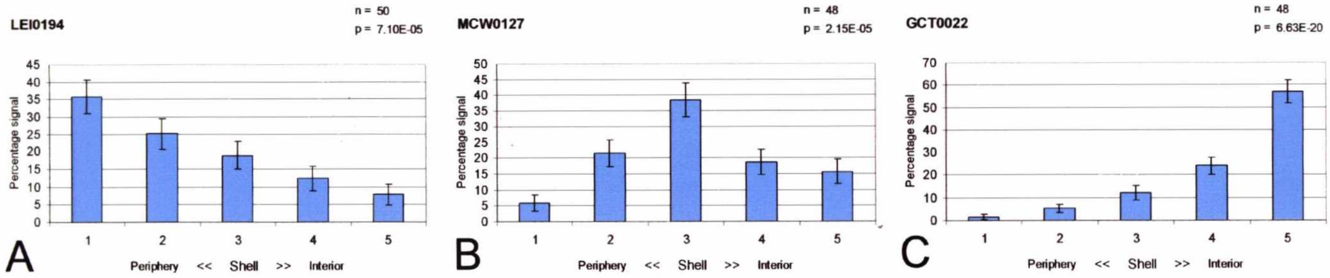


Figure 5.3: Signal distributions for A) LEI0194, GGA1, peripheral; B) MCW0127, GGA3, medial; C) GCT0022, GGA27, internal. Error bars represent standard error of the mean; n = number of nuclei analysed; p = probability that distribution is non-random by chi-square test (significance level p<0.05, 4 d.f.).

5.3.2.2. Chromosome territory positioning in chicken

Eighty chicken BACs for chromosomes 1-28 (except 25 for which no working BACs could be isolated) were successfully hybridised to chicken fibroblast interphase nuclei (Table 5.1). Due to a lack of available BACs, chromosome paints for GGAZ and W were used, with the caveat that results are less reliable than for the other chromosomes.

GGA	Marker	Analysable images produced?		
		Chicken	Turkey	Duck
1	LEI0101	✓	✓	✓
1	LEI0134	✓	✓	✓
1	LEI0169	✓	✓	✓
1	LEI0194	✓	✓	
1	MCW0112		✓	
1	MCW0188	✓	✓	
1	MCW0200	✓		✓
1	LEI0068	✓		
1	LEI0146	✓		
1	MCW0248	✓		
1	LEI0091	✓		
1	ADL0101	✓		
2	LEI0129	✓	✓	✓
2	MCW0082	✓	✓	
2	MCW0157	✓		✓
2	MCW0131	✓	✓	✓
2	ABR0008	✓		✓
2	LEI0147		✓	
2	MCW0239	✓		
2	MCW0358	✓		
2	ADL0176	✓		
2	MCW0274	✓		
2	ADL0120	✓		
3	ADL0370	✓	✓	✓
3	GCT0083	✓	✓	✓
3	MCW0127	✓	✓	✓
3	MCW0261	✓		✓
3	MCW0162	✓		
3	LEI0115	✓		
3	MCW0224	✓		
3	MCW0040	✓		
4	LEI0063	✓	✓	✓
4	MCW0295		✓	
4	ADL0203	✓	✓	✓
4	MCW0180	✓	✓	
4	ADL0317	✓		✓
4	ADL0246	✓		
4	COM0085	✓		
5	HUJ003	✓	✓	✓
5	MCW0193	✓	✓	
5	MCW0210	✓	✓	✓
5	ROS0013	✓	✓	✓
5	MCW0113	✓	✓	✓
6	ADL0040	✓	✓	
6	LEI0192	✓	✓	

GGA	Marker	Analysable images produced?		
		Chicken	Turkey	Duck
6	ROS0003	✓	✓	✓
7	LEI0064	✓	✓	✓
7	MCW0120	✓	✓	✓
7	MCW0201	✓	✓	✓
8	ADL0301	✓	✓	✓
8	ADL0302	✓	✓	✓
8	MCW0160		✓	
8	MCW0275	✓	✓	✓
9	ADL0191	✓	✓	✓
9	MCW0134	✓	✓	✓
9	ROS0078	✓	✓	✓
10	ADL0272	✓	✓	✓
10	MCW0003	✓	✓	✓
10	MCW0035		✓	✓
11	ADL0308	✓	✓	✓
12	SCW0019	✓	✓	
13	MCW0213	✓	✓	
13	MSX2	✓	✓	✓
14	ADL0200	✓		
14	MCW0296	✓	✓	✓
15	CRY3A4	✓	✓	
15	MCW0211	✓	✓	
16	LEI0258	✓	✓	✓
16	MCW0371	✓	✓	
17	MCW0151	✓	✓	
18	HUJ0010	✓		
19	CTG1740		✓	
19	CTG7040	✓		
19	SCW0024	✓		
20	ADL0193	✓	✓	
20	GCT20050	✓		
21	AGRIN	✓	✓	✓
21	PLOD	✓	✓	
22	ROS0073	✓		✓
22	TVSB3	✓		
22	ROS0022		✓	
23	LEI0339	✓	✓	✓
24	ROS0113A	✓	✓	✓
26	MCW0286	✓	✓	✓
27	GCT0022	✓	✓	✓
27	MCW0328	✓	✓	✓
28	ST28BE07	✓	✓	
Z	Paint	✓		
W	Paint	✓		

Table 5.1: Chicken markers within BACs hybridised to chicken, turkey and duck, indicating those which produced images suitable for ring analysis (i.e. appeared free from non-specific hybridisation)

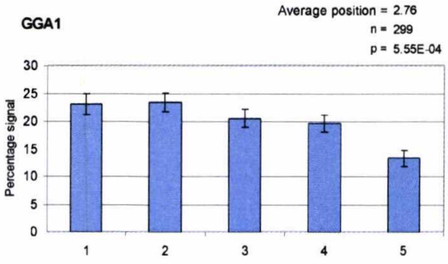
Twenty-four of the 29 chromosomes examined showed a non-random distribution (χ^2 test, 4 d.f., $p<0.05$); those that did not show a significant difference from a random distribution were chromosomes GGA4 ($p=0.19$), GGA5 ($p=0.89$), GGA11 ($p=0.31$), GGA20 ($p=0.25$) and GGAW ($p=0.053$). The signal distributions for each chromosome are shown in Figure 5.4, with the data summarised in Table 5.2.

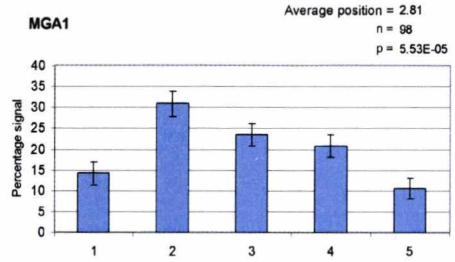
Figure 5.4 (following pages): Chromosome distributions in chicken (left column), turkey (centre column) and duck (right column). Orthologous chromosomes are aligned; n indicates the number of nuclei analysed, p indicates results of the χ^2 test against a random distribution. Non-significant values ($p>0.05$) are shown in red. Error bars indicate the standard error of the mean.

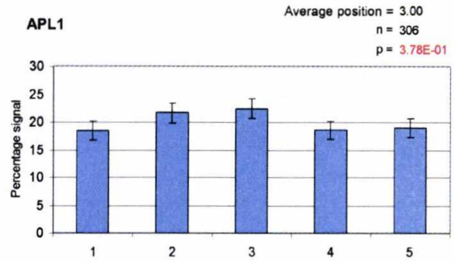
Chicken

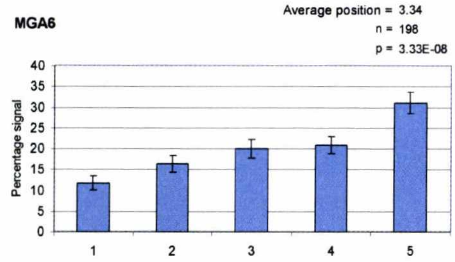
Turkey

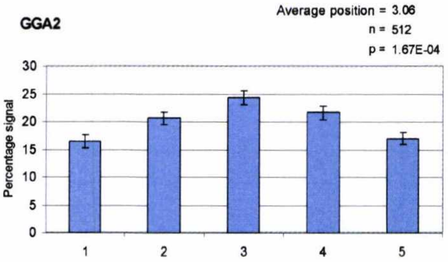
Duck

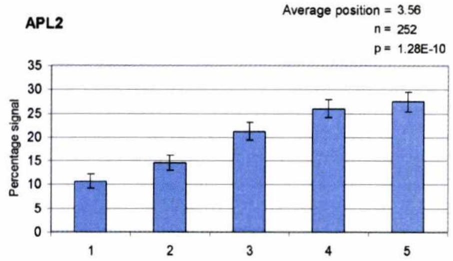


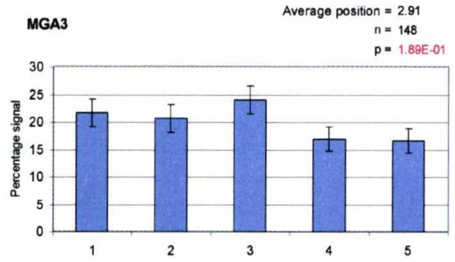


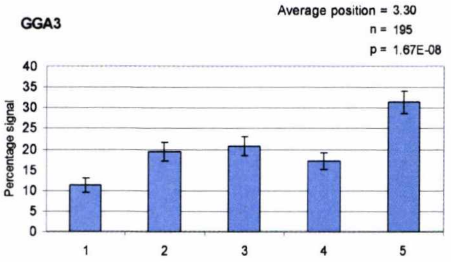


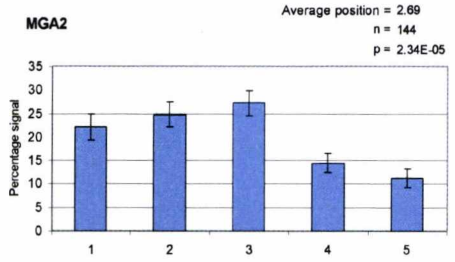


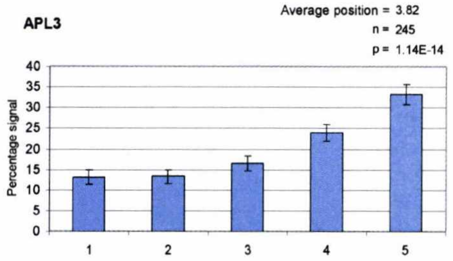


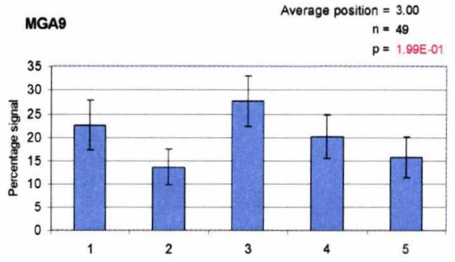


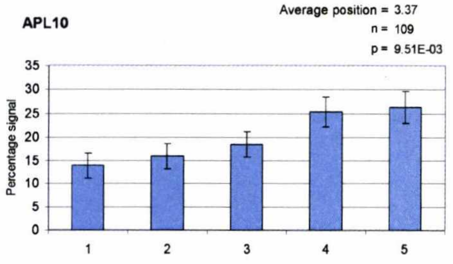


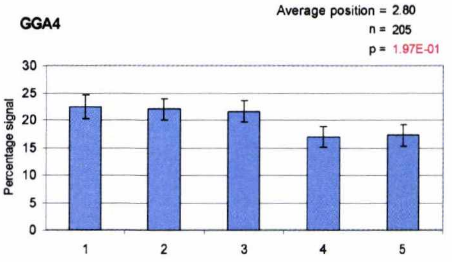


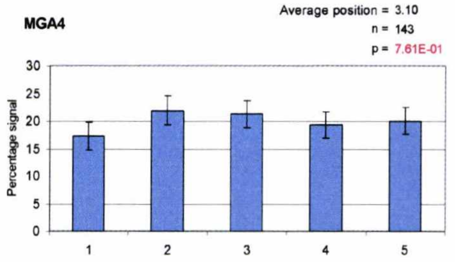


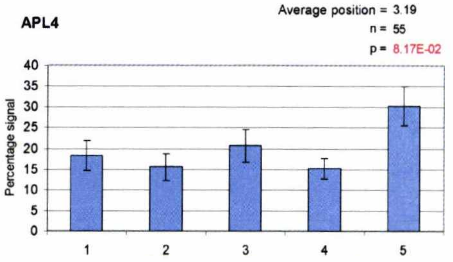




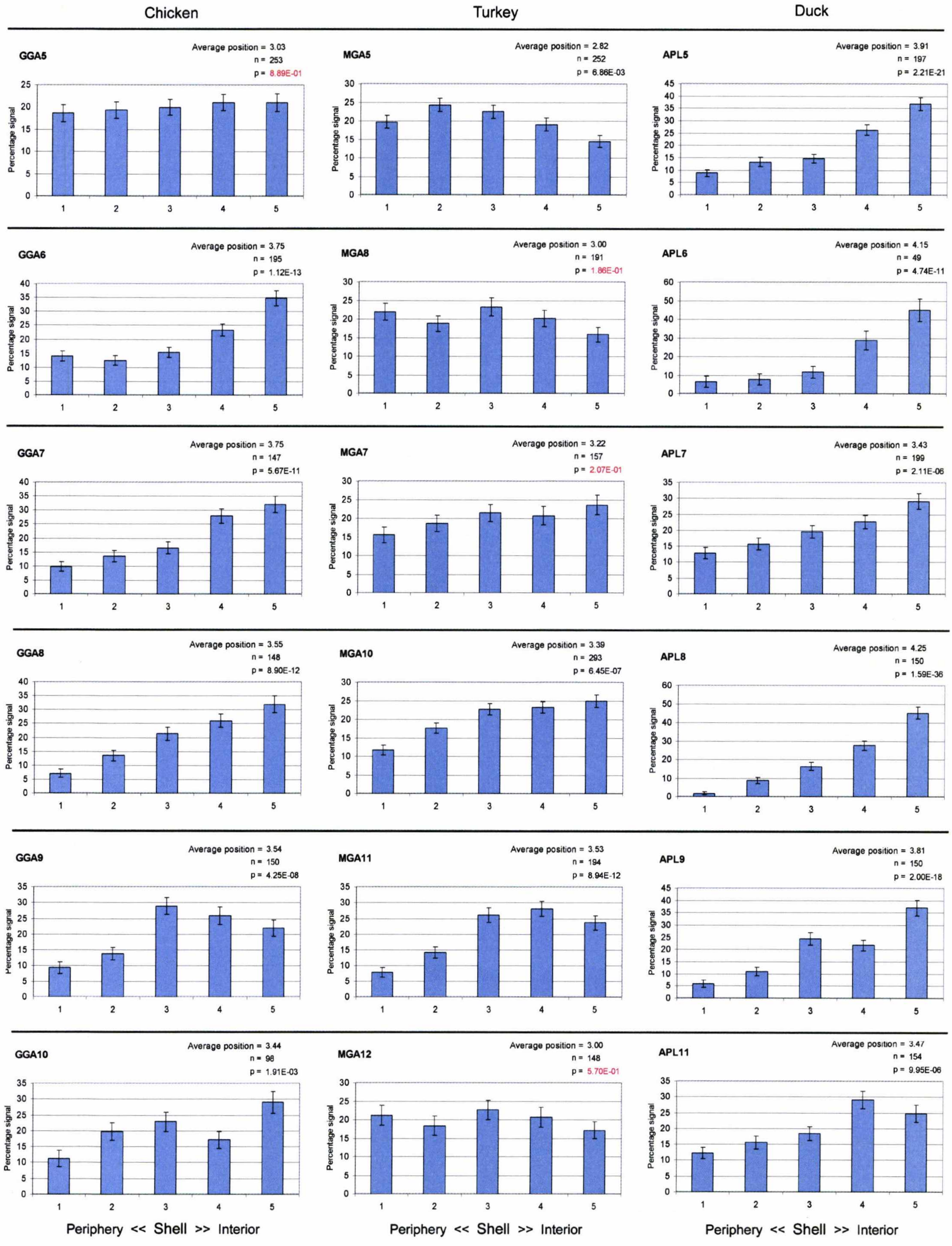








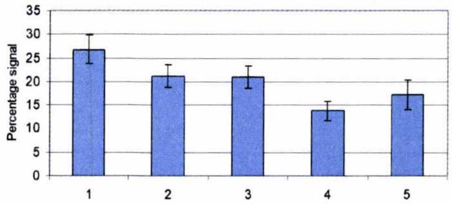
Periphery << Shell >> Interior   Periphery << Shell >> Interior   Periphery << Shell >> Interior


Chicken
Turkey
Duck
GGA5
Average position = 3.03
n = 253
p = 8.89E-01
MGA5
Average position = 2.82
n = 252
p = 6.86E-03
APL5
Average position = 3.91
n = 197
p = 2.21E-21
GGA6
Average position = 3.75
n = 195
p = 1.12E-13
MGA8
Average position = 3.00
n = 191
p = 1.86E-01
APL6
Average position = 4.15
n = 49
p = 4.74E-11
GGA7
Average position = 3.75
n = 147
p = 5.67E-11
MGA7
Average position = 3.22
n = 157
p = 2.07E-01
APL7
Average position = 3.43
n = 199
p = 2.11E-06
GGA8
Average position = 3.55
n = 148
p = 8.90E-12
MGA10
Average position = 3.39
n = 293
p = 6.45E-07
APL8
Average position = 4.25
n = 150
p = 1.59E-36
GGA9
Average position = 3.54
n = 150
p = 4.25E-08
MGA11
Average position = 3.53
n = 194
p = 8.94E-12
APL9
Average position = 3.81
n = 150
p = 2.00E-18
GGA10
Average position = 3.44
n = 98
p = 1.91E-03
MGA12
Average position = 3.00
n = 148
p = 5.70E-01
APL11
Average position = 3.47
n = 154
p = 9.95E-06
Percentage signal
1
2
3
4
5
Periphery << Shell >> Interior

Chicken Turkey Duck

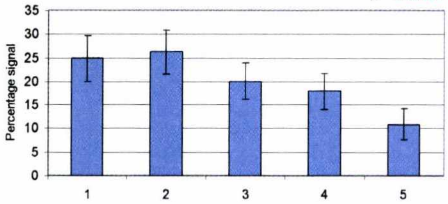
GGA11

Average position = 2.59
n = 50
p = 3.14E-01



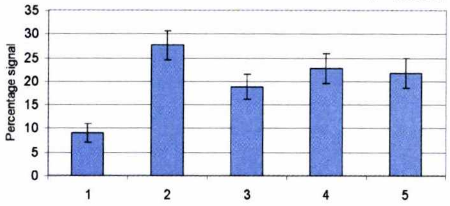
MGA13

Average position = 2.49
n = 50
p = 1.13E-01



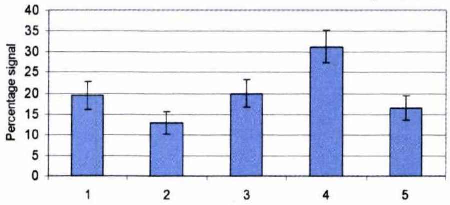
APL12

Average position = 3.24
n = 100
p = 7.93E-04



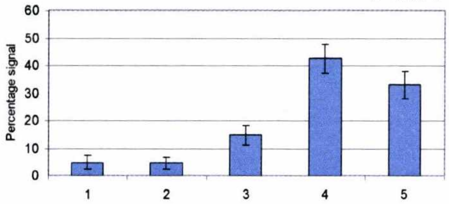
GGA12

Average position = 3.24
n = 65
p = 1.52E-02



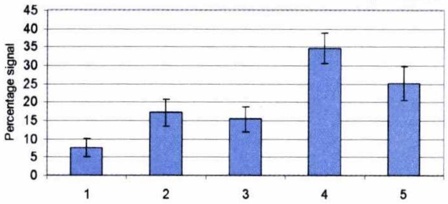
MGA14

Average position = 4.01
n = 48
p = 1.48E-11



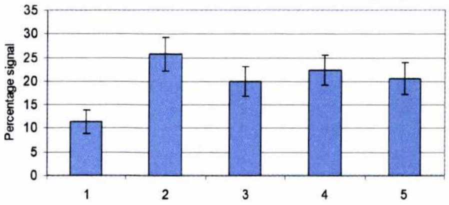
APL13

Average position = 3.48
n = 50
p = 3.19E-04



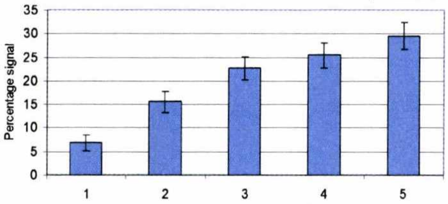
GGA13

Average position = 3.05
n = 99
p = 2.53E-02



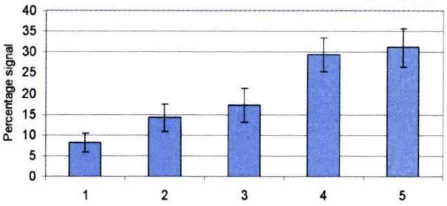
MGA15

Average position = 3.75
n = 148
p = 1.34E-09



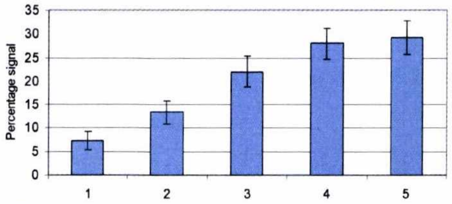
APL14

Average position = 3.56
n = 49
p = 6.81E-04



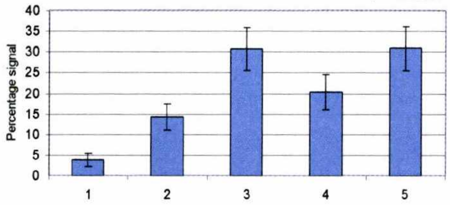
GGA14

Average position = 3.84
n = 100
p = 3.15E-07



MGA16

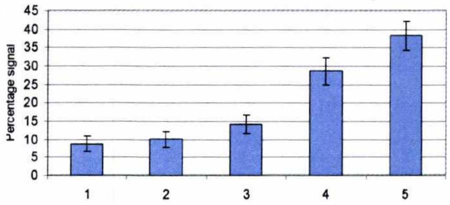
Average position = 3.46
n = 49
p = 3.23E-05



N/A

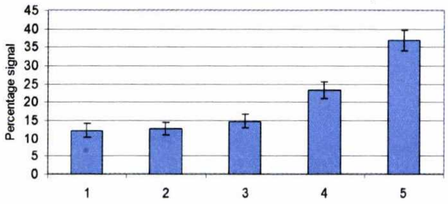
GGA15

Average position = 4.00
n = 102
p = 5.71E-14



MGA17

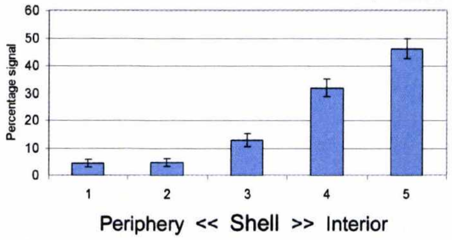
Average position = 3.74
n = 157
p = 5.00E-14



N/A

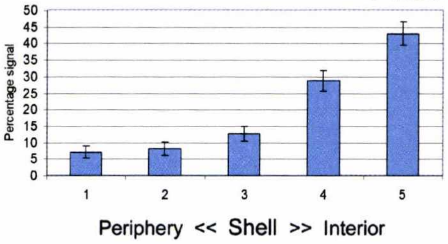
GGA16

Average position = 4.21
n = 108
p = 1.10E-30



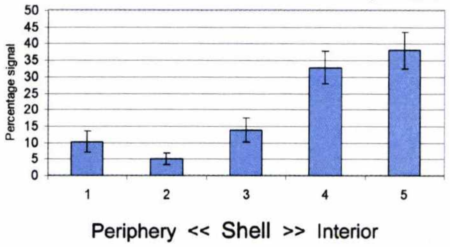
MGA18

Average position = 4.15
n = 100
p = 5.57E-20



APL17

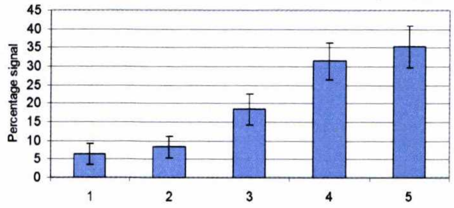
Average position = 4.00
n = 50
p = 1.42E-08



Chicken Turkey Duck

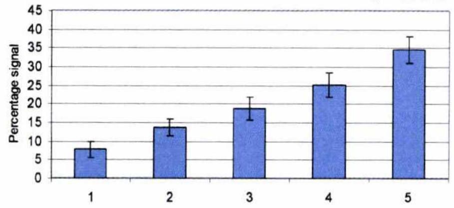
GGA17

Average position = 3.99
n = 50
p = 5.42E-07



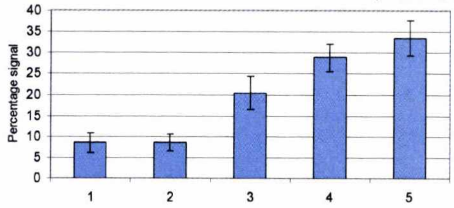
MGA19

Average position = 3.90
n = 99
p = 1.25E-08



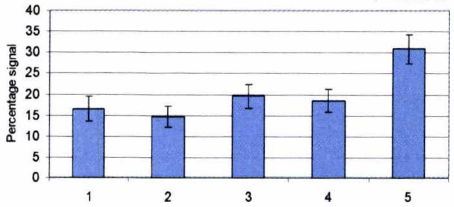
APL18

Average position = 3.85
n = 48
p = 4.71E-05



GGA18

Average position = 3.23
n = 103
p = 2.26E-03

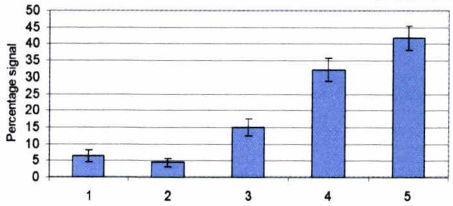


N/A

N/A

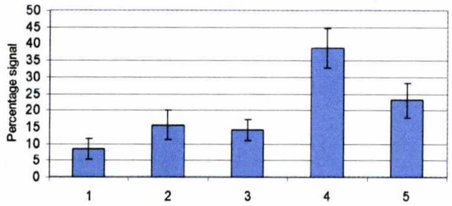
GGA19

Average position = 4.14
n = 97
p = 9.77E-22



MGA21

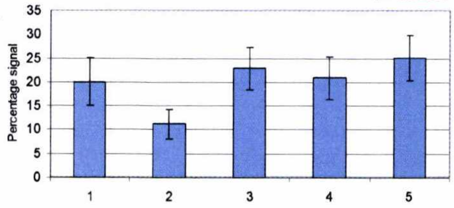
Average position = 3.92
n = 49
p = 2.14E-05



N/A

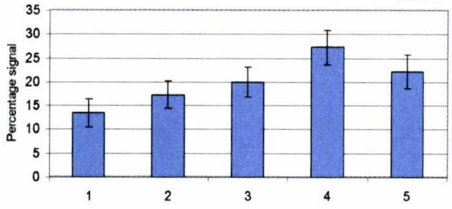
GGA20

Average position = 3.39
n = 48
p = 2.50E-01



MGA22

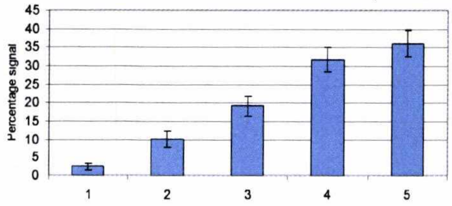
Average position = 3.34
n = 98
p = 3.22E-02



N/A

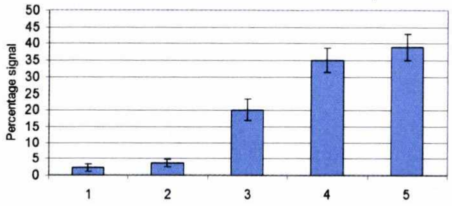
GGA21

Average position = 3.98
n = 100
p = 1.39E-16



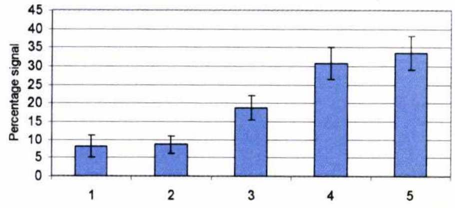
MGA23

Average position = 4.15
n = 95
p = 3.81E-23



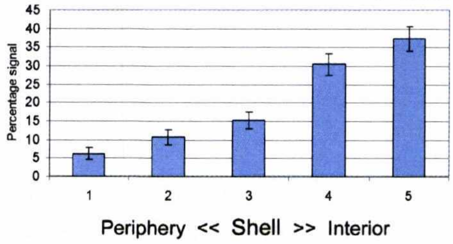
APL22

Average position = 3.92
n = 50
p = 8.78E-06



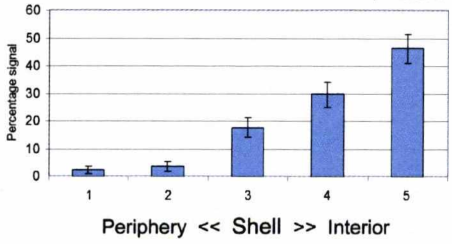
GGA22

Average position = 4.01
n = 100
p = 1.51E-14



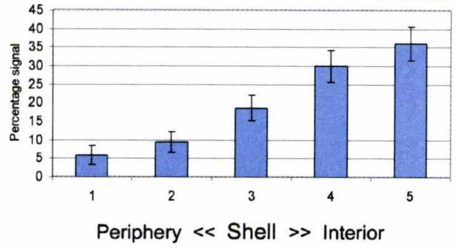
MGA24

Average position = 4.29
n = 50
p = 4.89E-14



APL23

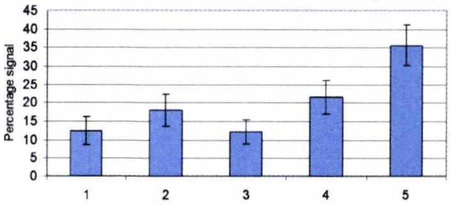
Average position = 3.93
n = 50
p = 9.12E-07

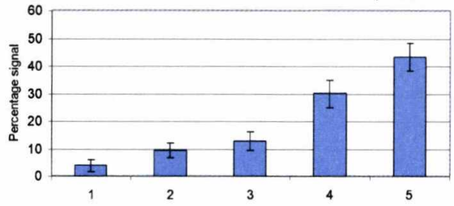


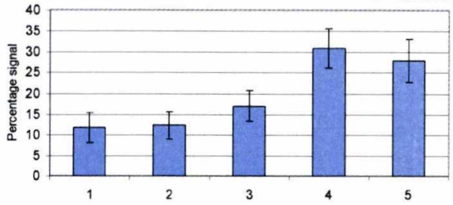
Chicken

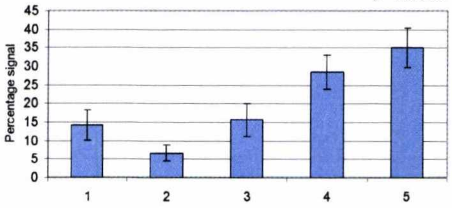
Turkey

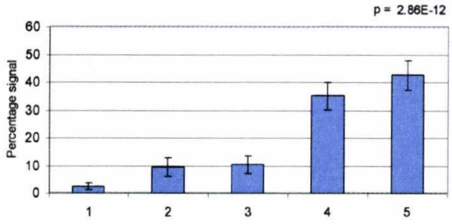
Duck

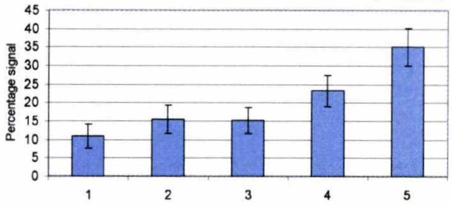

GGA23
Average position = 3.56
n = 44
p = 2.80E-03

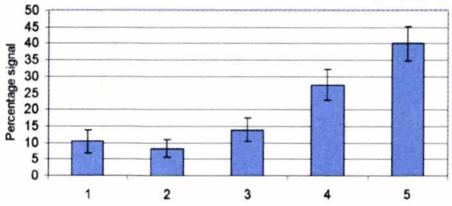

MGA25
Average position = 4.06
n = 50
p = 7.58E-11

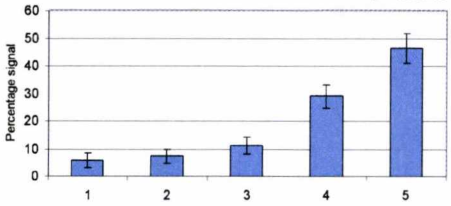

APL24
Average position = 3.83
n = 50
p = 3.26E-03

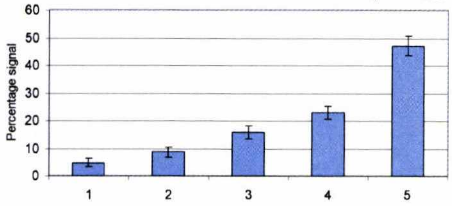

GGA24
Average position = 3.94
n = 50
p = 2.69E-05

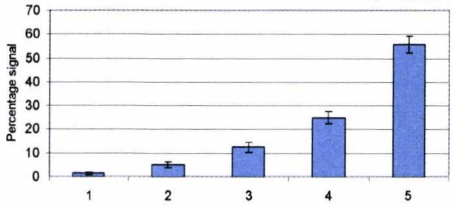

MGA26
Average position = 4.20
n = 48
p = 2.86E-12

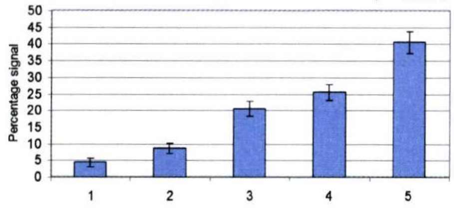

APL25
Average position = 3.82
n = 50
p = 1.18E-03

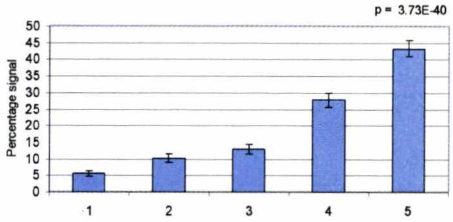

GGA26
Average position = 3.99
n = 46
p = 1.05E-06

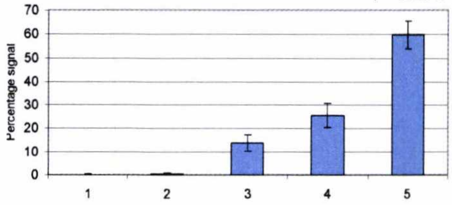

MGA28
Average position = 4.33
n = 50
p = 1.56E-12

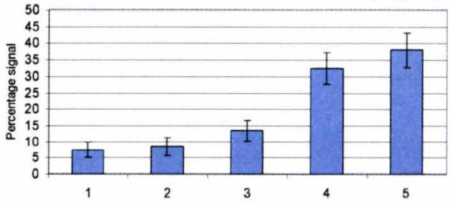

APL27
Average position = 4.15
n = 109
p = 1.60E-25


GGA27
Average position = 4.49
n = 96
p = 4.55E-39

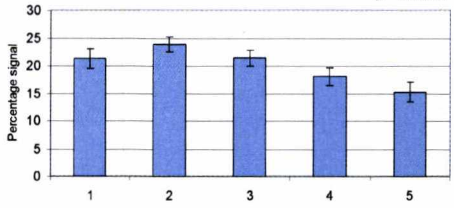

MGA29
Average position = 4.00
n = 143
p = 1.12E-24


APL28
Average position = 4.04
n = 200
p = 3.73E-40


GGA28
Average position = 4.46
n = 39
p = 1.12E-19


MGA30
Average position = 4.08
n = 48
p = 6.66E-08

N/A


GGAZ
Average position = 2.71
n = 193
p = 7.16E-02

N/A

N/A

Periphery << Shell >> Interior

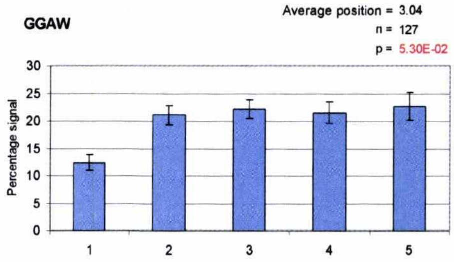
Periphery << Shell >> Interior

Periphery << Shell >> Interior

Chicken

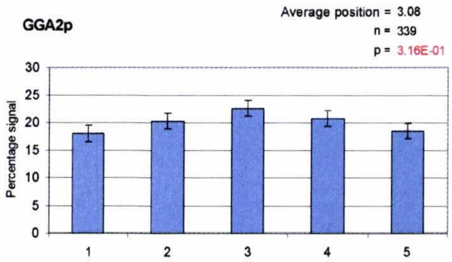
Turkey

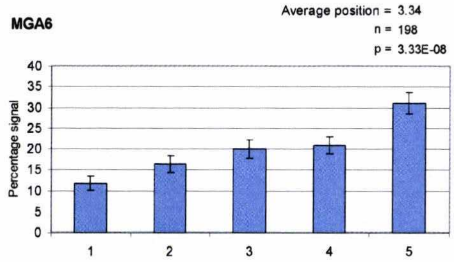
Duck

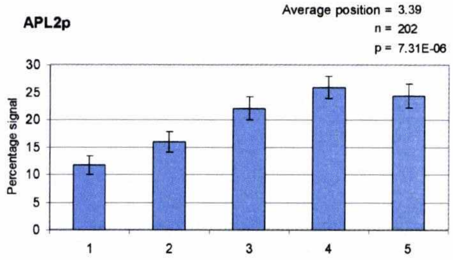


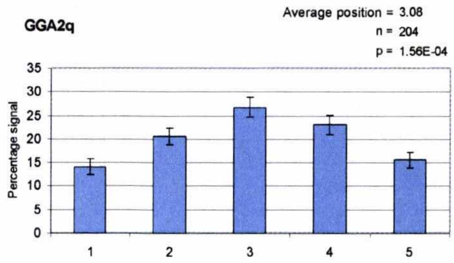
N/A

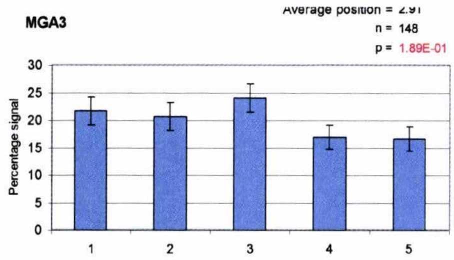
N/A

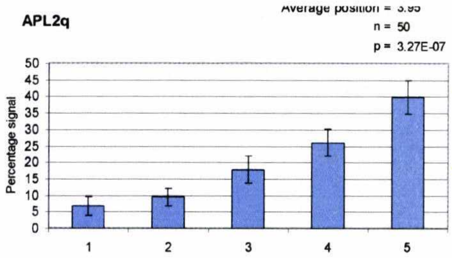


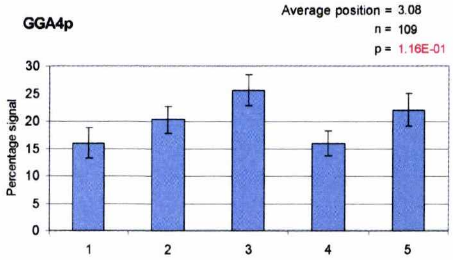


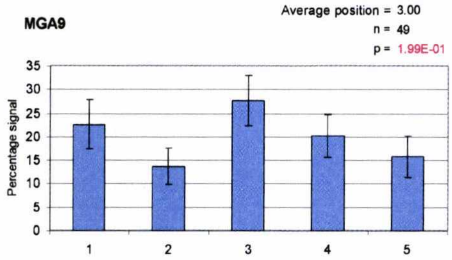


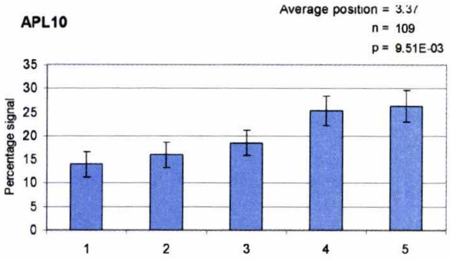


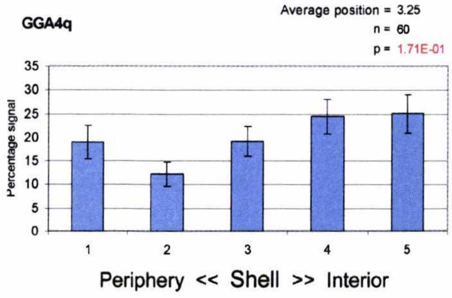


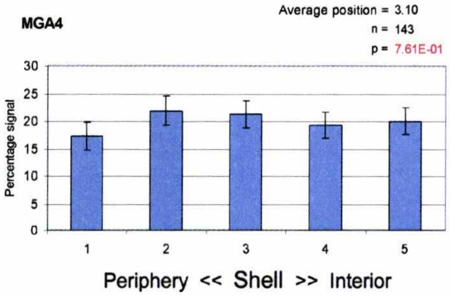


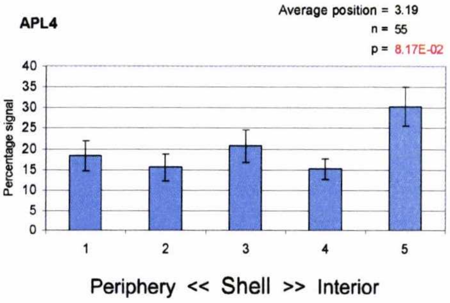












GGA	Median Position	IQR	MGA	Median Position	IQR	APL	Median Position	IQR
1	2.76	1.58	1	2.81	1.49	1	3.00	1.85
2	3.06	1.38	-	-	-	2	3.82	1.53
2p	3.08	1.45	6	3.34	1.96	2p	3.39	1.42
2q	3.08	1.20	3	2.91	1.62	2q	3.95	1.51
3	3.30	2.06	2	2.69	1.38	3	3.56	2.03
4	2.80	1.54	-	-	-	-	-	-
4p	3.08	1.28	9	3.00	1.91	10	3.37	1.88
4q	3.25	1.39	4	3.10	1.82	4	3.19	1.75
5	3.03	1.81	5	2.82	1.55	5	3.91	1.50
6	3.75	1.81	8	3.00	1.70	6	4.15	1.33
7	3.75	1.73	7	3.00	1.79	7	3.43	1.63
8	3.55	1.52	10	3.39	1.51	8	4.25	1.31
9	3.54	1.17	11	3.53	1.15	9	3.81	1.50
10	3.44	1.59	12	3.00	1.94	11	3.47	1.52
11	2.59	1.00	13	2.49	2.05	12	3.24	1.63
12	3.24	1.16	14	4.01	0.79	13	-	-
13	3.05	1.74	15	3.75	1.50	14	3.48	1.16
14	3.84	1.47	16	3.46	1.43	15	3.56	1.36
15	4.00	1.78	17	3.74	1.73	16	-	-
16	4.21	1.00	18	4.15	1.11	17	4.00	1.45
17	3.99	1.26	19	3.90	1.58	18	3.85	1.09
18	3.23	2.03	20	-	-	19	-	-
19	4.14	1.25	21	3.92	1.68	20	-	-
20	3.39	1.88	22	3.34	1.69	21	-	-
21	3.98	1.11	23	4.15	1.34	22	3.92	1.10
22	4.01	1.34	24	4.10	1.04	23	3.93	1.27
23	3.56	1.71	25	4.06	1.06	24	3.83	1.74
24	3.94	1.62	26	4.20	1.06	25	3.82	1.47
26	3.99	1.31	28	4.33	1.22	27	4.15	1.35
27	4.49	1.09	29	4.00	1.28	28	4.04	1.04
28	4.46	0.88	30	4.08	1.38	29	-	
Z	2.71	1.67	Z	-	-	Z	-	-
W	3.04	1.57	W	-	-	W	-	-

Table 5.2: Overall positions of chromosomes (from periphery, 1, to interior, 5) in chicken, turkey and duck; tabulation of data in Figure 5.4. Orthologous chromosomes are aligned. Positions which did not meet the significance threshold (χ^2,4 d.f, $p<0.05$) for a non-random distribution are indicated in red. IQR indicates interquartile range.

Overall, smaller chromosomes tended to be located towards the nuclear centre, while larger chromosomes were located more towards the nuclear periphery (Figure 5.5A). Likewise, gene-dense chromosomes were more frequently found towards the nuclear centre (Figure 5.5B). The correlation coefficient revealed a relationship between chromosome position and chromosome size (Spearman's rank, ρ= -0.71, n=24, p=0.0006). No significant correlation was seen between chromosome position and gene density (Spearman's rank, ρ=0.34, n=24, p=0.097).

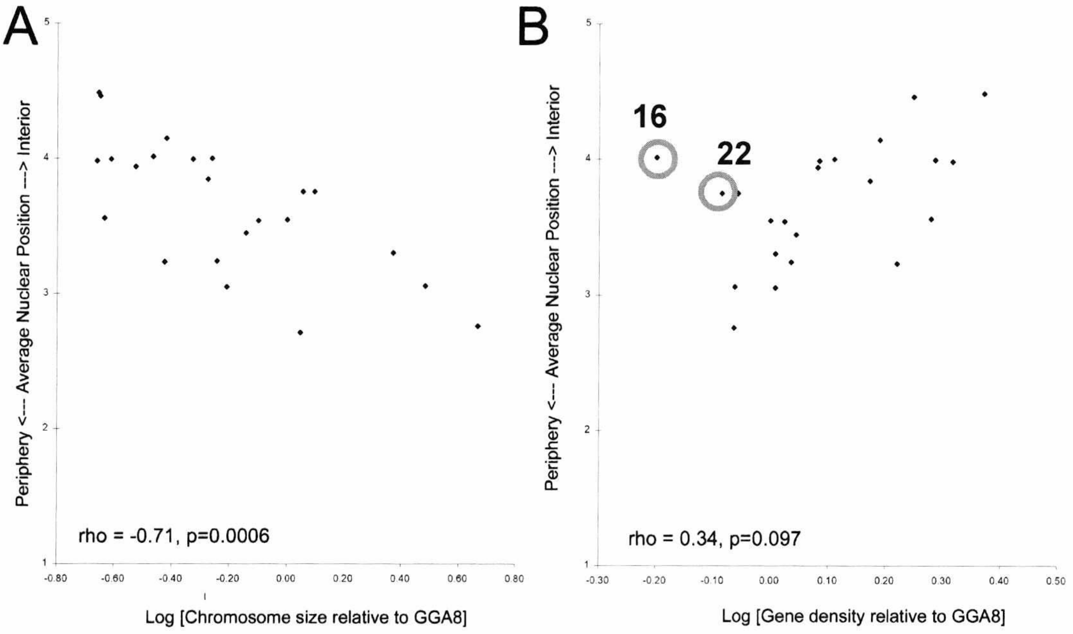


Figure 5.5: Chromosome positions in chicken A)plotted against chromosome size; B) plotted against gene density. Spearman's rho and associated p-values are included. Log values for size and density are shown to improve clarity for the smaller chromosomes; correlations are unaffected by this. GGA16 and 22 are circled in B.

Given that the reported number of genes for GGA16 is almost certainly an underestimate, it seems reasonable to remove this chromosome from the analysis, which improves the position-gene density correlation substantially (Spearman's rank, ρ=0.55, n=23, p=0.009). The position-chromosome size correlation is not greatly affected (Spearman's rank, ρ= -0.70, n=23, p=0.001).

Although an outlier, there is no evidence to suggest that the reported gene number for GGA22 is inaccurate; however, if this chromosome is removed from the analysis as well, the correlation between chromosome position and gene-density increases substantially (Spearman's rank, ρ=0.69, n=22, p=0.0016) and becomes equivalent to the correlation seen between chromosome size and position (Spearman's rank, ρ= -

0.69, n=22, p=0.0016). Therefore, the evidence appears to favour a chromosome size based distribution, the main discriminator being GGA22.

5.3.3. Nuclear location of chromosome territories in turkey and duck

Nuclear positions were assayed in turkey and duck as for chicken, though effects of background and non-specific hybridisation meant that fewer BACs provided reliable signals for interphase analysis. 59 BACs provided analysable signals in turkey, covering 27 chromosomes (MGA1-19, 21-26, 28-30; Table 5.1). 43 BACs provided analysable signals in duck, covering 20 chromosomes (APL1-12, 14, 15, 17, 22-28; Table 5.1). The overall pattern of nuclear organisation observed in turkey and duck was similar to that seen in chicken, with smaller chromosomes located towards the nuclear centre and larger chromosomes oriented towards the nuclear periphery (Figure 5.6C,D). The correlation between chromosome size and position was found to be statistically significant in turkey (Spearman's rank, ρ= -0.88, n=20, p=0.0002) and duck (Spearman's rank, ρ= -0.50, n=20, p=0.023).

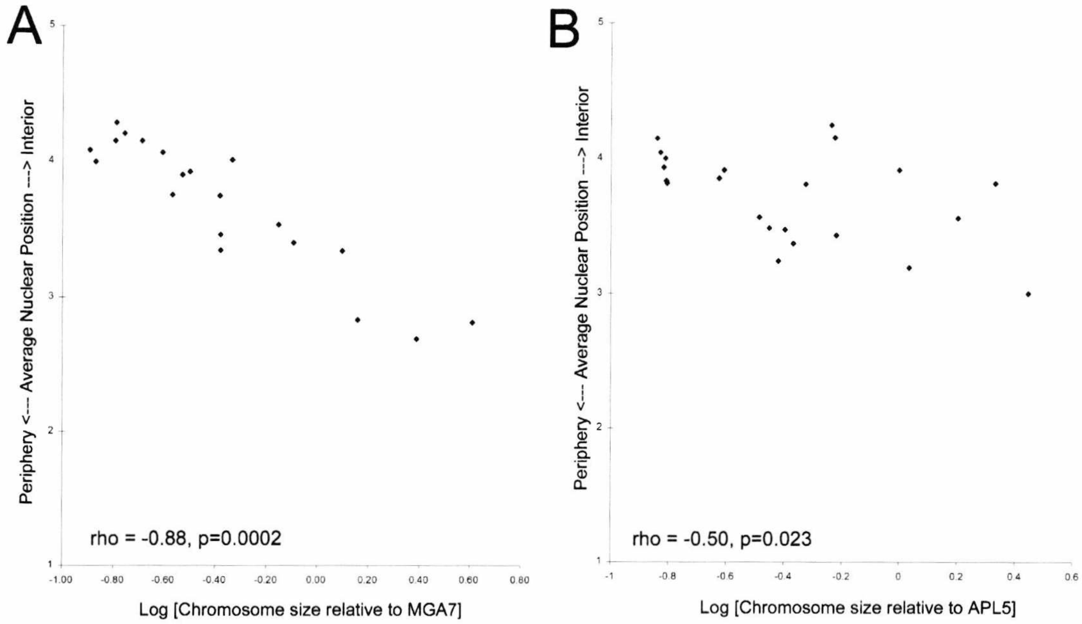


Figure 5.6: Chromosome positions plotted by chromosome size A) in turkey; B) in duck. Spearman's rho and associated p-values are included. Log values for size and density are shown to improve clarity for the smaller chromosomes; correlations are unaffected.

There are two interchromosomal differences between chicken and turkey; GGA2 is orthologous to MGA3 and 6, and GGA4 is orthologous to MGA4 and 9. GGA4 was found not to be different to a random distribution (χ^2, p=0.19). The positions of APL4 and MGA4 & 9 were not significantly different from random (χ^2, p=0.082, p=0.76 and p=0.20 respectively); the position of APL10 was somewhat towards the interior (χ^2, p=0.001), consistent with its size. If the data for GGA4p is considered there is also no evidence for a non-random distribution (χ^2, p=0.12). Similarly, separating the data for GGA2 and APL2, it can be seen that GGA2p is not distinguishable from a random distribution (χ^2, p=0.32). APL2p and MGA6 show non-random distributions, both similar and towards the interior. The data for GGA2q and APL2q show non-random distributions, but the data for MGA3 does not (χ^2, p=0.19). Thus, these results do not provide conclusive evidence for or against an association between patterns of nuclear organisation and evolutionary chromosomal rearrangements, albeit in this one somatic cell type.

5.3.4. Gene expression in stimulated versus un-stimulated macrophages

Chicken and duck macrophages were isolated from whole blood, and cultured in the presence of absence of lipopolysaccharide (LPS) for 24 hours. RNA was extracted from the chicken macrophages with and without LPS, and hybridised to the ARK-Genomics Affymetrix based chicken immune array in two experiments. Two technical replicates were performed for stimulated and un-stimulated RNAs, for a total of four experiments.

Based on a threshold value of a 25% increase or decrease in gene expression, a list of 46 genes determined to be induced or repressed is shown in Table 5.3. The gene lists of induced and repressed genes were input into the web program GOEAST (Gene Ontology Enrichment Analysis Software Toolkit, Zheng and Wang, 2008), which is designed to identify significantly enriched GO terms among given lists of genes, in order to visualise the biological processes represented by the differentially regulated genes (Figure 5.7). Enrichment can be seen among the induced genes for stress response and wound healing. Repressed genes are enriched for angiogenesis and phosphate transport.

Public ID	Gene description	+LPS / -LPS signal ratio	Chromosome	Sequence position
BX932384.1	cell division cycle associated 3	0.6840	1	80,411,783-80,413,885
BU448434	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	1.3152	1	
NM_205281.1	lysozyme (renal amyloidosis)	1.5810	1	37,298,007-37,301,735
BU116455	fatty acid binding protein 5	0.7205	2	126,080,810-126,085,868
BX931545.2	collectin sub-family member 12	0.7296	2	105,165,392-105,260,653
AJ445561	histone deacetylase 9	1.2511	2	29,265,256-29,534,926
NM_204628.1	interleukin 6 (interferon, beta 2)	1.2541	2	30,893,611-30,896,305
CK613373	Transcribed locus	1.2545	2	125142104-125142128
BX930076.2	carboxymethylenebutenolidase homolog (Pseudomonas)	1.3126	2	80,448,704-80,455,278
CR523336.1	uridine phosphorylase 1	1.3497	2	82,222,031-82,230,629
CK609569	interleukin 20 receptor, alpha	1.2572	3	56,808,836-56,816,532
ENSGALT00000022493.1	interleukin 20 receptor, alpha	1.3453	3	56,808,836-56,816,532
BU482672	interleukin 20 receptor, alpha	1.3595	3	56,808,836-56,816,532
CR385611.1	hypothetical gene supported by CR387685	1.7656	3	70654202-70654226
CR385834.1	hypothetical gene supported by CR387685	1.8747	3	70654202-70654226
CD729845	G protein-coupled receptor 34	0.7300	4	2,092,594-2,093,517
ENSGALT00000015877.1	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	1.2601	4	30,033,464-30,089,274
NM_205018.1	K60 protein	1.2935	4	52,434,109-52,437,187
CR386350.1	similar to K60 protein	1.3404	4	52,472,071-52,473,367
Y11273.1	deiodinase, iodothyronine, type III	1.4620	5	51,793,195-51,793,593
CR387544.1	Hermansky-Pudlak syndrome 5	1.5215	5	13,576,218-13,601,437
ENSGALT00000009304.1	ATP-binding cassette, sub-family G (WHITE), member 2	0.7339	6	18,627,461-18,640,727
CR387741.1	Deleted in malignant brain tumors 1	0.7418	6	32,869,836-32,883,415
NM_205259.1	leukocyte ribonuclease A-1	0.7452	6	10,376,970-10,386,988
ENSGALT00000009017.1	coagulation factor III (thromboplastin, tissue factor)	1.2994	8	14,226,411-14,231,816
BU302973	hepatitis A virus cellular receptor 1	0.4878	13	11,525,677-11,573,991
CR523673.1	Finished cDNA, clone ChEST840d13	1.2522	13	13:11585185-11585322
ENSGALT00000000109.1	L-amino-acid oxidase precursor	1.5103	16	163,943-166,969

Public ID	Gene description	+LPS / -LPS signal ratio	Chromosome	Sequence position
NM_205422.1	quiescence-specific protein	1.4829	17	881,078-883,977
NM_204961.1	nitric oxide synthase 2A (inducible, hepatocytes)	1.4107	19	9,162,701-9,181,877
BX934796.1	complement component 1, q subcomponent, B chain	0.6286	21	6,268,874-6,270,887
ENSGALT00000007607.1	complement component 1, q subcomponent, B chain	0.6901	21	6,268,874-6,270,887
BX929372.1	complement component 1, q subcomponent, A chain	0.7021	21	6,274,928-6,276,593
BU347270	complement component 1, q subcomponent, C chain	0.7086	21	6,272,270-6,273,886
NM_204524.1	interleukin 1, beta	1.2644	22	3,876,886-3,878,491
AJ720014	MARCKS-like 1	1.2582	23	5,377,330-5,379,580
ENSGALT00000001194.1	5-hydroxytryptamine (serotonin) receptor 1D	1.3102	23	1,606,652-1,607,782
BU261799	Finished cDNA, clone ChEST1005i19	1.3653	1, 23	23:1608529-1608553
BU415657	Glycerol-3-phosphate dehydrogenase 1 (soluble)	0.6571	E22C19W28_E50C23	269,080-269,804
CR352862.1	hypothetical gene supported by CR352862	1.2509	Un_random	
CR385967.1	MAS-related GPR, member H	1.3441	Un_random	27,669,857-27,670,741
BU424477	similar to carbonic anhydrase 9	1.2612	Z	8,562,779-8,570,341
CK612871	Transcribed locus	1.3906	Z	49215633-49215657
BU459954	---	1.2851		
BX262924	---	1.2879		
ENSGALT00000024608.1	similar to organic anion transporter OATP-M1	1.3142		

Table 5.3: Differentially regulated genes in chicken macrophages with and without LPS stimulation.

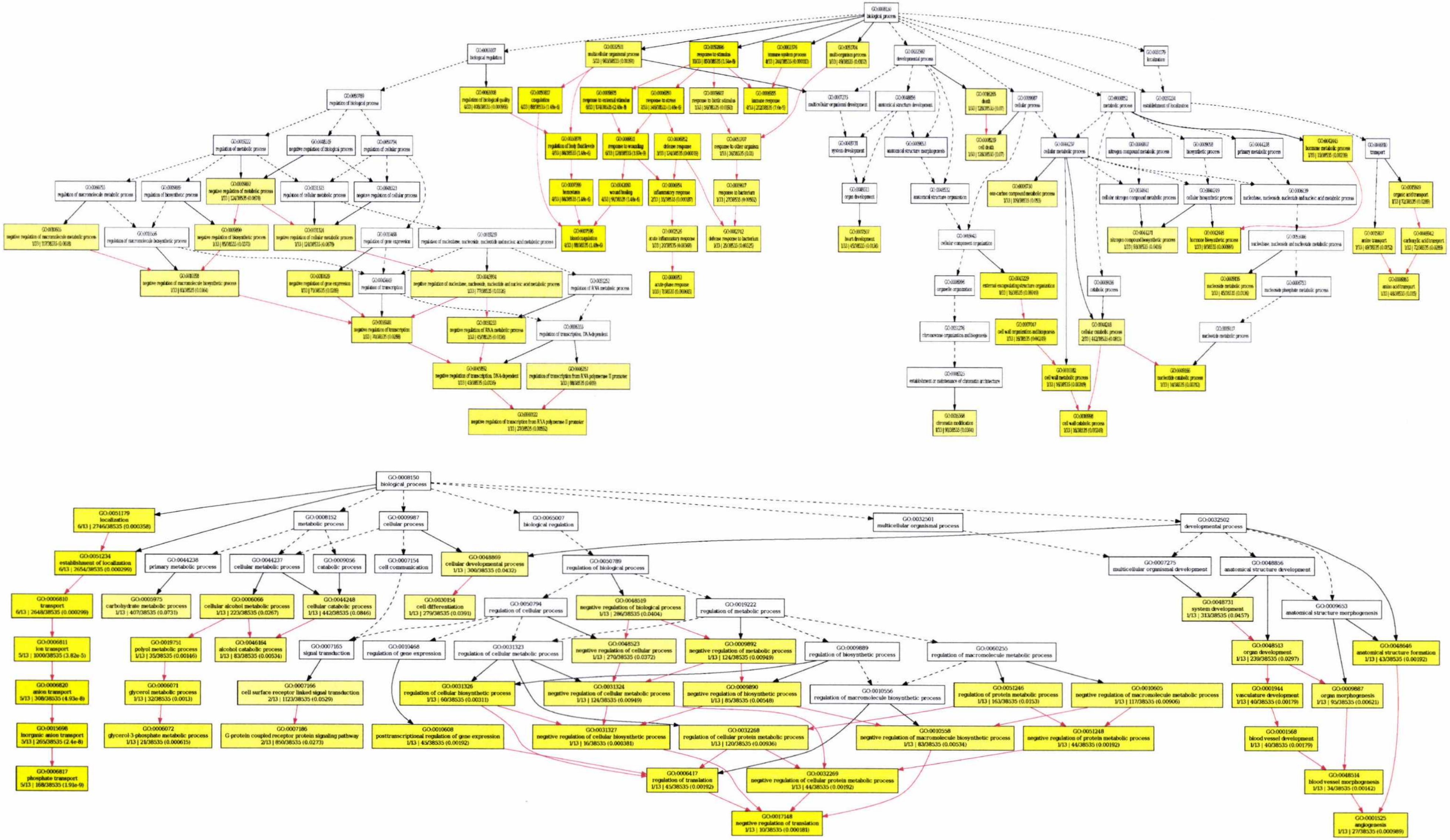


Figure 5.7: Output from GOEAST showing (top) up-regulated and (bottom) down-regulated biological processes significantly enriched in the microarray data in yellow. Darker colours indicate greater significance of the enrichment.

5.3.5. Hybridisation of BACs to LPS stimulated and un-stimulated macrophages

Chicken BAC clones on 11 chromosomes of interest (i.e. those containing up- or down-regulated genes) were selected and hybridised to LPS-stimulated and un-stimulated macrophages from chicken and duck. The BACs for which signals were produced analysable using the ImageJ macro are listed in Table 5.4.

Chicken chromosome	BAC clone	Clear hybridisation in chicken?	Clear hybridisation in duck?
2	WAG18C12	✓	✓
2	WAG39H3	✓	✓
2	WAG41C02	✓	✓
2	WAG41E24	✓	✓
3	WAG9P24	✓	
4	WAG13I05	✓	
4	WAG21J21	✓	
4	WAG23C04	✓	
5	WAG3O23	✓	
6	WAG21C4	✓	
6	WAG59L17	✓	
6	WAG6O17	✓	
16	WAG65G9	✓	✓
17	WAG40I7	✓	
17	WAG46F5	✓	
19	WAG20J24	✓	✓
22	WAG12K8	✓	✓
22	WAG32L22	✓	✓
23	WAG28L18	✓	✓
Z	WAG19N8	✓	
Z	WAG40K9	✓	

Table 5.4: BAC clones successfully hybridised and analysed in chicken and duck macrophages.

The overall chromosome positions were calculated, as described previously for fibroblasts. The distributions for each chromosome are shown in Figure 5.8 (next three pages). This figure shows the macrophage distributions alongside fibroblast distributions for ease of reference.

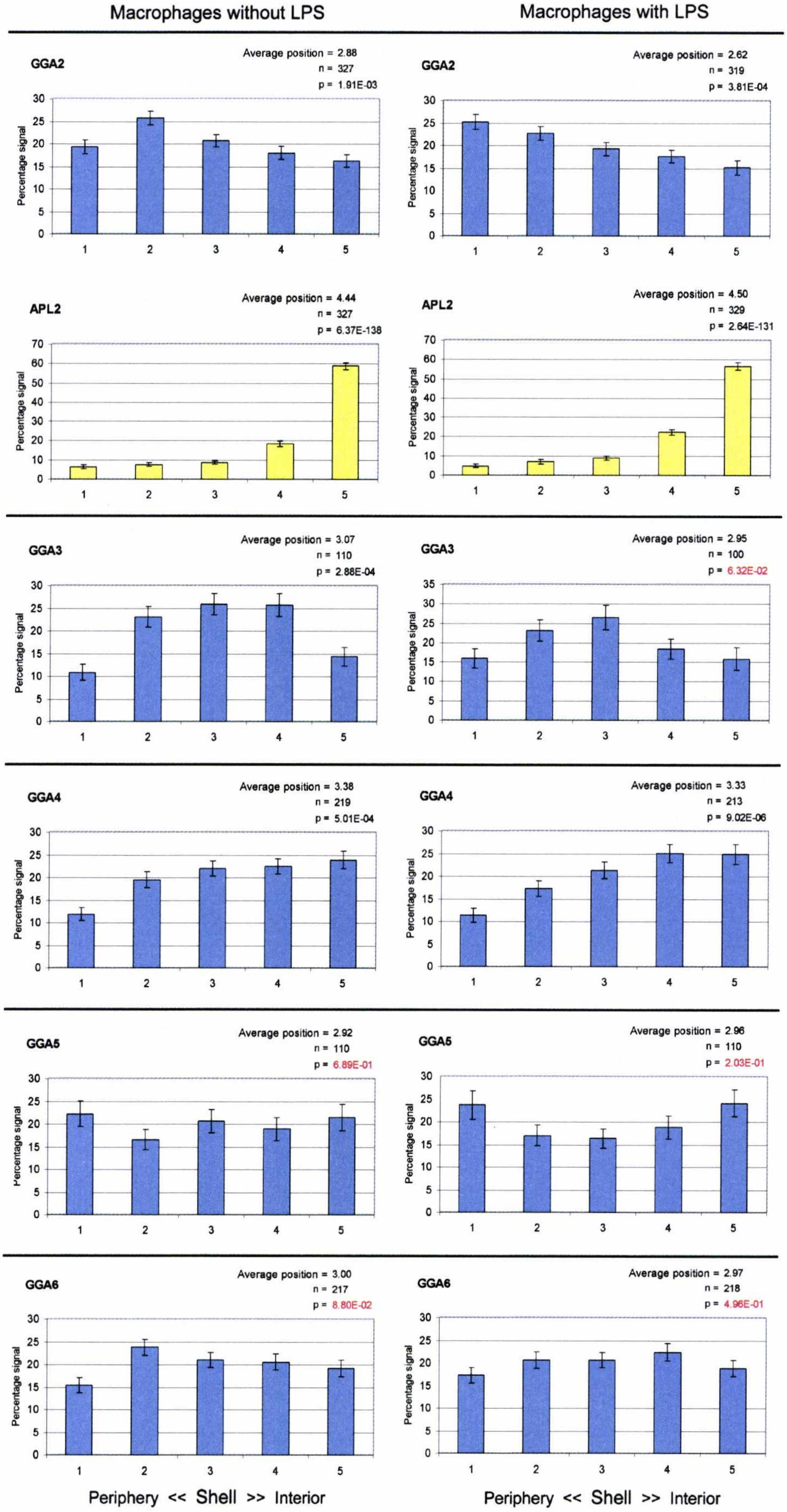


Figure 5.8: Signal distributions for chromosomes examined in macrophages with and without LPS stimulation. Blue indicates chicken, yellow indicates duck. Distributions not significantly different from random (chi-square tests, $p<0.05$) are indicated in red. Continued over the page.

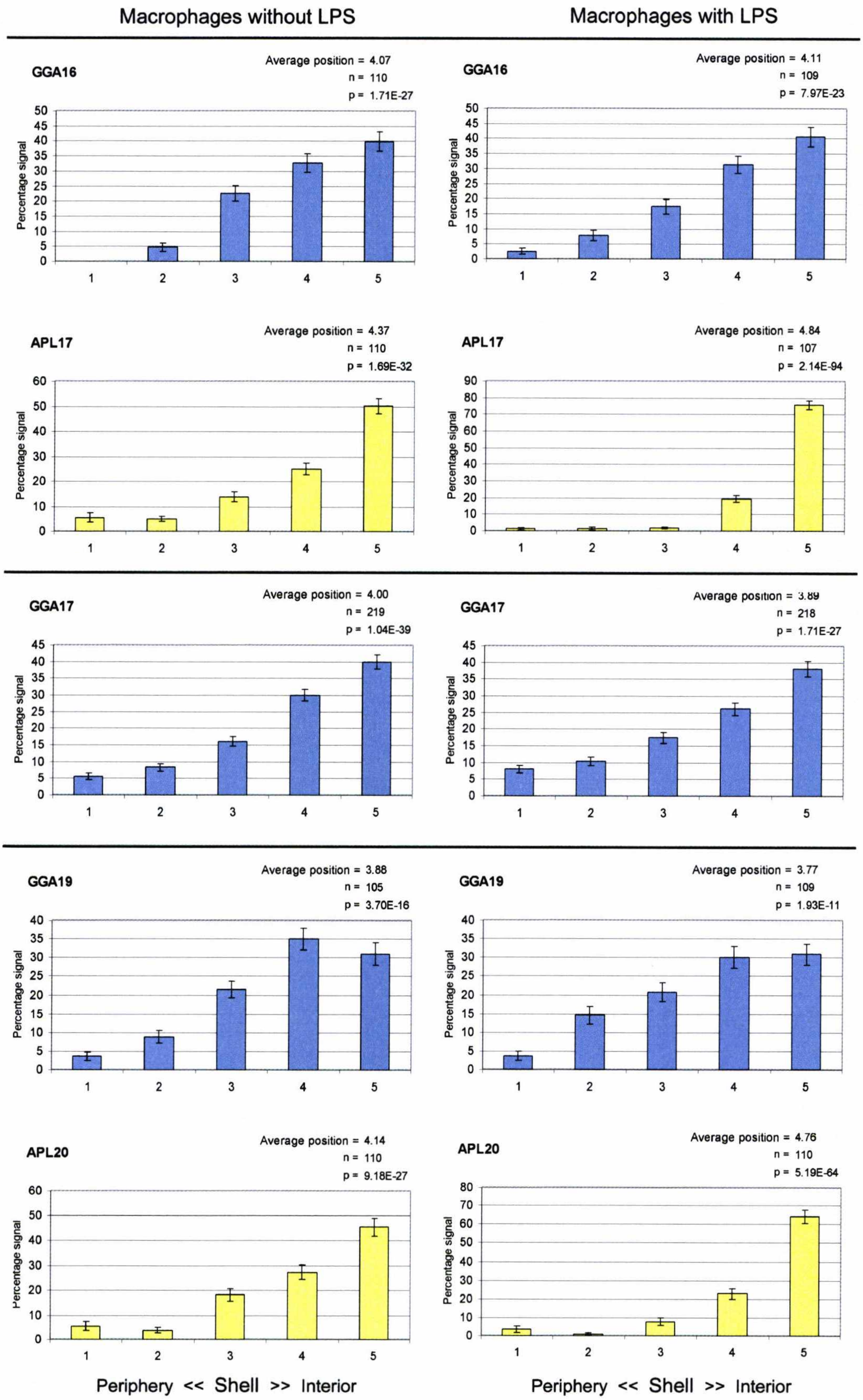


Figure 5.8 (continued): Signal distributions for chromosomes examined in macrophages with and without LPS stimulation. Blue indicates chicken, yellow indicates duck. Distributions not significantly different from random (chi-square tests, $p<0.05$) are indicated in red. Continued over the page.

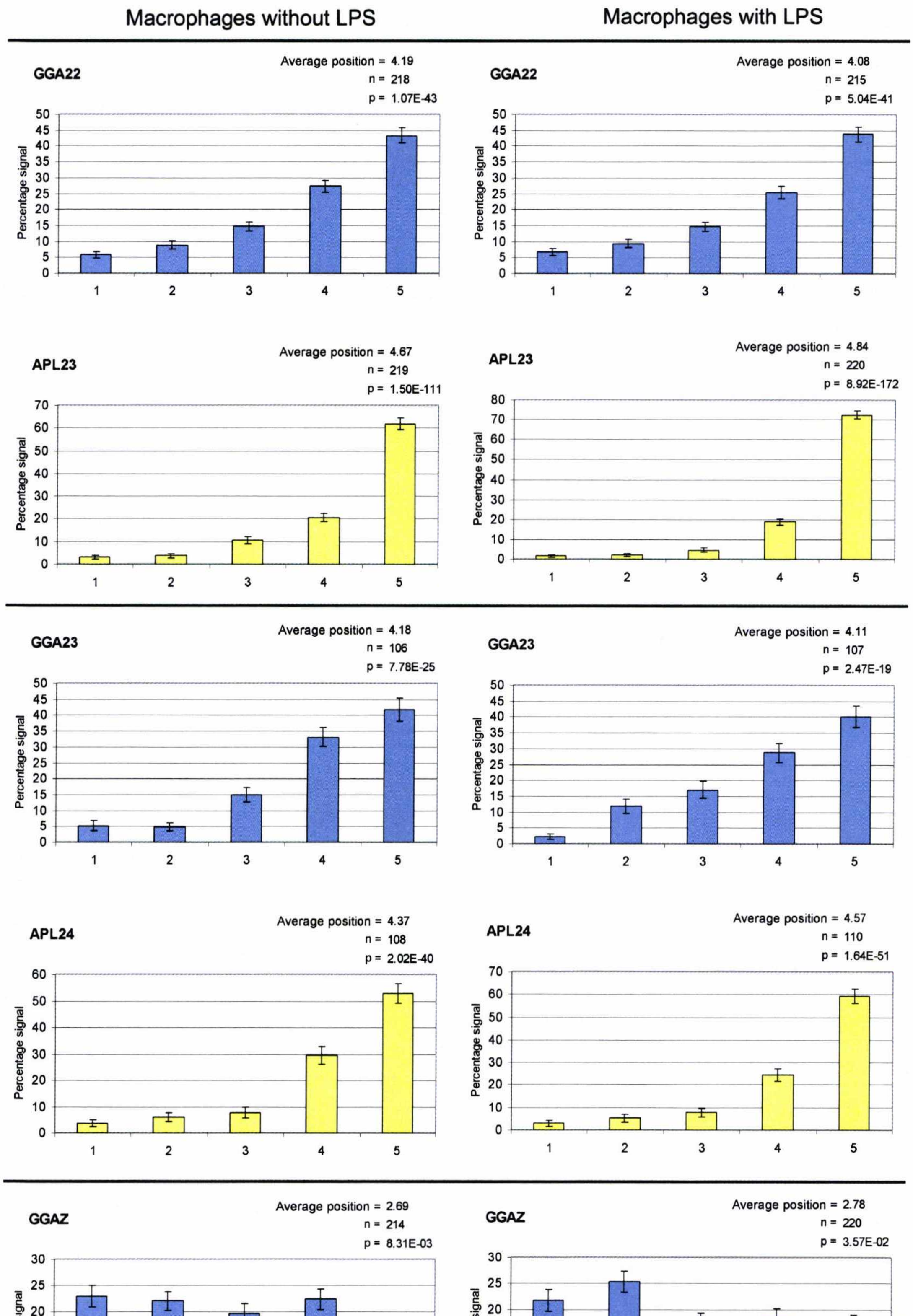


Figure 5.8 (continued): Signal distributions for chromosomes examined in macrophages with and without LPS stimulation. Blue indicates chicken, yellow indicates duck. Distributions not significantly different from random (chi-square tests, $p<0.05$) are indicated in red.

5.3.6. Positions of chromosomes in LPS-stimulated and un-stimulated macrophages versus fibroblasts

Visual comparison of the graphs in Figure 5.8 suggested that the broad patterns of nuclear organisation are similar between fibroblasts and macrophages, and between LPS stimulated and un-stimulated macrophages, although different probes were hybridised for fibroblasts and macrophages. The data are summarised in Table 5.5.

	Average Nuclear Position					
	Chicken			Duck		
Chromosome	Fibroblasts	Macrophages (without LPS)	Macrophages (with LPS)	Fibroblasts	Macrophages (without LPS)	Macrophages (with LPS)
2	3.06	2.88	2.62	3.28	4.44	4.50
3	3.30	3.07	N/S	N/A	N/A	N/A
4	N/S	3.38	3.33	N/A	N/A	N/A
5	N/S	N/S	N/S	N/A	N/A	N/A
6	3.75	N/S	N/S	N/A	N/A	N/A
16	4.21	4.07	4.11	3.73	4.37	4.84
17	3.99	4.37	3.89	N/A	N/A	N/A
19	4.14	3.88	3.77	N/A	4.14	4.76
22	4.01	4.19	4.08	3.73	4.67	4.08
23	3.56	4.18	4.11	N/S	4.37	4.57
Z	2.71	2.69	2.78	N/A	N/A	N/A

Table 5.5: Comparison of chromosome positions in chicken and duck for fibroblasts and macrophages. Note that BAC clones used are not identical. Summary of data presented in Figure 5.8. N/A indicates no data available; N/S indicates distribution not significantly different from random.

In chicken, comparison of the distributions by chi-square tests (4 d.f., $p<0.05$) revealed three chromosome distributions were not significantly different between fibroblasts and un-stimulated macrophages – GGA17 ($p=0.84$), GGA22 ($p=0.83$) and GGAZ ($p=0.58$). The remaining eight chromosomes showed significant differences in distribution patterns. These three chromosomes were also not significantly different in fibroblasts versus LPS-stimulated macrophages, nor was GGA2 ($p=0.08$).

In duck, two chromosomes showed no significant differences in distribution between fibroblasts and un-stimulated macrophages – APL17 (GGA16) ($p=0.19$) and APL23 (GGA22) ($p=0.47$). All four chromosomes showed a significant difference between fibroblasts and activated macrophages.

Comparing chromosome distributions in LPS-stimulated versus un-stimulated macrophages, one distribution was significantly different in chicken, GGA16. Three of the five distributions were significantly different in duck – APL17 (GGA16), APL20 (GGA19) and APL23 (GGA22). The chicken GGA16 distribution appeared to become more peripheral, while the three duck distributions became more internal in stimulated macrophages.

5.4. Discussion

5.4.1. Gene density and chromosome size correlation in chicken

There is a strong inverse correlation between chromosome size and gene density, which confirms previous findings based on the analysis of the chicken genome sequence (Hillier *et al.,* 2004). Two chromosomes measured did not fit the trend; GGA16 and GGA22. The deviation of GGA16 from the general correlation may be partially explained by the fact that this chromosome contains the nucleolus organiser region (NOR) (Masabanda *et al.,* 2004), which has been shown to contain between 80 and 400 tandem repeats of rRNA genes depending on the chicken breed (Delany and Krupkin, 1999). These genes are not represented in the Ensembl gene-build for GGA16. GGA16 also contains the MHC region in chicken (Dominguez-Steglich *et al.,* 1991), another rich source of repeats. Consequently the current Ensembl gene-build for GGA16 almost certainly underestimates the number of genes on this chromosome, leading to an underestimation of the gene density of GGA16 in the present study. Although exact data are not available for the chicken breed used, assuming the lower bound of 80 tandem repeats places GGA16 within the gene-density range seen for other chromosomes of that size (data not shown).

GGA22 also has a lower gene density than its size would predict (or a smaller size than its gene density would predict). It is presently unclear whether this is a real gene density difference or due to incomplete sequence assignment in the current Ensembl gene-build. Looking at the comparative chromosome sizes between chicken and turkey, and chicken and duck, GGA22 is larger than either its turkey or duck orthologues. A possible explanation for this is a heterochromatin accumulation or repeat expansion in the chicken lineage that increased chromosome size and reduced gene density. Heterochromatin accumulation has been documented in the chicken lineage on GGAZ (Hori *et al.,* 1999).

5.4.2. Nuclear location of chromosome territories in chicken

Previous studies by Habermann *et al.* (2001) in proliferating fibroblasts and neuronal cells provided the detailed chromosome positions for chicken chromosomes 1-5 and Z. Pooled chromosome paints for GGA1-5 and Z, for GGA6-10, and for 19 pairs of microchromosomes indicated that the largest chromosomes were found at the periphery of the nucleus, the intermediate sized chromosomes were slightly more internal, and the microchromosomes were predominantly internal, though with the possibility that some microchromosomes could adopt a peripheral position. However, there was no data to suggest whether the positions the microchromosomes adopt was correlated with their individual sizes, or whether they existed in a more flexible 'pool' of microchromosomes towards the centre of the nucleus. This study has demonstrated that there is indeed a correlation between size and position across all the measured chromosomes which showed a non-random position (chromosomes GGA1-3, 6-10, 14-19, 21-24, 26-28, Z). The fact that no microchromosomes were observed towards the periphery, in contrast to Habermann *et al.* (2001) may be explicable by the larger number of nuclei examined in this study; where 50 nuclei per BAC were examined here, their analysis looked at 21 neuronal nuclei and 28 fibroblast nuclei. Given that chromosome positioning appears to be dynamic, and that the overall positions found here are an average, it is not surprising that microchromosomes might be observed at the periphery in any single image though predominantly internal.

It is worth noting that some chromosomes (GGA4, 5, 11, 20, W) did not show a significant difference from a random distribution. In the case of GGAW, the available probe was a chromosome paint; as mentioned, initial work showed that chromosome paint signals overlapped a number of shells, even when at the nuclear periphery, simply due to the size of the signal. This meant that the ImageJ macro could never properly class paints as peripheral, even in cases obvious by eye, and prompted the use of BACs as probes. This could be a contributing factor for GGAW. In the case of the autosomes, the measured orthologues of GGA5, 12, 13 and 20 in turkey and duck all had non-random distributions; therefore either the analysis may simply have lacked the statistical power to detect a non-random signal distribution, or these chromosomes do not adopt preferred locations in the nucleus.

The strong inverse correlation between chromosome size and gene density makes it difficult to determine if nuclear organisation in the chicken follows a chromosome size-based or a gene density-based pattern. At first sight, the data appear to fit a size-based distribution better than a gene-density based distribution; the correlation between chromosome size and position is statistically significant, while the correlation between gene-density and chromosome position is not. However, given that the reported number of genes for GGA16 is almost certainly an underestimate, it seems reasonable to remove this chromosome from the analysis. This improved the correlation between position and gene density substantially (Spearman's rank, $\rho=0.55$, $n=23$, $p=0.009$). As noted above, it is not clear whether the reported gene number for GGA22 is accurate; with this chromosome removed from the analysis as well for the sake of example, the correlation between chromosome position and gene-density increased further (Spearman's rank, $\rho=0.69$, $n=22$, $p=0.0016$), and became equivalent to that between chromosome size and position (Spearman's rank, $\rho= -0.69$, $n=22$, $p=0.0016$). Given that there is no reason to consider the data for GGA22 to be inaccurate, it appears reasonable to retain this chromosome in the analysis. Therefore, the presently available data provide evidence favouring a chromosome size-based model of chromosome territory position over a gene-density based model in two of the three cases tested (i.e. including all chromosomes, excluding GGA16, excluding GGA16 and 22). Of these cases, the second (excluding GGA16 only) seems the most reasonable to use. Hence, chromosome position in chicken appears to follow a chromosome size based distribution over a gene-density based distribution, with the caveat that a single chromosome, GGA22 is the major discriminator.

A size based organisation is consistent with previous studies on human fibroblasts (Bolzer *et al.,* 2005, Sun *et al.,* 2000, Cremer and Cremer, 2001). Fibroblasts have elliptical nuclei; other cell types, such as lymphocytes, in which a gene density based organisation has been seen (Croft *et al.,* 1999), have spherical nuclei. It is still unclear precisely what the link is between nuclear shape and chromosome positioning. One suggestion is that a size based organisation in elliptical nuclei may be a functional consequence of mitotic spindle formation; that is, space constraints force the arms of the larger chromosomes towards the nuclear periphery (Bolzer *et al.,* 2005). This then posits a gene density based organisation as the default, disrupted in elliptical nuclei.

Another factor in chromosome positioning, in some instances, appears to be transcriptional status. Individual loci and gene clusters can move towards the interior of the nucleus upon transcriptional up-regulation (Stadler *et al.,* 2004), though locus re-positioning does not always correlate with gene activity (Meaburn and Misteli, 2007). In terms of entire chromosome territories, Bridger *et al.* (2000) describe a central shift of the gene poor human chromosome 18 as fibroblasts became quiescent, indicating that multiple different factors can affect chromosome position. The baseline developed here will facilitate such comparative studies in birds.

There is a theoretical possibility that the results are confounded by the analysis of flattened nuclei rather than 3D analysis. However, it is unlikely that this technical issue had severe effects on the results obtained. Although Edelmann *et al.* (2001) found that living cells had less variation in chromosome territory position over time than seen in fixed cells, which they ascribed to disruption from the FISH procedure, it has been shown that measuring large numbers of flattened nuclei gives equally accurate results as the analysis of 3D-preserved nuclei (Federico *et al.,* 2008).

5.4.3. Nuclear organisation in turkey and duck

Chicken BACs were cross-hybridised onto turkey and duck fibroblast nuclei, which previous studies had shown to successfully hybridise across species (Griffin *et al.,* 2008; chapter 4 of this thesis). The data for turkey and duck demonstrated a correlation for each between chromosome position and size (smaller chromosomes being more internal). The observed decrease in p-values in turkey and particularly in duck compared to chicken was likely due to an increased level of non-specific background. Cross-species FISH with chicken BACs is less frequently successful and produces more background in duck than in turkey (for example, 82 BACs successfully hybridising in chicken versus 59 in turkey and 43 in duck in this study).

Evolutionary conservation of nuclear organisation has also been seen among the primates studied to date (Müller *et al.,* 2003, Neusser *et al.,* 2007, Tanabe *et al.,* 2005), and a recent study demonstrated conservation in the positioning of gene rich and gene poor human chromosomes when placed in a mouse-human hybrid nucleus (Sengupta *et al.,* 2008). Taken together, these findings suggest that nuclear organisation in birds follows a general pattern that is also similar to patterns observed in other organismic groups, providing further evidence for the hypothesis that the principles underlying nuclear organisation are evolutionarily highly conserved (Neusser *et al.,* 2007).

5.4.4. Nuclear organisation and chromosomal rearrangements

It has been suggested that nuclear organisation may be related to chromosomal rearrangements by facilitating close spatial proximity between translocation partners (e.g. Cavalli, 2007, Gandhi *et al.,* 2008). Support for this hypothesis would come from chromosomes involved in evolutionary rearrangements that are found in close proximity in the interphase nucleus, while a large spatial distance between such chromosomes would argue against it. The karyotypes of the turkey and duck differ from that of the chicken by the retention of the ancestral bird chromosomes 4 and 10 (MGA4 & 9 and APL4 & 10), which are fused in the chicken (GGA4q & 4p). Besides, the ancestral chromosome 2, which is intact in chicken and duck, has undergone a fission in the turkey to produce MGA3 & 6. GGA4 was found slightly towards the periphery. The positions of APL4 & 10 and MGA4 were not significantly different from random; the position of MGA9 was more peripheral than expected given its microchromosomal properties (as seen from its fused state in chicken as GGA4p – that is, high GC content, high number of CpG islands, high recombination rate (Burt *et al.,* 1999)). Thus, these results unfortunately do not provide conclusive evidence for or against a functional role of nuclear organisation in chromosomal rearrangements. However, it would be interesting to pursue this question further using nuclear organisation studies in germ cells.

Such studies will certainly be of interest in other, more distantly related bird species; the Falconiformes, for example, contain some of the most extensive gross rearrangements of the ancestral karyotype seen in birds (e.g. de Oliveira *et al.,* 2005, Nanda *et al.,* 2006). Most of the macrochromosomes have undergone fissions and subsequent fusions with microchromosomes. Additionally, many microchromosomes have fused, resulting in a karyotype with mainly bi-armed medium sized chromosomes and few microchromosomes (e.g. de Oliveira *et al.*, 2005, Nanda *et al.*, 2006). Federico *et al.* (2005) used GC rich and GC poor isochore based probes from chicken in Falconiformes, and found that the GC rich isochores were internal at interphase and that the GC poor isochores were more peripheral in both chicken and falcons. Indeed, it has recently been suggested that 'gene-density' based organisations are 'GC content' based instead, masked by the typically high correlation between gene content and GC content (Hepperger *et al.*, 2008). How these relate to chromosomal positioning, and the implications thereof, are still open questions.

The results presented here provide information for comparisons with mammalian nuclear organisation given a divergence approximately 310 million years between the two classes (Blair and Hedges, 2005, Pereira and Baker, 2006). Moreover, the approaches described here present the basis for future nuclear organisation studies in addressing some aspects that are peculiar to the study of avian genomes.

5.4.5. Nuclear organisation in avian macrophages

Preliminary data is also presented extending nuclear organisation information to chicken and duck macrophages. The question was asked whether differences in transcriptional activity are linked to differences in chromosome territory positioning, using microarray based comparisons of LPS-stimulated and un-stimulated chicken macrophages to identify chromosomes containing genes with the highest levels of differential transcriptional regulation.

5.4.5.1. Microarray analysis of chicken macrophages

The effect of LPS on avian macrophages has previously been studied in the context of comparing LPS (simulating a bacterial infection) with an actual bacterial infection; Bliss *et al.* (2005) used a targeted chicken macrophage specific cDNA microarray as opposed to the immune cDNA array used in this study. Those results indicated that LPS was indeed activating many of the same pathways as a true infection. That data is largely corroborated by this study, in which genes identified as being subject to transcriptional regulation are significantly overrepresented in gene ontology categories representing stimulus and stress response; this is consistent with expectations of LPS stimulation activating pathways involved with response to infection. Two of the genes, for chemokine K60 and interleukin-1 beta that were up-regulated are also reported by Bliss *et al.* (2005). The presence of genes in both data sets provides further support for the successful stimulation of the macrophages. Upon activation, macrophages increase in size and motility, as well as bactericidal, tumouricidal and phagocytic activity (Quereshi *et al.,* 2000). Analysis of the gene ontologies of the genes involved shows enrichment for biological processes involving wound healing, stress responses, and bacterial defence among the up-regulated genes, consistent with macrophage function. Down regulated genes are seen to be involved with metabolic processes and angiogenesis, consistent with a redirection of resources towards combating infection. The successful identification of differentially regulated genes allowed chromosomes to be selected for investigation in the macrophage nuclear organisation study.

5.4.5.2. Comparison of nuclear organisation in fibroblasts and macrophages

Chromosome position was measured for 11 chromosomes in chicken macrophages. It must be noted that the BAC clones that were available for hybridisation were different from those used in the fibroblast experiments, and thus there may be some differences in the overall chromosome distributions obtained. With this caveat in mind, although the chi-square tests indicated that there are significant differences between chromosome distributions between fibroblasts and macrophages for eight chromosomes, the orientation of the distributions (i.e. whether towards the periphery or towards the interior) was maintained except for GGA4 (and perhaps GGA6, from visual inspection, although the macrophage distributions could not be said to be significantly different from random). This suggests that any positional differences are subtle, and should not be taken as definitive evidence for a different distribution. This is true of both chicken and duck, suggesting that the pattern of nuclear organisation is conserved in these two cell types. Notably, probes for GGA2 showed a peripheral tendency in chicken, but a slight interior tendency in duck, both in fibroblasts and macrophages, but far more pronounced in macrophages. It is unclear why this is the case from the present data.

The positions of chromosomes 1 and 8 were previously examined in LPS-stimulated chicken macrophages by Stadler *et al.* (2004). They found that these chromosomes were broadly in agreement with previous data from embryonic fibroblasts (Habermann *et al.*, 2001). However, they noted some cell type specific differences between LPS-stimulated macrophages and proerythroblasts, myeloblasts and precursor cells (Stadler *et al.*, 2004). On the basis of these data, it appears that patterns of nuclear organisation are likely conserved between un-stimulated chicken macrophages and fibroblasts, though cell-type specific differences in nuclear organisation (such as whether nuclear organisation in macrophages is chromosome size or gene density based) may emerge with more detailed investigation.

The positions of chromosomes in LPS-stimulated and un-stimulated macrophages were, in chicken, mostly not significantly different. The exception, GGA16, showed a slightly more peripheral distribution in stimulated macrophages. In duck, the opposite was observed; three of five distributions were significantly different between LPS-stimulated and un-stimulated macrophages, and appeared more towards the interior in stimulated macrophages. A shift towards the interior is consistent with previous descriptions of individual gene loci (e.g. Zink *et al.*, 2004, Williams *et al.*, 2006) and of whole chromosome territories (e.g. Bridger *et al.*, 2000) upon altered transcriptional activity. However, given that the same pattern was not seen in both chicken and duck, it is not possible to tell from the current data whether this reflects a biological difference between chicken and duck responses to LPS or technical effects from the cross-species hybridisation (as also mentioned above, 5.3.3).

5.5. Conclusions

This study has determined fibroblast nuclear organisation in three avian species with more detail than previously available. It has shown that there is a great deal of conservation among chicken, turkey and duck, which mirrors the strong conservation of karyotype structure and genome size reported in previous studies of the avian genome. It also provides some evidence for a size based chromosome organisation in elliptical fibroblast nuclei in birds, which has previously been described in mammalian nuclei only, and adds to the body of data suggesting that the principles governing nuclear organisation are conserved among vertebrate species. Moreover, the approaches described here provide tools for future nuclear organisation studies in avian species. Such studies may eventually determine whether or not nuclear organisation is a major player in avian embryology, disease phenotypes and genome evolution.

6. Specific aim 4: To test the hypothesis that inter-specific copy number variation (CNV) can be established between a range of avian species and speculate on the possible ramifications for genome evolution.

6.1. Background

As outlined in section 1.5, copy number variants (CNVs) contribute substantially to normal and disease related phenotypic variation in humans and other primates (Emanuel and Saita, 2007; Freeman *et al.*, 2006). Furthermore, copy number variable regions (CNVRs) may comprise about 12% of the human genome (Redon *et al.*, 2006). However, information is lacking on patterns of copy number variation in other vertebrate groups.

Birds represent a potentially informative group in which to study copy number variation; the 9600 species of birds, cover a wide range of lifestyles, feeding styles, locomotion styles and mate choice preference. In some cases, the genetic variations underlying phenotypic differences can involve large scale chromosomal rearrangements (e.g. Thomas *et al.*, 2008). However, a large proportion of the genetic contribution to phenotypic diversity, between the chromosomal scale and the sequence level scale, in birds is unaccounted for. Analysis of copy number variation across species in birds has been limited to turkey, and has suggested that there are low numbers of CNVs when compared with mammals.

Therefore, some general hypotheses can be made about copy number variation in bird genomes. The smaller genome size of birds compared to mammals and the smaller intergenic distances (Burt *et al.*, 1999) suggest that (1) there are fewer CNVs present within avian genomes than in mammalian genomes; (2) only CNVs with functional effects have been retained through evolution; (3) CNVs should be on average smaller than those seen in mammals. The association of segmental duplications with chromosomal rearrangements suggests that (4) specific avian lineages with a higher number of chromosomal rearrangements – such as the parrots and falcons – may have more segmental duplications and CNVs than other lineages. Furthermore, given the success of cross-species chromosome painting in birds, cross-species aCGH should prove successful across a wide range of birds, permitting comparative studies among phenotypically diverse species.

6.2. Specific Aims

With the above hypotheses in mind, the detailed specific aims of this chapter were as follows:

Specific aim 3a: To isolate and apply genomic DNA from feather pulp (and/or blood) from various avian species to a commercially available chicken whole genome tiling path oligonucleotide microarray.

Specific aim 3b: To test whether inter-specific CNVs can be determined in birds by this approach.

Specific aim 3c: To devise a means of analysing and expressing this data in a readily understandable format.

Specific aim 3d: To test the hypothesis that certain inter-specific CNVs are shared between multiple species.

Specific aim 3e: To speculate on the possible role of CNVs in avian genome evolution.

6.3. Results

6.3.1. Hybridisation of genomic DNA to a commercial microarray

Genomic DNA from nine avian species was successfully isolated from feather pulp or blood samples (see section 2.5.1.1). DNA was hybridised to the 384,000 oligonucleotide chicken tiling path microarray (Roche Nimblegen) by the company and data were returned for analysis. The results presented here incorporate a re-analysis of the raw data from turkey (Griffin *et al.*, 2008), for a total of 10 species. The species selected are detailed in Table 6.1.

Common name	**Binomial name**	**Individuals**	**Females**
Gyrfalcon	*Falco rusticolus*	5	5
Zebra finch	*Taeniopygia guttata*	2	1
Duck	*Anas platyrhynchos*	2	2
California quail	*Callipepla californica*	2	0
Bobwhite quail	*Colinus virginianus*	2	2
Peafowl	*Pavo cristatus*	1	0
Golden pheasant	*Chrysolophus pictus*	2	0
Lady Amhearst's pheasant	*Chrysolophus amherstiae*	2	0
Silver pheasant	*Lophura nycthemera*	4	2
Turkey	*Meleagris gallopavo*	1	1

Table 6.1: Species sampled for aCGH

The data returned by Nimblegen have had a segmentation algorithm applied to detect segments with different levels of hybridisation, as determined by $\log_2$ ratios of sample signal to reference signal. These are represented in GFF files viewable using software provided by Nimblegen; an example plot of the segmented $\log_2$ ratios for one zebra finch sample on GGA8 is shown in Figure 6.1; 5 segments are visible, one of which shows a significant enough $\log_2$ ratio (>0.5) for a CNV to be called (for more detail see Methods section 2.5.1.2). In this case, the CNV is a gain relative to chicken.

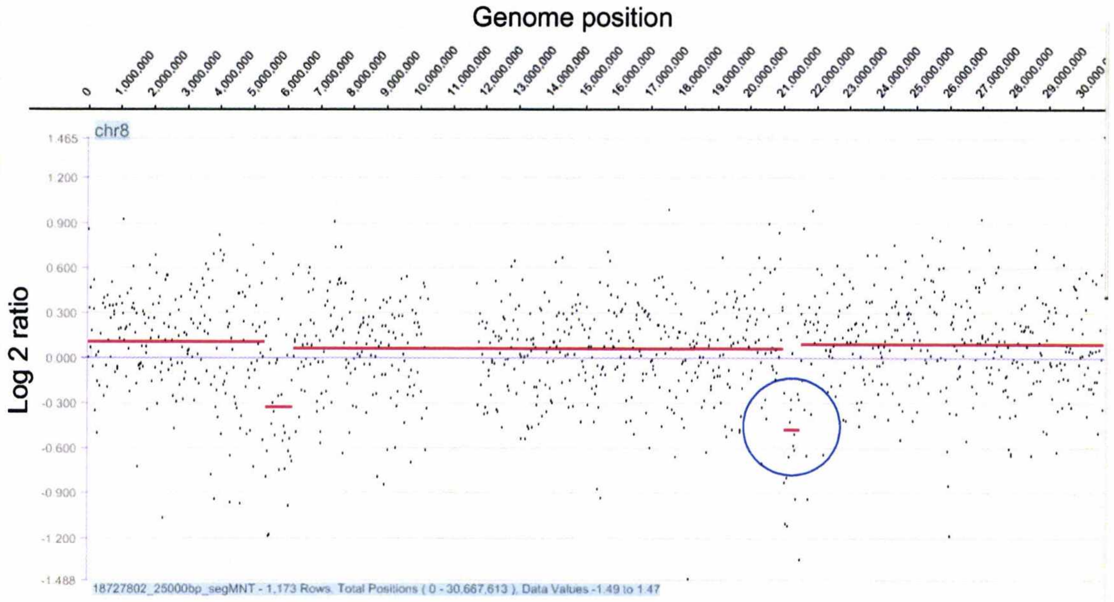


Figure 6.1: Screenshot from Nimblegen SignalMap software showing hybridisation of chicken and zebra finch DNA to GGA8. Individual oligonucleotide probes are represented by black dots. Detected segments are indicated by the red lines. Segmentation analysis shows 1 CNV (21.0-21.5Mb; within CNVR#44; circled in blue). The region 5.3-6.1Mb did not meet the significance threshold of a 0.5 change in $\log_2$ ratio.

Differential hybridisation of male and female genomic DNA to Z chromosome probes was observed, appearing as a gain in the homogametic males relative to the heterogametic reference DNA (Figure 6.2). Homo- or heterogamety was taken into account when CNVs were detected (hence no CNVs covering the entire Z chromosome of males are reported). Correct identification of sex also served as a technical validation of the hybridisations.

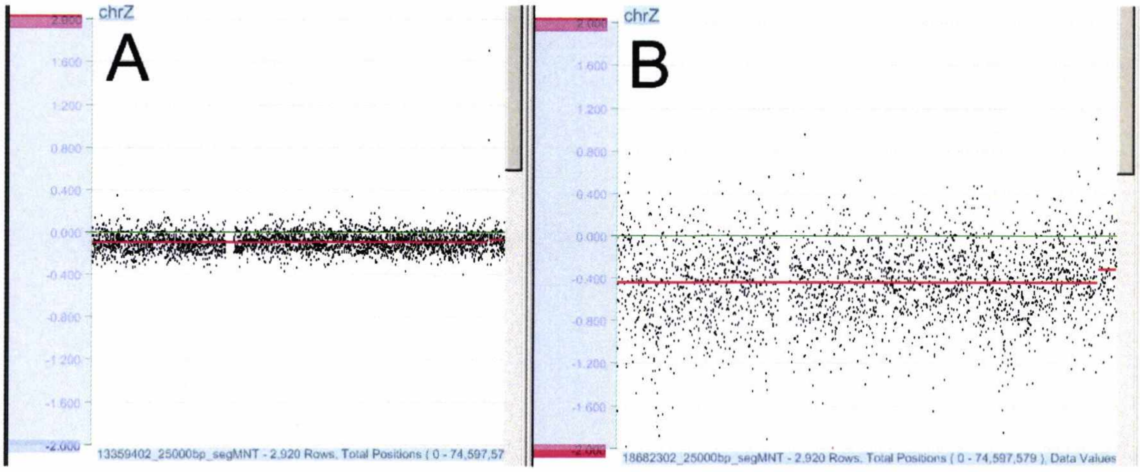


Figure 6.2: Screenshots from SignalMap showing hybridisation to GGAZ from A) ZW duck, log2 ratio = 0; and B) ZZ California quail, $\log_2$ ratio = -0.4 (gain relative to chicken).. Scale on the Y axis from +2 to -2.

6.3.2. Identification of CNVs by cross-species aCGH

Overall, 203 unique CNVs were found (summary data shown in Table 6.2). A template was designed in Microsoft Excel to analyse the raw data provided, calculate the positions of overlapping CNVs and generate a graphical representation of the data (see methods section 2.5.1.2). The overlapping CNVs were combined into 122 CNV regions (CNVRs, Table 6.3). A graphical representation of the CNVRs found in the 10 species is shown in Figure 6.3, indicating whether each CNVR is shared with other species, or is unique to that species.

	FRU	TGU	APL	CCA	CVI	PCR	CPI	CAM	LNY	MGA
Mean (kb)	186.37	237.37	297.70	776.72	231.45	202.81	430.16	286.78	186.70	299.80
Median (kb)	75	159.96	74.80	169.95	129.90	50	184.97	75	82.44	152.53
Min (kb)	7.34	15.04	12.58	12.58	7.81	15.16	7.34	14.93	7.34	7.40
Max (kb)	3409.98	1225	4439.67	8517.26	1400	2904.56	2810.05	2267.39	1360.11	900
Gains	30	22	6	21	28	7	5	6	13	9
Losses	23	11	24	16	19	21	21	23	23	1
Total CNVs	**53**	**33**	**30**	**37**	**47**	**28**	**26**	**29**	**36**	**10**

Table 6.2: Summary data of CNVs detected in the nine species from this study, and from turkey (Griffin *et al.*, 2008). FRU, gyrfalcon; TGU, zebra finch; APL, Pekin duck; CCA, California quail; CVI, Bobwhite quail; PCR, peafowl; CPI, Golden pheasant; CAM, Lady Amherst's pheasant; LNY, silver pheasant; MGA, turkey.

	FRU	TGU	APL	CCA	CVI	PCR	CPI	CAM	LNY	MGA
Gain in CNVR	23	17	6	17	25	7	4	6	12	8
Loss in CNVR	20	10	19	15	15	20	16	21	20	1
Total CNVRs	**43**	**27**	**25**	**32**	**40**	**27**	**20**	**27**	**32**	**9**

Table 6.3: Summary of gains and losses within CNVRs for each species (species codes as per Table 6.2). Despite increasing evolutionary distance from chicken towards the left, there is no trend towards a higher proportion of losses to gains, or of an increase in the total number of losses.

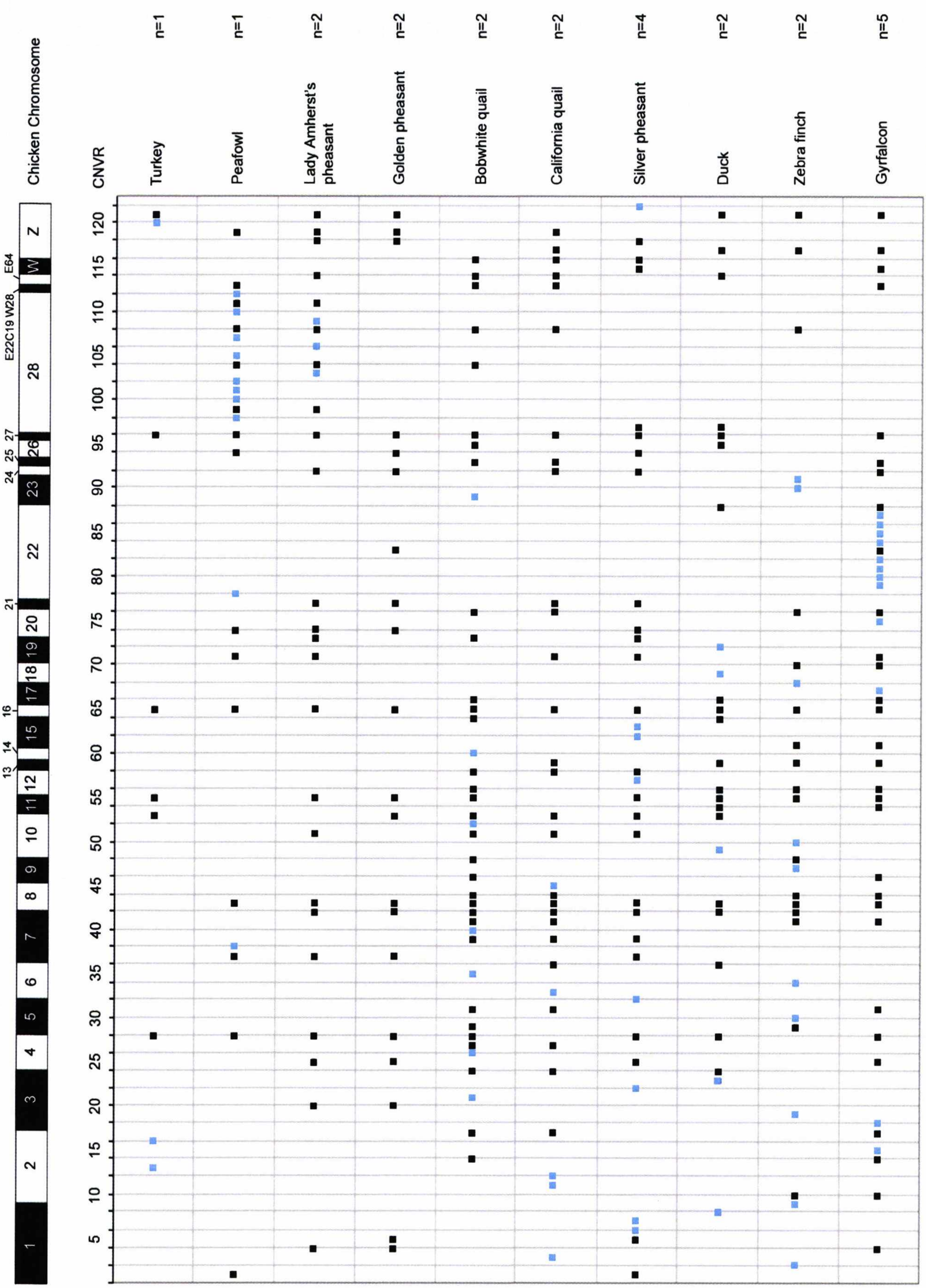


Figure 6.3: CNVRs detected in 10 bird species. Each dot represents a CNVR irrespective of size. Blue dots are CNVRs unique to one species; black dots are CNVRs shared by two or more species. n indicates the number of individuals sampled per species.

The CNVRs were examined for association with known or novel genes on the Ensembl database (release 52, Hubbard *et al.,* 2009). Of the 122 CNVs, 105 (86%) contained genes, or overlapped genes (Table 6.4). Both gains and losses were predominantly associated with genes, either known or novel; 69 of the CNVRs with gains (85%) and 39 of the CNVRs with losses (83%) had associated genes.

	CNVR shared between species		**CNVR seen in single species only**	
	Loss	**Gain**	**Loss**	**Gain**
No genes	8	3	3	9
Novel genes	12	4	7	5
Known genes	27	38	17	22
Total	**39**	**42**	**24**	**27**
Percentage associated with genes	93%	83%	75%	89%

Table 6.4: Proportion of CNVRs (unique and shared) associated with (i.e. overlapping) Ensembl genes. The majority of CNVRs contain known or novel genes, with a slightly higher proportion in CNVRs shared between species than in species specific CNVRs.

Sixty-three (52%) of the CNVRs were unique to a single species. The other 59 CNVRs (48%) were found to be shared between two or more species, up to all 10 of the species studied (Figure 6.4).

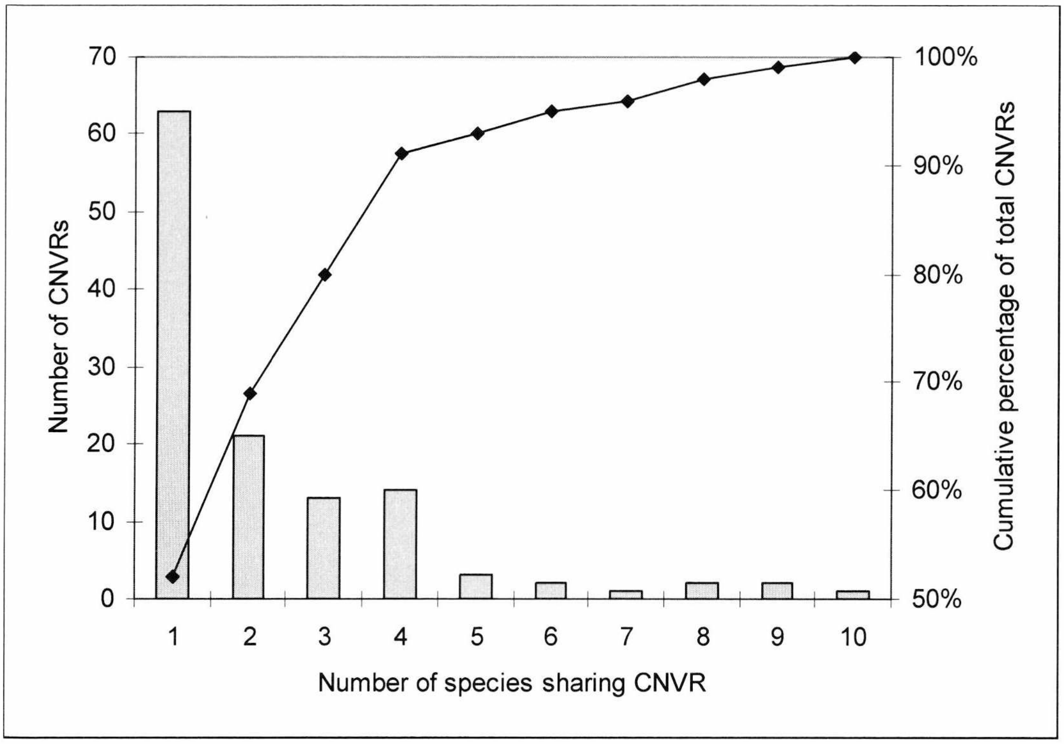


Figure 6.4: The number of species sharing CNVRs are shown against numbers of CNVRs (bars, left axis) and against cumulative percentage of total CNVRs (line, right axis). 48% of the CNVRs are shared in two or more species.

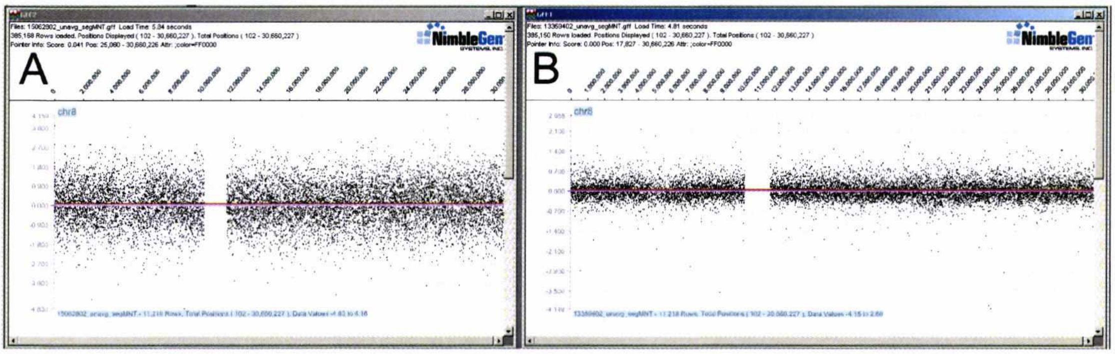
The CNVRs that were shared in 8 or more species are listed in Table 6.5, with their associated genes (see appendix table S1 for full list of CNVRs). There are three CNVRs (28, 65, 74) containing genes related to immune function, especially CNVR 65, which comprises almost the entirety of the available GGA16 sequence; two CNVRs (74, 96) contain genes which may relate to developmental processes. The remaining three CNVRs (43, 55 and 121) contain, respectively, no known genes, potential centromeric sequence, and novel genes of unknown function.

CNVR	No. species	Chromosome	Start	Stop	Gain/Loss relative to chicken	Genes	Comment
19	8	chr11	2670295	3192570	Loss	None	Possibly centromeric sequence
30	10	chr16	351	432851	Gain + Loss	All GGA16	MHC locus, NOR, rRNA genes, immune related
40	8	chr20	1450330	10662500	Gain	CDH22	Cadherin-22 precursor; involved in brain development
40						ITCH	Ubiquitin-dependent protein degradation
40						DUSP15	Dual specificity protein phosphatase
40						TGM2_CHICK	Protein cross-linking
40						CSK21_CHICK	Casein kinase in Wnt receptor signaling pathway
40						NP_989996.1	Involved in the immune response, apoptosis
40						NP_989998.1	Matrix metalloproteinase
54	10	chr27	2911	1005315	Loss	DAD1_CHICK	Negative regulator of apoptosis
89	8	chr04	88710224	89132504	Loss	NP_990566.1	Involved in the process of T-cell mediated killing
105	8	chr07	38335030	38380092	Loss	NAT5	N-acetyltransferase
106	8	chr08	102	35016	Loss	None	
121	10	chrZ	71510021	71885010	Loss	Novel	

Table 6.5: Genes found within CNVRs present in eight or more species. Three (30, 40, 89) relate to immune function; two (40, 54) may relate to developmental processes.

6.3.3. Assessment of noise in microarray data

The power of the segmentation analysis (section 2.5.1.2) to detect CNVs is in part dependent of the level of noise in the data (i.e. the spread of the data on the log 2 plot). Visually, one duck sample appeared to have a much lower amount of noise on the $\log_2$ ratio plots than the other samples (Figure 6.5).



```
Files: 15062802_unavg_segMNT.gff  Load Time: 5.34 seconds
385,158 Rows loaded. Positions Displayed ( 102 - 30,660,227 ). Total Positions ( 102 - 30,660,227 )
Pointer Info: Score: 0.041 Pos: 25,060 - 30,660,226 Attr: ;color=FF0000
```

```
Files: 13369402_unavg_segMNT.gff  Load Time: 4.81 seconds
385,150 Rows loaded. Positions Displayed ( 102 - 30,660,227 ). Total Positions ( 102 - 30,660,227 )
Pointer Info: Score: 0.000 Pos: 17,827 - 30,660,226 Attr: ;color=FF0000
```

Figure 6.5: Comparison screenshots from Nimblegen SignalMap software of A) zebra finch hybridisation on GGA8 with B) duck hybridisation on GGA8. The duck sample appears to have less noise, i.e. more constrained $\log_2$ ratios.

In order to compare the levels of noise between the species tested, standard deviations of $\log_2$ ratios were taken for every 10 probes across the unaveraged dataset (384,000 probes) – i.e. across 38,400 overlapping approximately 25kb windows (section 2.5.1.3). The resulting standard deviations were grouped into 0.01 size bins and plotted as a frequency distribution, with the interpretation that higher standard deviations would imply higher noise. All samples showed a similar or lower spread of data to turkey (Figure 6.6).

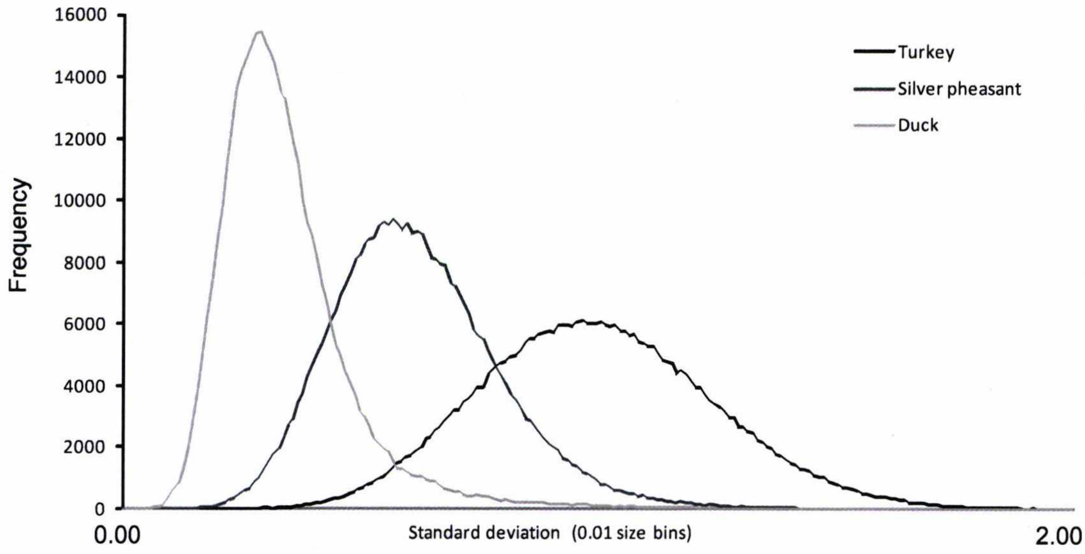


Figure 6.6: Assessment of noise in turkey, duck and silver pheasant data. Only the silver pheasant and duck samples were significantly different to turkey.

The differences between distributions were tested using (non-parametric) Mann-Whitney U tests; most of the samples showed no significant difference in distributions (significance level $p \leq 0.01$). The exceptions were a duck sample ($U=25063.5$, $n_{1,2}=200$, $p<0.0001$), and a silver pheasant sample ($U=17244.5.5$, $n_{1,2}=200$, $p=0.0087$) (shown on Figure 6.6). Hence, it is reasonable to assume that CNVs can be detected at least as readily as in turkey – differences in CNV numbers detected should be due to biological, not technical, causes.

6.3.3.1. Association of CNVRs with reported chicken segmental duplications

The segmental duplication data published for chicken genome galGal2 (2004) release (Hillier *et al.*, 2004, Chung *et al.*, 2003) was converted to match the galGal3 (2006) release from which the microarrays were designed, using the LiftOver tool in the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Of 49,166 segmental duplications, 28,789 were assigned to new positions in the Galgal3 build. The CNVR positions were compared to these updated segmental duplication positions. Overlaps were found for 57 of the 122 CNVRs (46.7%) (Appendix table S1).

6.4. Discussion

6.4.1. Cross-species application of aCGH in birds

Genomic DNA was successfully extracted from 9 bird species, and hybridised to the Nimblegen oligonucleotide array. The resulting dataset provided by the company yielded CNV information for all species tested. This data provides support for the hypotheses outlined in the background to this chapter.

Furthermore, the successful hybridisation of genomic DNA from zebra finch and gyrfalcon to a chicken microarray represents the most distant cross-species array-CGH to date. The divergence between the Galloanserae (chicken) and Neoaves (zebra finch and gyrfalcon) is thought to have occurred around 100 million years ago (van Tuinen *et al.*, 2001; Pereira and Baker, 2006). These successful hybridisations, despite the large evolutionary distance, may for the most part reflect the general conservation of genome structure in birds (e.g. the lack of large scale genomic rearrangements in avian karyotypes (Griffin *et al.*, 2007)).

6.4.1.1. Validation of data

The inclusion of both male and female DNA test samples provided a measure of validation of the cross-species hybridisation. In all cases, the expected gain on the Z chromosome in male (ZZ) samples against the female (ZW) reference DNA was observed. A concurrent loss against the W chromosome could not be confirmed, due to the small amount of euchromatic sequence (~260kb) available for the W in the current sequence assembly (Ensembl release 52). This also possibly contains the chicken pseudoautosomal region (and hence may be subject to cross-hybridisation of Z chromosome DNA).

A basic assessment of the amount of noise in the data sets, represented by the variation in $\log_2$ ratios between adjacent probes, showed no relationship between the evolutionary distance of the sample species from chicken and the spread of the data;

the most closely related species to chicken, turkey, had the highest spread of data, and most of the remaining samples were not significantly different. This suggests that CNVs, if present, are as readily detectible in all species as in turkey.

Independent validation of CNVs (such as by RT-PCR) was not performed; when performing cross-species hybridisations over such an evolutionary distance, such experiments would not necessarily yield informative data due to the dependency on accurate sequence data in both species for primer design. Where such validation has been performed cross-species (e.g. Dumas *et al.*, 2007, Marques-Bonet *et al.*, 2009) there have been draft genome sequences for some of the species in question. Moreover, even within species, RT-PCR experiments do not always agree with microarray based results (e.g. Fadista *et al.*, 2008). Nonetheless, although no validation technique agrees with aCGH data all of the time, all techniques show broad agreement between aCGH data and true copy number (e.g. Marques-Bonet *et al.*, 2009, Fadista *et al.*, 2008, Cutler *et al.*, 2008). That is, even if individual CNVs are not accurately called, the overall pattern of CNVs described remains valid. Furthermore, validation of CNVs becomes more important when attempting to match specific CNVs to phenotypic differences than when describing broad patterns. The availability of the zebra finch genome sequence, and those of other avian genomes will allow such validations to be performed.

6.4.2. Patterns of CNVs in birds

The 122 CNVRs detected had a mean length of 294kb and a median length of 70kb. A study of CNVs in humans only, using a similar platform, found 913 CNVRs with a mean length of 341kb and a median length of 228kb (Redon *et al.*, 2006). Though this study is based on a low sample size, it supports the hypotheses outlined above that CNVs in birds are less frequent, and smaller than those in mammals. Specific points of interest noted among the entire dataset, and among the more evolutionarily distant (from chicken) non-Galliformes are detailed below.

6.4.2.1. Association of CNVs with genes

Ensembl genes were found within the majority (80%) of the CNVs (and hence CNVRs). This is higher than that seen by Redon et al (2006), who found 58% of CNVRs in the human genome overlapping known RefSeq genes. The higher proportion of gene associated CNVs could be related to the smaller genome size of the chicken, in comparison to mammals; the chicken genome contains a similar number of genes to human, with the majority of the difference accounted for by a lower number of repeats and intergenic sequence (Hillier *et al.,* 2004). Beyond the direct associations reported here, CNVs can have effects on flanking genes, even those not directly overlapping the CNV (Reymond *et al.,* 2007), for example through disruption of promoters or enhancers.

Of the genes in the most commonly shared CNVRs (those appearing in 8-10 species), there are losses and gains in GGA16, which contains the MHC locus as well as other rRNA genes. Other immune related genes in CNVRs are found on GGA4 and GGA20, one showing a gain, the other a loss. Two genes are involved in regulation of apoptosis, and one is involved in brain development, showing a gain. A more detailed investigation into these CNVs would be of interest.

6.4.2.2. CNVs in Duck

Examining the CNVs in duck in more detail, all copy number gains in duck compared to chicken were located in coding regions. Genes in regions of copy number gain relative to chicken included transcription factors, neural proliferation control and neurotransmitter activity, and a predicted MHC class I gene. This is consistent with previously described duplication of the MHC class I locus in the mallard duck, followed by subsequent inactivation of some of the extra gene copies (Moon *et al.,* 2005).

Due to the vastly different levels of resolution afforded by cytogenetic mapping and microarray analysis, it is difficult to correlate the results of these two methods directly. Nevertheless, it is interesting to note that two of the CNVs revealed in the present study appear to coincide with rearrangements detected from cytogenetic mapping (section 4.3.2). These are CNVRs #37 and #43, on chromosomes GGA7 and 8 respectively. Further studies are necessary to examine this link between chromosomal rearrangements and CNVs in more detail.

6.4.2.3. CNVs in Zebra Finch

The zebra finch and gyrfalcon are the birds most distantly related to chicken that were examined in this study. Despite them also therefore having the highest amount of sequence divergence from chicken, there was no apparent difference in the number of CNVRs seen in zebra finch, or in the proportion of gains versus losses. Nor is there a noticeable difference in the number of 'unique' CNVRs (i.e. those that were seen only in zebra finch and are not shared with any other species). Given the caveats of sample size, and the underrepresentation of intraspecies variation, it seems reasonable to assume that with further sampling the number of 'unique' CNVRs will decrease further for each species. This in turn may indicate that certain genomic regions exist which are prone to copy number variation (i.e. hotspots), and the pattern of hotspot regions then harbouring CNVs is species specific. Comparison with the sequence data and segmental duplication data from the zebra finch genome project will be of significant interest in this regard.

6.4.2.4. CNVs in Gyrfalcon

The gyrfalcon has substantial rearrangements compared with the 'typical' avian karyotype (2n=52 (Schmutz and Oliphant 1987) c.f. 2n~80). The reduction in chromosome number, typical of the Falconiformes, was mediated by fusions of microchromosomes with macrochromosomes, microchromosomes with other microchromosomes, and some fissions of the largest ancestral chromosomes (Bed'Hom *et al.*, 2003, de Olivera *et al.*, 2006). In primates, there is a correlation between the locations of segmental duplications (SDs) and breakpoints of chromosomal rearrangements, and suggestion that chromosomal fusions may also be facilitated by the presence of segmental duplications (Kehrer-Sawatzki and Cooper, 2008). If this holds true for birds, one might expect to see an increase in the number of segmental duplications – and hence CNVs – in the falcon lineage, and in other avian orders with atypical karyotypes (e.g. the Psittaciformes (Nanda *et al.,* 2007)). The five falcon samples tested here show some evidence in favour of this – the falcons show the highest number of CNVs and CNVRs (with the caveat that this is also the group with the largest number of individuals tested, and therefore more CNVs would be expected to be detected). Notably, there are not more unique CNVRs in the falcons than in the other species (as also seen with zebra finch). This further supports the idea of CNV hotspots in birds, and can be tested in future experiments following these proof-of-principle successful hybridisations.

6.4.3. Shared inter-specific CNVs

The presence of shared CNVRs in different bird species has been previously suggested to represent sites of potential copy number hotspots (i.e. regions with clusters of copy number polymorphisms (Sebat *et al.,* 2007) or regions enriched for segmental duplications) (Griffin *et al.,* 2008). The data from this study show 43% of CNVRs shared between two or more species, a substantial proportion. Additionally, the number of gains and losses in CNVs comprising the CNVRs were similar. This suggests that the shared CNVRs are not merely regions where hybridisation to the microarray failed.

Shared CNVRs as detected here could represent either an independently occurring copy number change, or a shared ancestral copy number change. From the standpoint of identifying genomic regions prone to copy number variation, independent occurrence is of greater interest. The question of whether putative CNV hotspots are recurring independently can only be answered with a dataset comprising a large number of individuals per species, over a number of species, and an accurate phylogeny. However, it can be predicted that these shared CNVRs represent regions of recurrent variation. Given the divergence times between these species, it would be surprising for neutral, or advantageous, polymorphisms to have not been fixed (Clark, 1997), even with the typically higher effective population sizes in birds as compared with primates and other mammals. Only a few stable shared polymorphisms, similar to the heterozygous advantage for the MHC locus seen in humans and chimpanzees

(Lawlor *et al.*, 1988), would be expected. One would expect therefore that shared CNVRs have undergone independent recurrent variation. This again is an area in which research in birds might inform future studies on mechanisms and processes of CNV generation.

6.4.4. Association of CNVs with segmental duplications

Current models of CNV generation suggest that ancestral segmental duplications can in part drive copy number variation through non-allelic homologous recombination (Perry *et al.*, 2006, Repping *et al.*, 2006), and that there may be genomic regions that are inherently unstable, or prone to frequent variation. Recent cross-species work in great apes demonstrated both lineage specific segmental duplication events and recurrent independent duplications associated with gene duplications (Marques-Bonet *et al.*, 2009).

The segmental duplication data available for chicken (Hillier *et al.*, 2004, Cheung *et al.*, 2003) suggests that SDs tend to be small, mostly less than 10kb, and none larger than 50kb (Hillier *et al.*, 2004). Fewer segmental duplications have been detected in the chicken genome than in the 'typical' mammalian genome. Based on the current data set, examining the coincidence of CNVRs with SDs, it was found that 57 (47%) of the CNVRs overlap with SDs to some degree. However, the genomic locations of chicken segmental duplications are based on the February 2004 galGal 2 sequence assembly. The latest March 2006 galGal 3 assembly, from which the microarray was designed, does not overlap exactly with the galGal 2 data. The SD data was converted to match the latest genome build (see methods 2.5.1.4), but a large number of the SDs remained unmatched; the results are therefore subject to error. A more in-depth study will of course be of significant interest once updated information becomes available.

6.4.5. Distinguishing between copy number loss and sequence divergence

It must be noted that of the 277 CNVs identified, 152 show losses of signal relative to chicken. This could be due to a deletion in the test lineage (or a copy number increase in the chicken lineage), or it could be caused by lack of hybridisation to the microarray through sequence divergence. That is, although the copy number of the orthologous regions may not have changed, their sequence identity could be low for the probes to hybridise. While this is a concern, if observed losses were predominantly due to sequence divergence, one would expect to see an increase in the number of losses as evolutionary distance increased, which is not the case. Indeed, the fewest CNVR losses and highest number of gains observed were in the zebra finch. Additionally, many apparent copy number losses were observed in coding regions; hence, the observed loss in hybridization efficiency is likely associated with functional consequences, regardless of whether it is due to copy number change or sequence divergence.

Nonetheless, the question of how much apparent loss is due to true losses and how much is due to sequence divergence can be addressed in part through the use of reciprocal hybridisation of original reference DNA onto a microarray for an original target species. While tiling path arrays complementary to that used here for chicken are not available for any other birds other at present, it is likely that they will be established in the near future. This is especially likely for zebra finch, given the upcoming publication of the zebra finch genome sequence, and the interest in zebra finch genomics for wide range of studies – for example, as a model for adult neurogenesis and learning (Itoh and Arnold, 2005, Marler, 1990). The method would involve the hybridisation of chicken DNA to a zebra finch microarray – a reciprocal of the zebra finch DNA on chicken microarray described in this thesis. Comparing the two data sets would allow for some losses to be distinguished from sequence divergence.

A copy number loss in the zebra finch would appear as a loss in a zebra finch DNA on chicken array experiment. However, in a chicken DNA on zebra finch array experiment, either no signal will be seen (if a probe containing that sequence is not on the array at all) or a gain will be seen (if a probe is present and the copy number is higher in chicken). Now consider a sequence divergence without copy number change. In both experiments a loss will be seen, as the relevant probes are present on both arrays. When choosing zebra finch to compare to chicken, an assumption can be made that it is unlikely for sequence divergence to be greater in species more closely related to chicken. Hence, this would put a rough upper boundary on the number of losses expected due to sequence divergence in other species.

6.5. Conclusions

Copy number variation is increasingly being recognised as an important, and largely unexplored, area of genetic variation, contributing to both disease states and normal phenotypic variation. This work represents a broad survey of copy number variation in avian species, and established a base from which future studies will build. Initial hypotheses on patterns of CNVs in birds appear to be supported. Undoubtedly, the utility of cross-species aCGH in birds will prove valuable to investigations into copy number variation in general and avian genetic variability in particular.

7. General Discussion

7.1. Interpretation of this study in the context of avian genomics

The evidence describing the features of avian genomes from multiple sources – chromosome level through to sequencing level has shown that they have many unique characteristics when compared with the genomes of other species, in particular mammals. For example, genome size is about one third that of the typical mammalian genome (Burt, 1999), karyotype structure is radically different to that seen in other vertebrates and the number of chromosomes is both greater and more constrained than seen in most other vertebrate classes (Griffin *et al.,* 2007). For these reasons, birds represent a special and extremely interesting group for genomic study. Despite this, work in avian genomics is still at an early stage, and there are many open questions. This thesis has attempted to address some of these questions, and has been largely successful at not only answering them, but also at opening new areas of study that can inform future research.

The data from chromosome size measurements confirms what had previously been suspected, but never directly demonstrated; that orthologous chromosomes have similar sizes, and that large scale additions or deletions of genetic material (e.g. heterochromatin) are rare or absent within birds (Burt, 2002). Furthermore, it seems that chromosomal rearrangements are accompanied by at most only minor changes in overall genome content – fitting with existing theories of genome size constraint in birds (Gregory *et al.,* 2005). The specific rearrangements detailed in this thesis, between chicken, turkey and duck, cover species separated by approximately 90 million years of divergent evolution (van Tuinen *et al.,* 2001). What emerges, above the fine scale detail covered in (for example) the comparative cytogenetic map presented in chapter 5, is that large scale rearrangements over this distance are apparently extremely rare. When compared with the whole scale rearrangements common in other vertebrate groups – mice being an obvious example (Graphodatsky *et al.,* 2008) – the paucity of genomic rearrangement is both surprising and interesting.

Two fundamental questions are then posed by this: Why are rearrangements so infrequent in birds when compared with mammals? – and: Where does the genetic contribution to genetic variation lie? Answering these questions requires work beyond the scope of this thesis; nonetheless some attempt has been made at filling in the blanks in our knowledge.

The emerging picture in genomics research is that genomic rearrangements are mediated and facilitated by segmental duplications (Kehrer-Sawatzki *et al.,* 2008, Repping *et al.,* 2006, Redon *et al.,* 2006), through the mechanism of non-allelic homologous recombination (NAHR) (Lupski *et al.,* 2004). One would therefore expect a correlation between the number of genomic rearrangements, and the number of segmental duplications – or a measure thereof. Given that there is a significant correlation in humans (and other primates) between segmental duplications and CNVs (Redon *et al.,* 2006, Marques-Bonet *et al.,* 2009, Perry *et al.,* 2006), it follows that there should exist an association between genomic rearrangements and CNVs. These associations have been investigated and demonstrated in a small number of mammalian species, mainly primates (Kehrer-Sawatzki *et al.,* 2008). Preliminary evidence presented here suggests that they may also exist in birds.

The initial study on copy number variation between avian species presented in this thesis has shown that CNVs are much less frequent than those detected in humans, when using similar platforms (Redon *et al.,* 2006). This is true even given the smaller genome size of chicken when compared with human. The CNVs that are present are also smaller than those seen in humans and other primates. Together, these are consistent with a lower level of NAHR, lower numbers of segmental duplications, and fewer chromosomal rearrangements.

With a consistent picture of how these genomic feature correlate, the next question is why birds have low numbers of segmental duplications, chromosomal rearrangements and CNVs. A potential explanation can be found in the processes of avian genome evolution. The proposed principle of biased gene conversion (Galtier *et al.,* 2001) holds that there is a selection pressure to repair T-G mismatches to C-G (rather than T-A). In this case, areas of high recombination will gradually develop an isochore structure – that is, regions of high GC content. However, regions with high recombination rates are more susceptible to NAHR if repetitive sequences are present (Lupski, 2004). Yet, the proposed description of the ancestral vertebrate genome consists of a small genome size, a low repeat content, and the presence of microchromosomes (Burt, 2002). With no selective pressure against genome size, these mechanisms can generate increased numbers of repeats, and thus act as substrates for NAHR, segmental duplication and copy number variation (Figure 7.1). Yet there is convincing evidence to show that a pressure existed towards small genome sizes even in the dinosaurs, probably associated with the energy requirements of endothermy (Organ *et al.,* 2006).

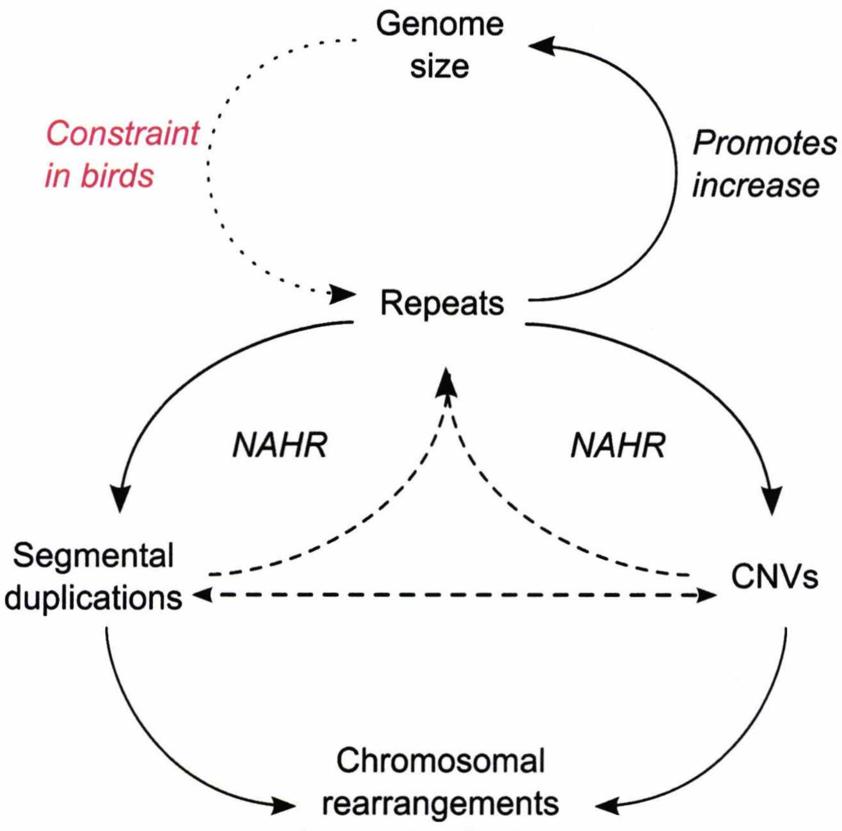


Figure 7.1: Relationship between CNVs, segmental duplications, chromosomal rearrangements and genome size. The genome size constraint in birds also imposes a constraint on the number of repeats, and thus on the number of SDs, CNVs and chromosomal rearrangements that can occur. The 'engine' for repeat expansion, SD and CNV generation is non-allelic homologous recombination (NAHR - note that this can also drive repeat excision given a size constraint as in birds).

A model of genome evolution in birds studied to date may then be proposed. Whereas in other vertebrate lineages, genome sizes generally increased, either through repeat content or polyploidy, in dinosaurs they appear to have been more constrained. About 150 million years ago, birds first appear in the fossil record (von Meyer, 1861); it is hypothesised a second constraint on genome size developed in at least some avian lineages, be it through energy requirements for flight, neuron size in learning (Gregory 2002, 2009), or indeed other factors. For these lineages, we can speculate that the genomic features leading to, and associated with, copy number variation (repetitive content, segmental duplications) were selected against. That is not to say that copy number variation could not develop under such a model; selection for genome size would likely be extremely subtle, and it seems improbable that the levels of copy number variation seen in the birds studied here (on the order of a few tens of megabases) could be selected for or against, the phenotypic effect being simply too small in the context of genome size constraint. Of course, sampling of a greater number and diversity of bird species will be needed at all levels (genome sizes, karyotypes, copy number variants), to properly establish whether this model is consistent (1) in the orders for which most information is available and (2) in other orders for which very little genomic data are available.

Although small in terms of effects on genome size, CNVs can have a significant phenotypic effect when associated with genes, as found in humans and other primates (e.g. Perry *et al.*, 2007). This then is a potential answer for the second of the 'fundamental questions' posed above – Where does the genetic variation in birds lie? At the time of writing, the zebra finch genome will soon be published. It will no doubt reveal numerous sequence level differences that will have phenotypic effects. Yet, subtle copy number changes are likely to be revealed as having a substantial contribution to avian phenotypic diversity with future in depth studies.

All of the features described in the first three chapters of this thesis then, appear to be connected by the unique characteristics of avian evolution. Does this then indicate that all aspects of avian genomics show considerable differences to other vertebrates? Not necessarily – in aspects of nuclear organisation, birds appear to follow the same global patterns as mammals.

The baseline provided by the analysis of chromosome position in embryonic fibroblasts in chicken, turkey and duck has demonstrated the apparent overall conservation of nuclear organisation in birds. It also suggests that the principles underlying nuclear organisation may date back to over 310 Myrs, when birds and mammals diverged (Burt *et al.*, 1999; Kumar and Hedges 1998). Besides the broad conservation of chromosome territory organisation, there are finer levels of organisation. Indeed, studies are linking isochore structure (i.e. GC content) with nuclear organisation (Hepperger *et al.,* 2008). This has been demonstrated to some degree in falcons and in mice. This again would place the origins of current nuclear organisation to between 310-360 Mya, when it is thought that isochores began to develop (Chojnowski *et al.,* 2007). If this is true, then birds are even more interesting for studies of nuclear organisation; the isochore structure in mammals is becoming less distinct via the homogenisation of GC content, and such differences may be reflected in patterns of nuclear organisation.

7.2. Further work arising

The work presented in this thesis will help to answer number of fundamental questions in avian genomics. However, many questions remain leading on from this work. Three areas immediately suggest themselves:

(1) The comparison between the resolution of cytogenetic maps and sequence maps. Although the cytogenetic map presented here is one of the most detailed in a bird to date, it still potentially overlooks fine scale rearrangements. Unresolved inconsistencies, such as the different morphologies of chicken and duck chromosomes yet identical marker orders, show chromosomal rearrangements that have yet to be characterised. The publication of genome sequences for other birds, for example the zebra finch and the duck, will allow direct *in silico* comparisons of syntenies, and identification of small translocations and inversions below the resolution of cytogenetic mapping.

(2) The study of CNVs can be extended to a larger number of individuals, with both chicken breeds and other birds, targeted at identifying fixed differences associated

with phenotypic traits. Studies within chicken will be facilitated by the greater reliability of validation techniques such as PCR and FISH. In other avian species, CNV studies will be especially of interest in birds with atypical karyotypes, such as the falcons and parrots, where CNVs may be more common. Furthermore, a detailed comparison of the chicken and zebra finch genomes has been performed as part of the upcoming publication of the zebra finch genome project. This highlights the breakpoints and rearrangements. Correlating these breakpoints with the positions of CNVs that have been discovered between the two species would be a useful test of the model outlined above – it would be predicted that they associate quite closely.

(3) Nuclear organisation information has now been established for chicken fibroblasts. Against this baseline, the question of chromosome size versus gene density (or other organisation) can still be investigated. Much remains to be studied on the patterns of nuclear organisation in the different cell types in chicken, how these patterns alter through development and cell status, and whether these patterns are evolutionarily conserved. Again, the falcons and parrots represent an ideal group for further study. A detailed analysis of chromosome position in falcons would inform future nuclear organisation work in birds, and could provide insights into the evolution of chromosome positioning.

7.3. General Conclusion

The work presented in this thesis includes both methods development and the subsequent use of these methods to investigate biological questions. In terms of the methodology, this thesis provides a valuable 'proof of principle' in the chapters on chromosome size, nuclear organisation and copy number variation for future work in these areas. Furthermore, this thesis has answered a number of outstanding questions in avian, and specifically duck genomics. Much novel data has been generated in the areas of comparative mapping, nuclear organisation and copy number variation. These data show avian genomes to be both unique and fascinating among vertebrate genomes. The prevailing view of birds having highly conserved genomes is supported, but a glimpse of the variety within may have been seen.

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9. Appendix

9.1. Supplementary table

Table S1 – This table lists the CNVRs found in chapter 6 with their position, associated genes, association with known chicken segmental duplications, and whether the CNVR contains gains, losses, or both relative to chicken.

CNVR	Chicken Chromosome	CNVR Start	CNVR End	Genes associated	SDs associated?
1	chr01	12500	87500	NP_001026572.1, GOLGB1	n
2	chr01	26847993	26917645	CAV1_CHICK, NP_001007087.1	n
3	chr01	29990403	30032862	None	n
4	chr01	81262500	81362500	Novel, NM_205385.1, XR_026667.1	n
5	chr01	86437500	86487500	Novel	n
6	chr01	104452890	104487555	Novel	y
7	chr01	126047778	126075193	Novel	n
8	chr01	140577735	140655038	None	y
9	chr01	169725133	169752564	None	n
10	chr02	162682	487500	AGAP3	y
11	chr02	49242621	49287943	None	n
12	chr02	51570302	51632686	AMPH_CHICK	n
13	chr02	54507726	54520395	TPK1	y
14	chr02	111362500	111687500	None	y
15	chr02	123580422	124210127	ZFHX4	y
16	chr02	148397711	148432599	None	n
17	chr02	154575479	154602505	Novel	y
18	chr03	20780115	20972919	SPATA17, NP_001026217.1	n
19	chr03	31462500	31637500	CANX_CHICK, HS90B_CHICK	n
20	chr03	43652597	43667533	None	y
21	chr03	50097761	50117849	None	n
22	chr03	50777886	50862913	C6orf97, RMND1	y
23	chr03	107012500	108262500	NP_990387.1, OTOF , EFR3B, NP_989898.1	n
24	chr03	113237500	113652668	FAM176A, MRPL19	y
25	chr04	12500	412500	KIAA1166, VSIG4, HSF3_CHICK, HEPH, EDA2R	y
26	chr04	5097963	5485088	XR_026989.1, Q9YGR0_CHICK, NP_001006433.1	n
27	chr04	51150035	51262979	LEG6_CHICK, ANKRD56, SEP11	n
28	chr04	88710224	89132504	JHD2A_CHICK, REEP1, NP_990566.1, NP_001006463.1	y
29	chr05	12500	62500	Q5ZMT2_CHICK	y
30	chr05	300133	395182	DDB1_CHICK, XR_026960.1 , PEPA_CHICK, NP_990252.1	y
31	chr05	12062500	12512500	SOX6	y
32	chr05	33262500	33862500	NP_990134.1, CO041_CHICK	n
33	chr06	12115135	12510079	COL13A1, P4HA1_CHICK, OIT3, CCDC109A, CBARA1	y
34	chr06	23650363	23970419	LCOR, SLIT1, RHG19_CHICK, MMS19, LOXL4, RRP12_CHICK	n
35	chr06	24472523	24532736	KAZALD1, SFXN3, PDZD7	n
36	chr06	37387597	37400184	None	y
37	chr07	352	485145	NP_989599.1, MFSD6, HIBCH_CHICK, GDF8_CHICK, NP_001006508.1	y
38	chr07	3632667	3695091	NP_989938.1	n
39	chr07	16200444	16485101	Q788U5_CHICK, CCDC141, Q07784_CHICK, Q90804_CHICK	n
40	chr07	21762500	22137500	FIGN	y
41	chr07	23612500	24037500	INHA_CHICK, NP_001006590.1, NP_990260.1, TBA5_CHICK, NM_204179.1, NP_001012937.1	n

42	chr07	38335030	38380092	NAT5	y
43	chr08	102	35016	None	y
44	chr08	21030081	21512500	IPO13, PRNPIP, ATP6V0B, DMAP1, NM_001031279.1	y
45	chr08	27717867	28062554	NFIA_CHICK	n
46	chr09	5887500	5937500	XR_026721.1, XR_027037.1, KIF1A, C2orf54	n
47	chr09	12720374	12770046	None	n
48	chr09	16962500	17137500	NP_001012952.1, EPHB3_CHICK, Q5ZIM4_CHICK	n
49	chr10	252	27706	Novel	y
50	chr10	3912500	3937500	Novel	y
51	chr10	16887500	18737500	COT2_CHICK, ARRDC4	y
52	chr10	21902944	22102758	CASC4_CHICK, BLM_CHICK	n
53	chr10	22287890	22537510	IQGAP1, TUBGCP4, TP53BP1, XR_026723.1, HISPPD2A	y
54	chr11	202	62500	Novel	y
55	chr11	2670295	3192570	None - centromere?	y
56	chr12	12500	105028	LIMS1	y
57	chr12	10387500	10537500	None	y
58	chr12	16237500	16665135	NP_001019998.1	y
59	chr13	18780447	18907544	EGR1_CHICK	y
60	chr14	7047885	7275252	HS3ST4, HS3ST2	n
61	chr15	917544	937693	NP_989796.1	n
62	chr15	7912500	8037500	A1EAT1_CHICK, NP_001006192.1	n
63	chr15	9337500	9412500	HNF1A_CHICK, RN185_CHICK, LIMK2_CHICK	n
64	chr15	10240354	10295196	Novel	n
65	chr16	351	432851	All GGA16 sequence	y
66	chr17	797987	867945	ABCA2, NPDC1, NP_001026647.1	n
67	chr17	8442527	8542643	NOTCH1	n
68	chr17	10137709	10170150	Novel	n
69	chr18	1812500	1837500	Novel	y
70	chr18	9537949	9920218	ENPP7 , CBX8, NP_989973.1, TBC1D16, CBX2, C1QTNF1, Q8JGT3_CHICK	n
71	chr19	12500	62500	NP_001001302.1	y
72	chr19	3665182	3845095	MLRB_CHICK	n
73	chr19	5337500	5612500	RFA1_CHICK, SMYD4_CHICK, Q5ZLQ7_CHICK	n
74	chr20	1450330	1480342	CDH22, TGM2_CHICK, NP_989996.1, NP_989998.1, NP_990541.2	y
75	chr20	5290431	5307740	Novel	n
76	chr20	9712500	10662500	Multiple, known and novel	n
77	chr21	6845130	6955012	DDI2, OST48_CHICK, NP_001006560.1, EIF4G3 (fragment)	n
78	chr22	3002	137500	NP_001006305.1, AAK1, NP_001026053.1	y
79	chr22	612500	662500	Q6RUW0_CHICK	y
80	chr22	1062500	1087500	Q5F3G5_CHICK	n
81	chr22	1212500	1237500	XR_027026.1	n
82	chr22	1987500	2037500	Novel	y
83	chr22	2087500	2437500	STAR_CHICK, Q8QGA0_CHICK, FGFR1_CHICK, NP_001026566.1	y
84	chr22	2762500	2787500	NM_001031397.1, NP_001026568.1, Novel	n
85	chr22	2962500	2987500	None	n
86	chr22	3137500	3162500	None	n
87	chr22	3462500	3512500	None	n
88	chr22	3887500	3935246	NM_001031382.1, XR_026841.1	n
89	chr23	5403	20091	Novel	n
90	chr23	1077949	1307784	SCMH1, CTPS	y
91	chr23	5500034	5887500	SYYC_CHICK, MACF1, PPIE, ADC	n
92	chr24	2752	82612	C2CD2L, HMBS, VPS11, SNF1LK2	y
93	chr25	772742	902648	NTRK1_CHICK, Q9YI41_CHICK, SH2D2A	n
94	chr26	437500	587500	XR_027082.1, KLHL12, TBA8_CHICK, JAD1B_CHICK, LZTR1	y
95	chr26	4387500	4762500	Q2YHU1_CHICK, FOXP4, MDFI, NP_001026093.1	n

96	chr27	2911	1007965	DAD1_CHICK, Lots of novel proteins	y
97	chr28	2	62500	MVP_CHICK	y
98	chr28	762500	787500	NDUFA11 , Novel	n
99	chr28	887500	937500	Novel	n
100	chr28	1062500	1087500	ATCAY	n
101	chr28	1312500	1337500	AN32B_CHICK	n
102	chr28	1662500	1687500	DOT1L, AP3D1	n
103	chr28	1737500	1762500	Novel	n
104	chr28	1862500	2412500	Q5R1T0_CHICK, NP_001034409.1, NP_989860.1, NP_001006338.1, FAM148C, SHC2, MP2K2_CHICK, NP_001026106.1	n
105	chr28	2487500	2512500	STK11_CHICK, CAMK4	n
106	chr28	2662500	2687500	Novel	y
107	chr28	2762500	2837500	SFRS14, HOMER3, NP_001026109.1	n
108	chr28	2900219	2945357	CRTC1	n
109	chr28	3037500	3537500	None	n
110	chr28	3637500	3662500	ARHGEF18, SIN3B	n
111	chr28	3812500	3862500	TMEM38A	n
112	chr28	4487500	4505197	TP8L1_CHICK	n
113	chrE22C19W28_E50C23	832581	895034	N/A	y
114	chrE64	402	45047	XR_026966.1	n
115	chrW	502	7842	None	n
116	chrW	10035	257807	Q6Y2W3_CHICK	n
117	chrZ	2090459	8587500	SETBP1, SYT4, RIT2, PIK3C3, A5A2G2_CHICK	n
118	chrZ	13017626	15827681	ISL1, ITA1_CHICK, ITGA2, MOCS2, FST_CHICK, Q8QGH0_CHICK	y
119	chrZ	53937833	62530427	Multiple, known and novel	n
120	chrZ	69222729	69230138	Novel	n
121	chrZ	71510021	71885010	Novel	y
122	chrZ	74542777	74595159	NP_001026680.1	n

9.2. Publications and activities arising from work presented in this thesis

9.2.1. Publications

Griffin DK, Robertson LB, Tempest HG, **Skinner BM**. (2007) The evolution of the avian genome as revealed by comparative molecular cytogenetics. *Cytogenet Genome Res,* **117**(1-4), 64-77.

Morris WB, Stephenson JE, Robertson LB, Turner K, Brown H, Ioannou D, Tempest HG, **Skinner BM**, Griffin DK (2007) Practicable approaches to facilitate rapid and accurate molecular cytogenetic mapping in birds and mammals. *Cytogenet Genome Res*, **117**(1-4):36-42.

Ioannou D*, Tempest, HG*, **Skinner BM**, Thornhill AR, Ellis M, Griffin DK. Quantum Dots as new-generation fluorochromes for FISH: An appraisal. *Chromosome Research, in press* (* joint first author).

Skinner BM, Völker M, Ellis, ME, Griffin DK. An appraisal of nuclear organisation in embryonic fibroblasts of chicken, turkey and duck. *Cytogenet Genome Res, in press.*

Skinner BM, Robertson LB, Tempest HG, Langley EJ, Ioannou D, Fowler KE, Crooijmans RPMA, Hall AD, Griffin DK, Völker M. Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis. *BMC Genomics, in press.*

9.2.2. Presentations and published abstracts

Skinner BM, Robertson LB, Perera J, Tempest H, Hall AD, Griffin DK (2007). Genome organisation in the nuclei of avian cells: Implications for chromosome evolution. *Chromosome Research*, 15 (S2):35.

Skinner BM, Volker M, Fonseka GL, Ellis M, Griffin DK. Nuclear (genome) organisation and comparative genomics in birds (2008). *Chromosome Research*, 16 (7):1054-1054.

Fowler KE, **Skinner BM**, Robertson LBW, Tempest HG, Volker M, Griffin DK (2008). Molecular cytogenetic maps of turkey, duck and zebra finch and their implications for genome evolution. *Chromosome Research*, 16 (7):1043-1044.

Fonseka GL, Ioannou D, **Skinner BM**, Ellis M, Griffin DK. Manual vs. automated methods to assess nuclear organization (2008). *Chromosome Research*, 16 (7):1050-1050.

Volker M, **Skinner BM**, Tempest HG, Griffin DK (2008). Evolution of the avian genome as revealed by molecular cytogenetics. *Chromosome Research*, 16 (7):1053-1054.

9.2.3. Prizes and invitations

Best oral presentation, 18th International Colloquium on Animan Cytogenetics and Gene Mapping (ICACGM), Romania, June 2008

Best final year PhD presentation, University of Kent Postgraduate Symposium, June 2008

Invited oral presentation, British United Turkeys (BUT) Research Seminar, Chester, August 2007.

Invited oral presentation, Genesis-Faraday Annual Symposium, Cambridge, September 2007.

9.3. Accepted conference abstracts

International Chromosome Conference (ICC) XVII (Boone, USA):

Skinner BM, Robertson LB, Tempest HG, Langley EJ, Ioannou D, Fowler KE, Crooijmans RPMA, Hall AD, Griffin DK, Völker M. Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis.

Skinner BM, Völker M, Al Mutery A, Griffin DK. An overview of copy number variation in birds.

Skinner BM, Völker M, Ellis, ME, Griffin DK A detailed appraisal of nuclear organisation in embryonic fibroblasts of chicken, turkey and duck.

Völker M, **Skinner BM**, Langley EJ, Bunzey SK, Gera C, Griffin DK. How conserved are bird genomes? Insights from the chicken and zebra finch genome projects.

Cytogenet Genome Res 117:64–77 (2007)
DOI: 10.1159/000103166

Cytogenetic and Genome Research

The evolution of the avian genome as revealed by comparative molecular cytogenetics

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Abstract. Birds are characterised by feathers, flight, a small genome and a very distinctive karyotype. Despite the large numbers of chromosomes, the diploid count of $2n \approx 80$ has remained remarkably constant with 63% of birds where $2n = 74–86$, 24% with $2n = 66–74$ and extremes of $2n = 40$ and $2n = 142$. Of these, the most studied is the chicken ($2n = 78$), and molecular cytogenetic probes generated from this species have been used to further understand the evolution of the avian genome. The ancestral karyotype is, it appears, very similar to that of the chicken, with chicken chromosomes 1, 2, 3, 4q, 5, 6, 7, 8, 9, 4p and Z representing the ancestral avian chromosomes 1–10 + Z; chromosome 4 being the most ancient. Avian evolution occurred primarily in three stages: the divergence of the group represented by extant ratites (emu, ostrich etc.) from the rest; divergence of the Galloanserae (chicken, turkey, duck, goose etc.) – the most studied group; and divergence of the 'land' and 'water' higher birds. Other than sex chromosome differentiation in the first divergence there are no specific changes associated with any of these evolutionary milestones although certain families and orders have undergone multiple fusions (and some fissions), which has reduced their chromosome number; the Falconiformes are the best described. Most changes, overall, seem to involve chromosomes 1, 2, 4, 10 and Z where the Z changes are intrachromosomal; there are also some recurring (convergent) events. Of these, the most puzzling involves chromosomes 4 and 10, which appear to have undergone multiple fissions and/or fusions throughout evolution – three possible hypotheses are presented to explain the findings. We conclude by speculating as to the reasons for the strange behaviour of these chromosomes as well as the role of telomeres and nuclear organisation in avian evolution.

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The unique avian karyotype

Birds have a number of peculiarities: They are the only extant phylogenetic class to possess feathers; flight is near ubiquitous (despite being extremely rare in other vertebrates) with all representatives having, or having lost, the ability to fly. Moreover, they have a characteristically small genome (one third the size of mammals i.e. 0.97–2.16 pg with a mean of 1.45 pg ± 0.1 pg, Burt et al., 1999, www.genomesize.com), which has, it is suggested, evolved in response to the energy conservation requirements associated with the evolution of flight (Hughes and Piontkivska, 2005). A further distinguishable avian feature is the gross organisation of the genome (i.e. karyotype), which is readily identifiable to a relatively trained eye. The ancient and consistent haploid number of around 40, in combination with the large number of microchromosomes is a pattern that is quite distinct in nature. That is, although many reptiles (including lizards, snakes and crocodiles) are known to have microchromosomes, and karyotypes of particular species of turtle (where $2n = 66$) are quite similar to avian ones, the 'so many, so small' pattern (where $2n \approx 80$) is a uniquely avian feature. The relatively unchanged nature of the diploid chromosome number among the majority of avian species further implies that such a karyotype was, and is, a highly successful means of genome organisation. Like flight, feathers and a small genome, this characteristic karyotype, once it had appeared in birds, remained relatively constant (with few exceptions) to the present day.

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The complete chicken karyotype – a baseline for comparative genomics

Chicken *(Gallus gallus domesticus)* is the domesticated descendent of the Red Junglefowl *(Gallus gallus)*, on which most genomic and hence cytogenetic studies have been performed. Its pivotal role in agriculture and as a classical embryology model – as well as a model for human disease – made it the primary species from which nearly all comparative vertebrate genome analysis in birds have ensued. Our group recently published a complete chicken karyotype (2n = 78) (Masabanda et al., 2004), which was the first avian karyotype to be completely described. The purpose of this review is to describe our findings and, more importantly, to indicate how they have, through collaborations with colleagues worldwide, led to a greater understanding of avian genome evolution.

Perhaps a convenient starting point is in the terminology. The terms macro- and micro-chromosome have been common parlance for many years when describing chicken chromosomes. In many ways though, these terms are somewhat of a misnomer. Closer inspection reveals that there is no clear dividing line between the smaller and the larger chromosomes, which may explain why different authors give different accounts of the relative numbers of macro- and micro-chromosomes. In an attempt to reconcile the confusion (but admittedly running the risk of adding to it), we suggested a different classification system (Masabanda et al., 2004). This was related to the ability to resolve the chromosomes in a flow karyotype and to the newly emerging genome sequence. Thus we assigned group A as the chromosomes that could be resolved in a flow karyotype (which included chromosome 10 which, although slightly smaller that 11 and 12, was resolved alone where 11 and 12 sorted together). Groups B and C comprised the remainder of the chromosomes (11–32) that had known markers from the genome project assigned to them at the time of writing. The groups were separated by the NOR chromosome which, despite its relatively small size, had previously been assigned number 16. Even two years after the original publication, the smallest 'group D' chromosomes (33–38) have yet to be anchored to the genome assembly and this raises the question of whether this is simply due to their small size and thus 'bad luck' or a more fundamental biochemical reason, e.g. a large proportion of repeats.

The classical way of studying any karyotype is, of course, by G-banding. In general terms however, G-band information is limited in birds firstly because bands on the group A chromosomes are less distinct than in mammals (presumably due to less distinct differences between the molecular correlates of G-banding along the genome) and secondly because the group B–D chromosomes are too small to visualise any banding pattern (Ladjali-Mohammedi et al., 1999). In defining the full karyotype, our group thus developed chromosome-specific FISH probes for each chicken chromosome (Griffin et al., 1999), and we subsequently set up a resource centre (www.farmachrom.net) for, among other things, comparative genomics. Using these probes (both chromosome paints and BACs) it has become possible, through zoo-FISH experiments, to establish chromosome orthologies among birds. This review focuses on the molecular cytogenetic studies and makes reference to the G-banding studies only when a general point is made or when the zoo-FISH studies have added extra information. We might add therefore that conclusions drawn from banding information alone, which is at best limited, should be viewed with caution until confirmed by molecular methods.

Chromosome numbers in birds – an overview

The most complete account of the chromosome number in birds is given by Christidis (1990), including 723 species with relatively accurate chromosome numbers and partial karyotypes. Rodionov (1997) suggested that there are nearly 800 published avian karyotypes in existence and cites several not quoted by Christidis. As mentioned the diploid number is very consistent with around 63% of birds where 2n = 74–86 and 24% with 2n = 66–74 (Christidis, 1990). Considering the more rapid rate of change in mammals (Wienberg, 2004), the relative lack of variation in birds is remarkable. That is, chromosomal changes are commonplace in the genome evolution of mammals and the best known examples include comparisons of the Chinese and Indian Muntjacs, *Muntiacus reevesi* (2n = 46) and *Muntiacus muntjak* (2n = 6 in females and 7 in males, Yang et al., 1997), and gibbons where gross rearrangements are commonplace (Wienberg, 2004). Examples of birds with significantly less than the norm include the Laridae (gulls and terns, where 2n = 66–70), the Pelecaniformes (pelicans etc. where 2n = 66–70 with only one known exception, the Little Cormorant, *Phalacrocorax niger* (Christidis, 1990) and the Psittacidae (parrot family) where 2n = 60–72. Interestingly the Psittacidae are rare examples of where clear differentiation between macro- and micro-chromosomes can be seen; for instance *Platycerus elegans* (the Crimson Rosella) has seven pairs of macrochromosomes (including Z and W) with the rest at least ten times smaller than that the smallest pair of macroautosomes (Christidis, 1990). In this instance the most likely mechanism therefore is a fusion of several pairs of group B–D chromosomes to form larger chromosomes and/or fusion on to group A chromosomes. In the absence of FISH studies the precise nature of these changes remains to be determined. In the Falconiformes chromosome number varies from 2n = 50 to 2n = 72 in all but the Cathartidae (new world vultures), Sagittariidae (Secretary bird) and selected Falconidae (falcons and caracaras). Indeed, it is the Falconidae that shows the most variation among the Falconiformes with *Falco jugger, peregrinus* and *subbuteo* (Laggar, Peregrine and Hobby Falcons respectively) with 2n = 50 and *Polyborus plancus* (the Crested Caracara) with 2n = 84–86 (Christidis, 1990). Unlike in parrots however, rather than a tendency to form large chromosomes, chromosome fusion seems to have been more uniform across the karyotype. Recent molecular cytogenetic studies

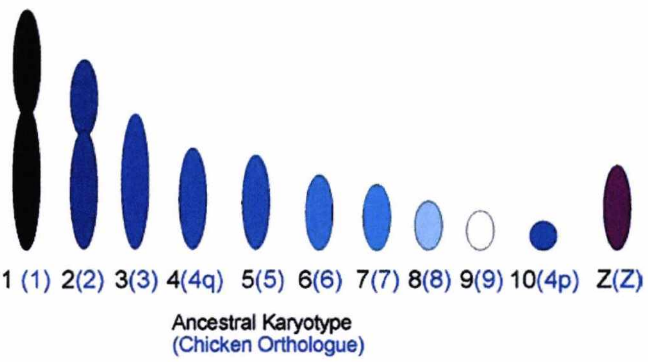


Fig. 1. Schematic depicting the ancestral avian karyotype and its chicken orthologues. The only difference is chicken chromosome 4, which is represented by ancestral chromosomes 4 and 10.

have shed light on the nature of these changes and this is dealt with in detail in a subsequent section.

For the sake of completeness it is appropriate to mention the extremes at both ends of the spectrum. Smallest among known diploid chromosome numbers are the stone curlew *Burhinus oedicemus* (2n = 40), the trumpeter hornbill *Ceratogyman bucinator* (2n = 40), the beach thick knee *Burhinus magnirostris* (2n = 42), and the black and white casqued hornbill *Ceratogymna subcilindrica* (2n = 42) (Christidis, 1990). On the other end of the scale the hoopoe *Upupa epops* has a diploid number of 126. The greatest number of reported chromosomes in a bird, however, is either (appropriately for a review of molecular cytogenetics) the common kingfisher *Alcedo atthis* where 2n = 132 or 138 depending on which paper you read (the Azure kingfisher *Ceyx azurea* has a mere 2n = 122) and the strangely named Gray or Southern Go-away-bird *Corythaixoides concolor* (2n = 136–142) (Christidis, 1990). These birds are rare examples indeed however, since the next highest number is cited as 108–110 in several unrelated species (Christidis, 1990).

Early evolution of birds and the ancestral karyotype

It has been thought that avian species existed in the Triassic approximately 200 million years ago since discovery of two nearly complete fossil skeletons of *Protoavis* (Rodionov, 1997) which pre-date the Jurassic *Archaeopteryx* by some 50 million years. Mitochondrial evidence suggests the common ancestor of birds (synapsids) and mammals (diapsids) diverged 310 million years ago (Kumar and Hedges, 1998; Burt et al., 1999), while the common ancestors of birds and crocodilians may have diverged 210–250 million years ago (Muller and Reisz, 2005). The presence of turtles in the scheme of things appears less certain, however they too are thought to have diverged from birds over 210 million years ago and recent molecular evidence from both mitochondrial and nuclear sources places birds, crocodilians and turtles in the same group (Archosaurs) with lizards and snakes (Lepidosaurs) separate (Hedges and Poling, 1999; Kumazawa and Nishida, 1999). Matsuda et al. (2005), through the isolation of cDNA libraries from soft-shelled turtles and comparison with chicken sequences provided compelling evidence that there was highly conserved linkage homology between birds and turtles (specifically chickens and soft-shelled turtles); moreover bird and turtle chromosomes 1–5 (as well as turtle 6 and avian Z) appear to be precise counterparts of one another. Of these chromosomes the Z chromosome is thought to be an ancient sex chromosome (Marshall Graves and Shetty, 2001) and sequence comparisons from the human and chicken genome projects reveal a remarkable degree of synteny of chicken chromosome 4q and (coincidentally) human chromosome 4 (Chowdhary and Raudsepp, 2000). That is, although unsurprisingly, there is extensive inter-chromosomal rearrangement between all other chromosomes but none between human chromosome 4 and chicken chromosome 4q other than a tiny segment of another chicken chromosome in the p-terminus of human chromosome 4 (Chowdhary and Raudsepp, 2000). Taken together then, the ancestral avian chromosomes 1, 2, 3, 5 and Z will have appeared at least 210 million years ago with the ancestral chromosome 4 appearing at least 310 million years ago.

Attempts to depict the ancestral karyotype of birds by examining banding patterns date back to at least 1982 (Stock and Bunch). The ancestral karyotype for the Galliformes as predicted by Shibusawa et al. (2004a) by comparative chromosome painting appears to be conserved throughout the avian lineages. Hence, for the purposes of this paper we will make reference to chromosome rearrangements in relation to the putative avian ancestor, rather than the chicken. The chicken chromosome 4p has been shown to be, most likely, a fusion of the ancient ancestral chromosome 4 to another ancestral chromosome (ICGSC, 2004). Our own banding comparisons of hybridised paints suggest that this chromosome is chromosome 9 in turkey and thus a group A chromosome. For the purposes of this study therefore we will refer to the orthologue of chicken chromosome 4p as 'ancestral chromosome 10'. Taking the studies as a whole it seems clear that the pattern of the chicken orthologues of chromosomes 1, 2, 3, 4q, 5, 6, 7, 8, 9, 4p and Z represent the ancestral chromosomes 1–10 + Z for all birds, illustrated in Fig. 1. The timing of appearance of extant chromosomes 6–9 remains to be determined (e.g. by means similar to that employed by Matsuda et al. (2005); it is not unfeasible however to suggest that they appeared at a similar point to their larger counterparts. Moreover, we have not yet seen any direct evidence of the chicken W chromosome orthologues in other birds (indeed we have been unsuccessful in hybridising chicken W paint to turkey metaphases), yet it seems reasonable to assume that this evolved by previously described mechanisms of sex chromosome divergence from a Z chromosome ancestor (Marshall Graves and Shetty, 2001). As will become clear in subsequent sections, it seems to be chromosomes 1, 2, 4 and 10 that are more prone to inter-chromosomal rearrangements (fissions and/or fusions) and the Z more prone to intra-chromosomal rearrangements.

Moreover selected orders and families have multiple rearrangements (mostly microchromosomal fusions) and these are also reviewed in detail presently.

The appearance of the microchromosomes (groups B–D) seems to have been a gradual rather than sudden event. Molecular and fossil data suggest that the divergence of all the major amniotes (reptiles, birds and mammals) occurred around 300–310 million years ago (Kumar and Hedges, 1998; Burt et al., 1999). The presence of microchromosomes in birds, lizards, snakes, crocodiles and turtles but not in mammals or amphibians suggests the first appearance of microchromosomes was after this time (Fig. 2). The presence of a 2n = 66 karyotype (including a large number of microchromosomes) in turtles (Stock and Mengden, 1975) might suggest that it is turtles rather than crocodilians that are the closest avian relative although this thesis may be challenged by other evidence. That is, if, as mitochondrial DNA data would suggest, the crocodilians are more closely related to birds than turtles, then it seems likely that the crocodilians underwent a series of microchromosomal fusions that was a feature of their own evolution. It is likely also that the genomes of the reptilian/avian ancestors continued to fragment over a period of 100 million years or so, reaching at least 2n = 66 by the time the lineages that led to turtles and birds diverged and became fixed in the classic 2n $\approx$ 80 pattern around the emergence of the first birds around 200 million years ago. An alternative explanation is that the Lepidosauria (lizards, snakes etc.) and birds evolved microchromosmes separately and the crocodilians retained a more ancestral karyotype however this seems very unlikely particularly as bird and turtle macrochromsomes are precise orthologues of one another and the crocodilians are a monophyletic group.

There are thought to be three major events involved in bird evolution: 1) the divergence of Palaeognathae (surviving members include the Struthioniformes or ratites such as emu, ostrich, rhea, kiwi etc.) and Neognathae (others) approximately 100–120 million years ago (van Tuinen and Hedges, 2001); 2) the divergence from the Neognathae of the Galloanserae (e.g. chicken, turkey, goose, duck etc.) approximately 100 million years ago (van Tuinen and Hedges, 2001); and 3) the divergence of the remainder of the Neognathae into 'higher land' and 'higher water' birds approximately 70–80 million years ago (van Tuinen and Hedges, 2001). These divergences can be seen in Fig. 2.

The first divergence

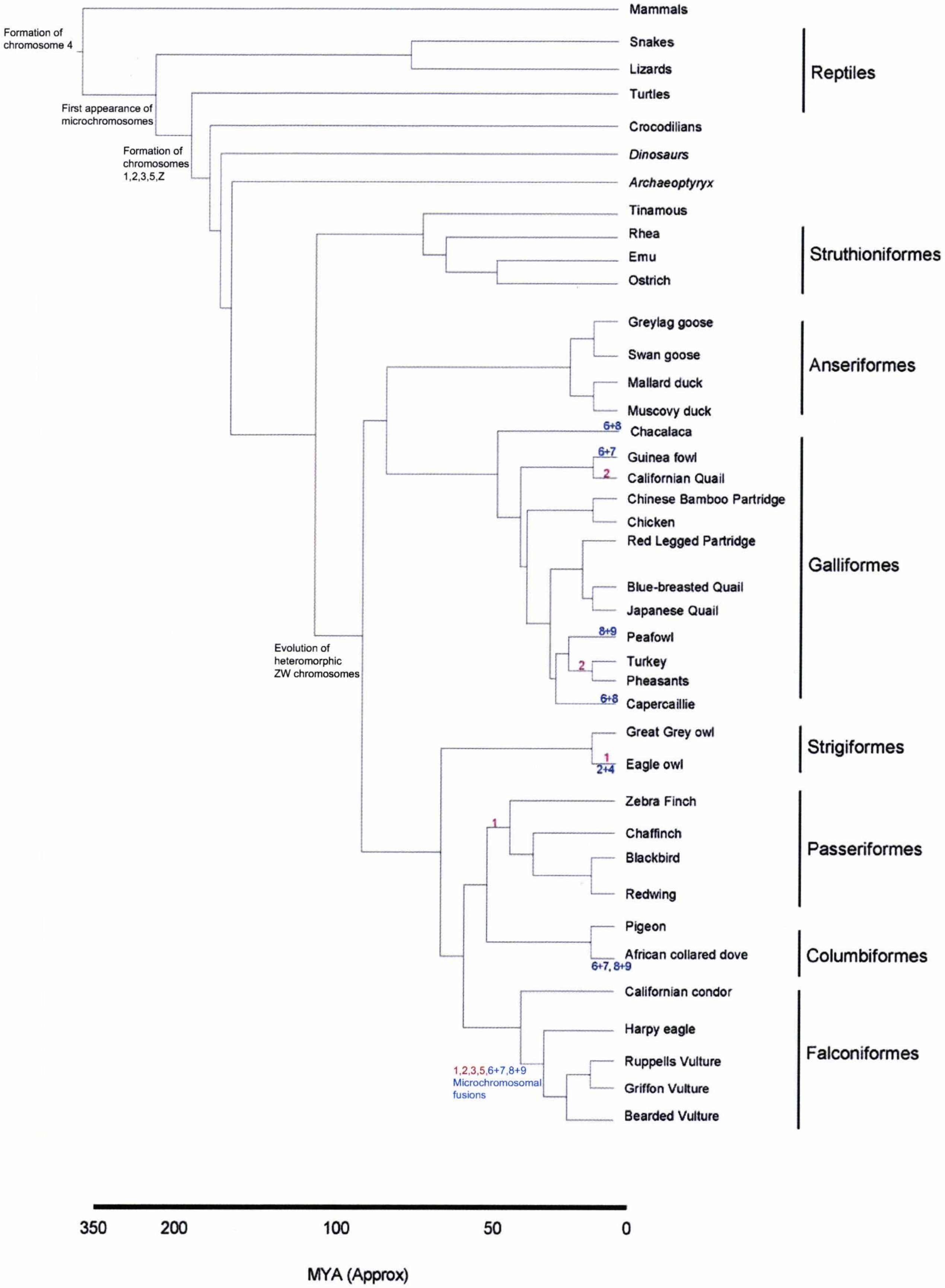
Modern day representatives of the divergence of Palaeognathae comprise only two extant orders; the flightless Struthioniformes (ratites; e.g. emu, ostrich, rhea, cassowary and kiwi) and the Tinamiformes (tinamous). One of the first successes of our avian chromosome resource centre (and for avian comparative genomics in general) was the confirmation of what had been long expected by classical studies; i.e. at least for the group A chromosomes, synteny is remarkably conserved. This was revealed by experiments using chicken whole chromosome paints from chromosomes 1–9 + Z on to emu *(Dromaius novaehollandiae)* metaphases (Shetty et al., 1999). All but one chromosome appeared not to display interchromosomal rearrangements; the exception being the extant chicken chromosome 4 represented by the aforementioned ancestral chromosomes 4 and 10. A similar pattern was more recently noted by Guttenbach et al. (2003) in the American Rhea *(Rhea americana)*. Given that chickens share a very similar karyotype (at least for the Group A chromosomes) to that of ratites, it seems clear that the first divergence was either not accompanied by a major autosomal change or was accompanied by a change in the smaller chromosomes that has yet to be discovered. The diploid number of 2n = 80–82 in all extant ratites suggest the former to be the case. A unique feature of the ratites is that they have homomorphic sex chromosomes, indicative of an ancestral autosomal origin and sex chromosome differentiation after the divergence of this group (Guttenbach et al., 2003).

The second divergence – Galloanserae (Galliformes and Anseriformes)

Galliformes are an order comprising the turkey, grouse, pheasants and quails and they contain the species in which the most genomic sequencing information is available i.e. chicken. Moreover, due to their status as agricultural birds, they are among the most studied avian orders in many other areas of science and, since the availability of chicken chromosome paints (Griffin et al., 1999; Masabanda et al., 2004), have been natural targets for comparative studies.

(For Fig. 2 see next pages.)

Fig. 2. Phylogenetic tree for which comparative genomic data (zoo-FISH) exists. The tree has been collated from consensus studies of DNA hybridisation studies, mitochondrial DNA sequencing and comparative protein sequencing (Edwards et al., 2005; Schmid et al., 2005). Only interchromosomal changes are shown, fissions are represented in red, fusions are represented in blue. All numbers correspond to the ancestral avian karyotype (Fig. 1). Chromosome 4 is omitted since it appears in Fig. 3. To the right is a representation of which ancestral chromosomes have split, fused or remained unchanged compared to the ancestral one in all the species thus far studied. Data from Takagi and Sasaki, 1974; Burt et al., 1999; Hedges and Poling, 1999; Shetty et al., 1999; Chowdhary and Raudsepp, 2000; van Tuinen et al., 2000; Zardoya and Meyer, 2001; Dimcheff et al., 2002; Donne-Gousse et al., 2002; Shibusawa et al., 2004a; Matsuda et al., 2005; Summers, 2005; Kohn et al., 2006. Known divergence dates have been taken from the following sources: Birds/mammals: 300–310 Myr (Kumar and Hedges, 1998; Burt et al., 1999); Birds/crocodilians: 210–250 Myr (Muller and Reisz, 2005); Birds/turtles: 210 Myr (Hedges and Poling, 1999; Kumazawa and Nishida, 1999, Matsuda et al, 2005); Palaeognathae and Neognathae: 100–120 Myr (van Tuinen and Hedges, 2001); Neognathae/ other Galloanserae 100 Myr (van Tuinen and Hedges, 2001); Neognathae 'higher land'/'higher water': 70–80 Myr (van Tuinen and Hedges, 2001); Anseriformes and Galliformes 90–96 Myr, Ciconiiformes (New world vultures) and Falconiformes: 75 Myr (van Tuinen and Hedges, 2001). Other divergence dates can be regarded as speculative only.


Formation of chromosome 4
First appearance of microchromosomes
Formation of chromosomes 1,2,3,5,Z
Evolution of heteromorphic ZW chromosomes
1,2,3,5,6+7,8+9 Microchromosomal fusions
Mammals
Snakes
Lizards
Turtles
Reptiles
Crocodilians
Dinosaurs
Archaeoptyryx
Tinamous
Rhea
Emu
Ostrich
Struthioniformes
Greylag goose
Swan goose
Mallard duck
Muscovy duck
Anseriformes
6+8
Chacalaca
6+7
Guinea fowl
2
Californian Quail
Chinese Bamboo Partridge
Chicken
Red Legged Partridge
Blue-breasted Quail
Japanese Quail
8+9
Peafowl
2
Turkey
Pheasants
6+8
Capercaillie
Galliformes
Great Grey owl
1
2+4
Eagle owl
Strigiformes
1
Zebra Finch
Chaffinch
Blackbird
Redwing
Passeriformes
Pigeon
African collared dove
6+7, 8+9
Columbiformes
Californian condor
Harpy eagle
Ruppells Vulture
Griffon Vulture
Bearded Vulture
Falconiformes
350
200
100
50
0
MYA (Approx)


1
2
3
4
5
6
7
8
9
10
Z
Mammals
Turles
Rhea
Emu
Greylag Goose
Swan Goose
Mallard
Muscovy
Chachalaca
Guinea fowl
Californian Quail
Chinese Bamboo Partridge
Chicken
Red Legged Partridge
Blue Breasted Quail
Japanese Quail
Peafowl
Turkey
Pheasants
Capercaillie
Great Grey Owl
Eagle Owl
Zebra Finch
Chaffinch
Blackbird
Redwing
Pigeon
African Collared Dove
Californian condor
Harpy Eagle
Rueppells Vulture
Griffon Vulture
Bearded Vulture
A
B
C
D
Ancestral chromosome
Ancestral chromosome, with rearrangements
Fusion of ancestral chromosomes
Fission of ancestral chromosome
No Data
A – Pericentric inversion
B – Centromere repositioning
C – Unknown fusion
D – Fusion of ancestral 4 and 9
NB Rearrangements of Z chromosome not shown as ancestral form as yet unclear

Indeed the largest body of comparative genomic studies has been performed with reference to this order. As mentioned, all birds examined other than the ratites have heteromorphic sex chromosomes, however there is no evidence that the divergence of the Galloanserae was accompanied by any other major chromosomal change (although isolated individual changes are apparent), due to the relatively stable chromosome number in the majority of species and the clearly established orthology of the group A chromosomes.

Chromosome number is conserved and ranges from 2n = 78 (chicken) to 2n = 82 (golden pheasant, *Chrysolophus pictus*, Guttenbach et al., 2003), and zoo-FISH with chicken whole chromosome paints reveals the interchromosomal rearrangements that have occurred in the Galliform lineage. The majority of the changes from the ancestral form are found in chromosomes 2 and 4. Chromosome 2 is represented as two separate telocentric chromosomes (3 and 6) in the five species of pheasant, turkey *(Meleagris gallopavo)* and California quail *(Callipepla californica)* (Shibusawa et al., 2004b). It is also represented as two telocentric chromosomes (3 and 7) in the capercaillie *(Tetrao urogallus)* (Shibusawa et al., 2004a).

The ancestral chromosome 4 (chicken chromosome 4q) is conserved intact in all the Galliformes and indeed most birds, albeit fused to smaller chromosomes on certain occasions. In the guinea fowl *(Numidea meleagris)* a fusion has occurred between it and ancestral chromosome 9 (Shibusawa et al., 2004a). However the most common fusion is between ancestral chromosome 4 and ancestral chromosome 10; this is seen in chicken, the partridges; peafowl and two quail species (Blue breasted and Japanese) (Shibusawa et al., 2004b). The pheasants, capercaillie, turkey, California quail and chachalaca *(Ortalis vetula)* all show the chicken 4p arm hybridising to ancestral chromosome 10 although it is usually described as an unassigned microchromosome (Shibusawa et al., 2004a). Interestingly, molecular evidence (ICGSC, 2004) has suggested that, despite the fusion in chicken, ancestral chromosome 10, when it appears as chicken chromosome 4p still retains the properties (e.g. gene density, recombination rate, CpG island distribution) of the smaller chromosome it once was.

In addition to the rearrangements of chromosomes 2 and 4, there are only four other interchromosomal changes detected to date for the remainder of the karyotype. In Guinea fowl *(Numidea meleagris)*, a fusion has occurred between ancestral chromosomes 6 and 7 (Shibusawa et al., 2002); the capercaillie shows a fusion of ancestral chromosomes 6 and 8 (Shibusawa et al., 2004a); and the common peafowl *(Pavo cristatus)* has both a fusion of ancestral chromosomes 8 and 9 and fusion of ancestral chromosome 7 to a microchromosome (Shibusawa et al., 2004a).

With regard to intrachromosomal rearrangements, in the Japanese quail *(Coturnix japonica)*, pericentric inversions have occurred between it and the ancestral type (Shibusawa et al., 2001) for chromosomes 1 and 2 (Schmid et al., 2000). An apparent pericentric inversion in Red-Legged Partridge *(Alectoris rufa)* has been revealed by Kasai et al. (2003) using comparative BAC mapping to in fact be the repositioning of the centromere to the p terminus. That is, the gene order has not changed along the length of the chromosome though the position of the centromere has. The reported 'pericentric inversion' seen in the Blue Breasted and Japanese Quails (Shibusawa et al., 2004b) should be viewed with caution therefore until further BAC studies are performed. One rearrangement that is probably a pericentric inversion however is the ancestral chromosome 8 which is metacentric in chicken and Chinese bamboo partridge but telocentric in other birds. BAC mapping experiments with turkey chromosomes (Robertson et al., unpublished results) have all but confirmed this.

On a related theme, recent studies of lampbrush chromosomes (Galkina et al., 2006) suggest that, while all chicken microchromosomes are telocentric, Japanese quail microchromosomes are all metacentric. The mechanism by which this occurred remains a mystery, however we are currently investigating whether this phenomenon occurred by pericentric inversion or centromere relocation. The reasons why it occurred are as yet unclear.

The Anseriformes are the nearest extant relatives to the Galliformes, diverging 90–96 million years ago, and the only other surviving order from the second divergence. Among them the Greylag goose *(Anser anser)* studied by Guttenbach et al. (2003) shows a fusion of the ancestral 4 and 10, an identical pattern to that seen in chicken. The swan goose *(Anser cygnoides)* studied by Jaszczak et al. (2002) shows evidence of rearrangements on chromosome 4, having a metacentric chromosome pair. Though painting data is not yet available to confirm that this is the ancestral 4, the accepted diploid number of 80, equal to that of the Greylag, plus the ease of hybridisation suggest conservation of the ancestral form as well. The Mallard duck *(Anas platyrhynchos)* shows the ancestral form (with chicken chromosome 4 paint hybridising to two chromosomes (Schmid et al., 2000, 2005), though mapping of chicken BAC clones reveals intrachromosomal rearrangements on the smaller macrochromosomes. The Muscovy duck *(Cairina moschata)* has not yet been painted with chicken chromosome paints, however banding studies by Denjean et al. (1997) showed few rearrangements in the macrochromosomes, and the diploid number is believed to be the same (2n = 80).

The final divergence

During the final divergence (into 'higher land' and 'higher water' birds) there is no evidence to suggest a change characteristic of either clade. Following this event, however, many birds on both sides of the divide clearly underwent a series of microchromosomal fusions and, to a smaller extent, macrochromosomal fissions with a net result of less chromosomes in the karyotype. In other words, a tendency to reduce the chromosome number has been an independent, convergent event happening in several unrelated families and orders significantly after the last major divergence

Table 1. Summary of comparative genomics between chicken and three Falconiformes established by painting of chromosome paints 1–10 + Z of chicken to metaphases of these species. Note that comparisons in this case are with chicken and not with the ancestral karyotype and thus the orthologies with chicken chromosome 4 are probably ancestral in the Falconiformes.

Paint	Chromosome		
Chicken (*Gallus gallus* 2n = 78)	Griffon vulture (*Gyps fulvus*)/ Ruppells vulture (*Gyps rueppelli* 2n = 66)[a]	Bearded vulture (*Gypaetus barbatus* 2n = 60)[a]	Harpy Eagle (*Harpia harpyia* 2n =58)[b]
1	7, 12, 15, 19, 20, 22	7, 8p, 11, 12q	5, 6, 19, 21, 24
2	2, 3, 23	1q, 2, 14q, 23q	1, 2
3	8, 16q, 21, 24	8q, 12, 21q, 22q	2p, 18, 23
4	1, 13	3, 16	4, 14
5	14q, 17	15q, 20	2q, 20
6	4q	4q	8
7	6q	6q	
8	10	10	
9	9q	5q	
10	18q	9q (interstitial)	
Z			Z

[a] Nanda et al. (2006).
[b] de Oliveira et al. (2005).

of the birds. As mentioned, 24% of all birds have an average of 2n = 66–74: the Laridae (gulls and terns), the Pelecaniformes (pelicans etc.) and the Psittacidae (parrot family). Perhaps the most striking example however is seen in the Falconiformes (e.g. vultures, falcons, hawks, eagles etc.) on which the most zoo-FISH studies have been performed.

Evolution in Falconiformes and Ciconiiformes

Falconiformes have a low chromosome number ranging from 2n = 50 (American Kestrel, *Falco sparberius*) to 2n = 68 (Red tailed Hawk, *Buteo jamaicensis*) (Shields, 1982) and an atypical chromosome morphology suggestive of several fissions and fusions among both the macro- and microchromosomes. The Ciconiiformes (New World vultures) were formerly classed as part of the Falconiformes, but are now separated following a divergence approximately 75 million years ago (van Tuinen and Hedges, 2001). They have 2n = 80, and are thus closer to the ancestral avian karyotype (Nanda et al., 2006). Cytogenetic findings therefore along with other lines of evidence would suggest that members of the Accipitridae family including old world vultures, eagles, hawks and kites are more closely related to one another than they are to the new world vultures, and that the major chromosomal changes are characteristic of the Accipitridae rather than the Falconiformes as a whole. Accipitridae that have been studied by comparative painting are the Griffon vulture *(Gyps fulvus)*, Ruppels vulture *(Gyps rueppelli)* and the Bearded Vulture *(Gypaetus barbatus)* by Nanda et al. (2006) and the Harpy Eagle *(Harpia harpyja)* by de Oliveira et al. (2005). There are no large macrochromosomes in these birds, rather ~25 pairs of medium sized chromosomes and 4–6 pairs of microchromosomes suggesting frequent and whole scale microchromosomal fusion; the black-winged kite *(Elanus caeruleus)* has only a single microchromosome pair (2n = 68; Bed'Hom et al., 2003). There are also several fissions of the larger chromosomes apparent when chicken whole chromosome paints are applied to these species; chicken chromosome 1–5 paints show extensive rearrangements; for example chicken chromosome 1 hybridises to six separate chromosomes ranging in size from 7 to 22 in *G. fulvus* and *G. rueppelli*; to four chromosomes in *G. barbatus* ranging from 7 to 12 and five chromosomes ranging from 5 to 24 in the Harpy Eagle (for all rearrangements from chicken chromosomes 1–10 see Table 1). In contrast, where data is available, chromosomes 6–10 hybridise only to a single chromosome or a larger, fused chromosome. An apparent exception is chromosome 4. The chicken chromosome 4 paint hybridises to only two chromosomes in all four species, a larger (~1–4) and a smaller (~13–16) chromosome suggesting conservation of the ancestral karyotype.

Among the Ciconiiformes the best studied example is the California Condor *(Gymnogyps californianus)*, which also has the distinction of being the largest flying bird. Raudsepp et al. (2002) found few rearrangements with chicken. The chicken 2 paint hybridises to chromosome 2 of this species with weak cross hybridisation to chromosome 3. Moreover, the chicken chromosome 3 paint hybridises to California condor chromosome 3 with a weak signal on chromosome 2. Chromosome 4 has a p arm and is therefore sub-metacentric, however the chicken chromosome 4 paint detects two chromosomes (4 and 9 – presumably the ancestral 4 and 10) in the California condor suggestive of a pericentric inversion or centromere relocation on the California condor chromosome 4.

Evolution in the Passeriformes

The Passeriformes are the largest avian order, comprising about half of all known bird species. The most studied is the zebra finch *(Taeniopygia guttata)*, an emerging model organism for study of many issues relevant to human health and disease mainly because of its ability to communicate via complex learned vocalisations (Arnold, 2004). Zebra finch has been used as a model species for sex differences in neural structure and function, influences of steroid hormones on neural networks, adult neurogenesis, steroid hormone synthesis in the brain, the neural basis for learning and complex auditory processing and auditory-motor integration (Arnold, 2004). It seems likely for these reasons that zebra finch will be the second bird (after chicken) to have a complete genome sequencing effort. Comparative genomics (zoo-FISH) has also been carried out on the chaffinch, redwing (Derjusheva et al., 2004) and blackbird (Guttenbach et al., 2003). Chicken chromosome paints 1–10 and Z reveal a few distinct rearrangements. The redwing *(Turdus iliacus)* and blackbird *(Turdus merula)* belong to the family Turdidae, and both display a fission of the ancestral chromosome 1 near or at the centromere. The chaffinch *(Frigilla coelebs)* is in the family Fringillidae; it and the zebra finch (Estrildidae) show a similar fission (Itoh and Arnold, 2005). Ancestral chromosome 4 is conserved in all four birds; however, due to the chromosome 1 fission it is referred to as chromosome 5 in the chaffinch and zebra finch.

Strigiformes and Columbiformes (Owls and Doves)

The Great Grey Owl *(Strix nebulosa)* and the Eagle Owl *(Bubo bubo)* from the family Strigidae were studied by Schmid et al. (2000) and Guttenbach et al. (2003) respectively. There are no interchromosomal changes from the ancestral form in the Great Grey Owl. The Eagle Owl shows a similar fission to that seen in the Turdidae i.e. that of ancestral chromosome 1. The chicken (ancestral) chromosome 2 paint hybridises to the long arm of the largest chromosome in the Eagle Owl with the short arm of the same chromosome orthologous to ancestral chromosome 4. Finally, the ancestral 5 has undergone a fission event in Eagle Owl and split to a macro- and microchromosome.

Among the Columbiformes, the Pigeon *(Columbia livia)* retains the ancestral karyotype (Derjusheva et al., 2004), but the African collared dove *(Streptopelia roseogrisea)* has a fusion of ancestral 4 and 10 (the same as chicken and goose), as well as two fusions of ancestral chromosomes 6, 7, 8 and 9 forming two larger macrochromosomes (Itoh and Arnold, 2005).

The sex chromosomes

Despite being an ancient conserved chromosome, the Z chromosome is subject to some of the most extensive intrachromosomal rearrangements within the lineages; the ancestral monomorphic pair seen in emu and rhea have been mentioned already. Thereafter, however, not only has a W chromosome evolved but the Z seems to have undergone numerous intrachromosomal changes. The metacentric chromosome of the chicken appears as a submetacentric chromosome in many of the other Galliformes, as well as in the Anseriformes, and there are indications of additions of heterochromatin accumulation in the q arms of chicken, blue-breasted and Japanese quails (Shibusawa et al., 2004b). Chicken Z paints hybridise to a single turtle chromosome (Marshall-Graves, personal communication), suggesting that chicken (apart from the heterochromatin on the q arm) represents the ancestral form. This form is also found in the ratites, where Z and W are homomorphic (Shetty et al., 1999). Detailed comparisons of BAC order in other birds are hampered by the paucity of markers in the sex chromosomes on the chicken assembly and it seems clear that the overall role of inversions, centromere relocation and heterochromatin accumulation is yet to be determined.

Chromosome 4

Independent convergent changes are not entirely unusual in avian karyotype evolution; for instance both chromosomes 1 and 2 have displayed individual fission events around the centromere. This has occurred in both the Turdidae and the Eagle owl for chromosome 1 and in the Californian Quail and the turkey/pheasant group for chromosome 2. These pale into insignificance however when compared to the story of chromosome 4. The ancestral chromosome 4 (chicken chromosome 4q) is the most ancient of all the avian chromosomes – appearing intact even in humans (Chowdhary and Raudsepp, 2000). It is somewhat ironic then that, when coupled with its counterpart as it appears on chicken chromosome 4 (i.e. as a submetacentric chromosome from fusion with ancestral chromosome 10) it is subject to the most puzzling of conundrums in avian evolution.

In the majority of species the ancestral pattern (separate chromosomes 4 and 10) is maintained, however the exceptions to this rule include chicken, goose and African collared dove. The most parsimonious explanation of these findings (Fig. 3) is three independent fusion events (in goose, dove and a recent ancestor of turkey, pheasants and some quail; nodes 5, 22 and 10) and one fission in the turkey/pheasant ancestor (node 15) (hypothesis 1, Fig. 3). Perhaps the least parsimonious involves a fusion before the second major divergence (node 2) and then at least eight different independent fission events (nodes 6, 9, 15, post 7, 20, 21, 22; hypothesis 3, Fig. 3). While ordinarily such a scenario might be disregarded as highly unlikely, it might be argued that it is equally unlikely that three independent fusion events involving the same chromosomes have occurred in the background of very few rearrangements occurring overall – a recurring fission event might at least be explained by the region being particularly fragile and prone to breakage. There is also an interim explanation (hypothesis 2, Fig. 3), for instance if we assume that there was not a fusion before the second major divergence, then a fusion in the Galliform/Anseriform ancestor could have been followed by four independent fission events a) in the turkey/pheasant group, b) in the group leading to the ducks, c) in the group leading to the Guinea fowl/Californian quail and d) leading to the chacha-

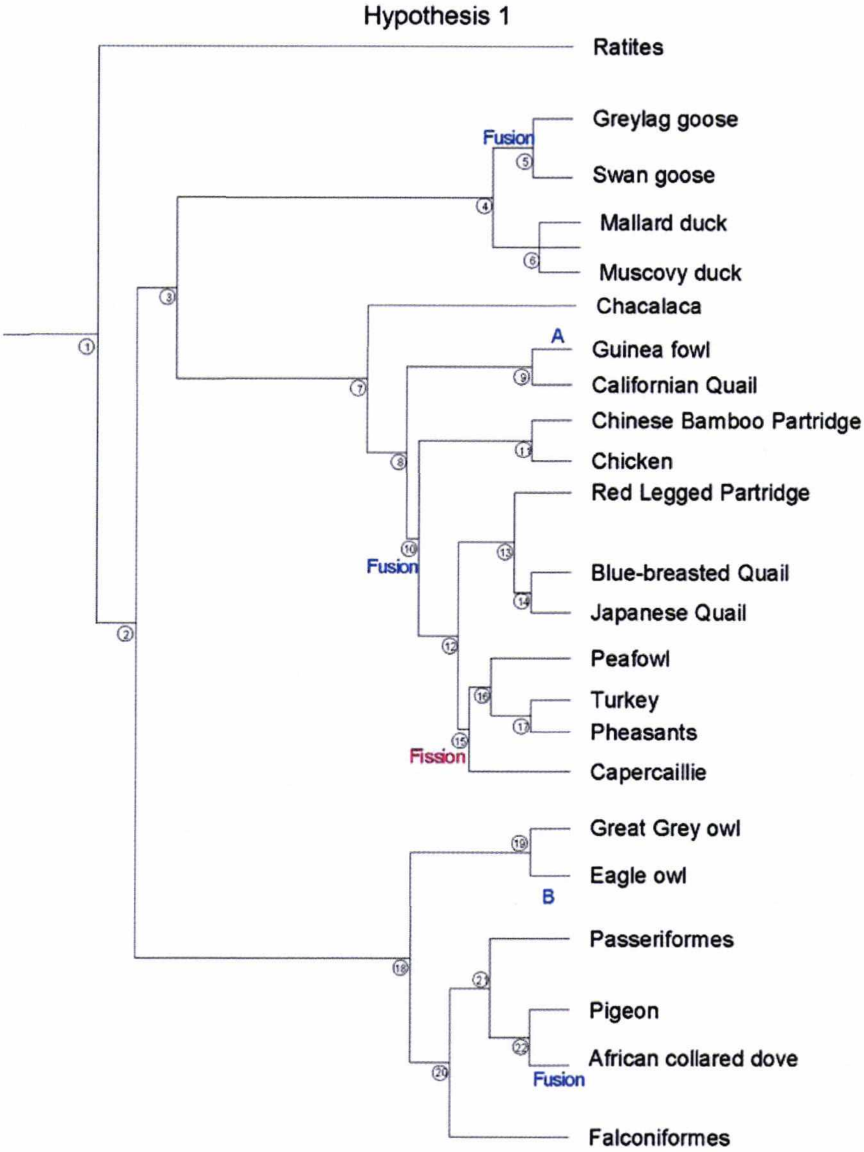
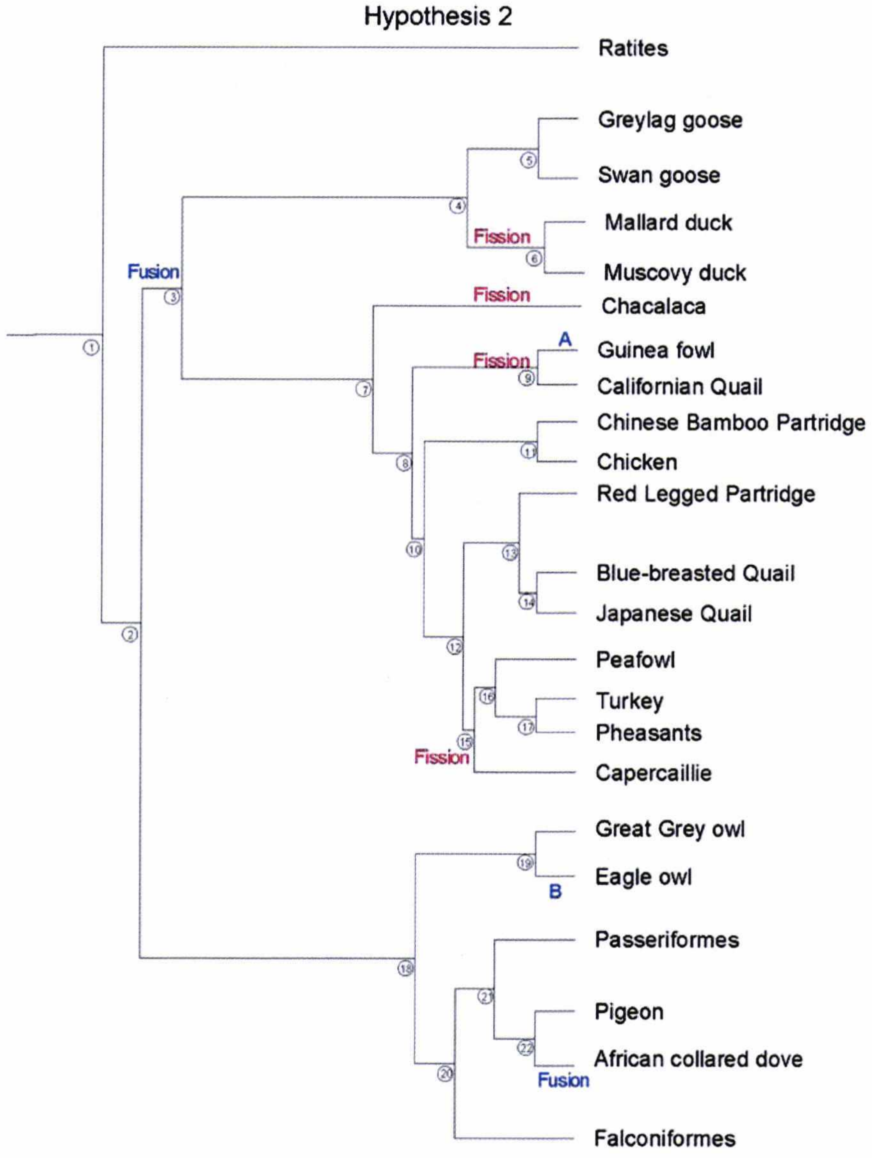


Fig. 3. Representation of the same tree but with three interpretations of the likely scenarios to explain the fission and fusion involved in the evolution of ancestral chromosomes 4 and 10. Fissions (of chromosomes 4 and 10) are in red, fusions are in blue and assumed to be fusions of chromosome 4 and 10 unless otherwise stated, i.e. A = fusion of 4 + 9 and B = fusion of 4 + 2. The numbers on the nodes of divergence are referred to in the text for the purposes of easier reading.
(For Hypothesis 2 and 3 see next pages.)

laca (nodes 6, 9, 16, post 7). Of course this still requires an explanation for the pattern seen in the higher birds of which the most parsimonious is an independent fusion event (African Collared dove) – leading to a total of two fusions (nodes 3, post 22) and four fissions (nodes 6, 9, 15, post 17; hypothesis 2, Fig. 3). We have no evidence to suggest that these fissions and fusions are any other than centric although this requires further testing using BAC mapping and/or chromosome paints from non-chicken species on tiling path microarrays (which have recently become available for the chicken) and/or lampbrush chromosomes. In a class where there are so few changes overall, having two chromosomes that are constantly splitting and joining is a mystery. A particularly fragile region of the genome (in this case perhaps the centromere) might explain multiple fissions, however genomic reasons as to why two chromosomes might be prone to fusion are more difficult to explain. In mammals, evolutionary breakpoints are more common at sites of segmental duplications, though to the best of our knowledge no such duplications exist in birds (ICGSC, 2004). Similar sequences at the centromeres of the two chromosome might provide one explanation (c.f. the acrocentric chromosomes in humans are more prone to fusion because of their association with the nucleolus) and/or proximity of the two chromosomes in the interphase nucleus might provide another.

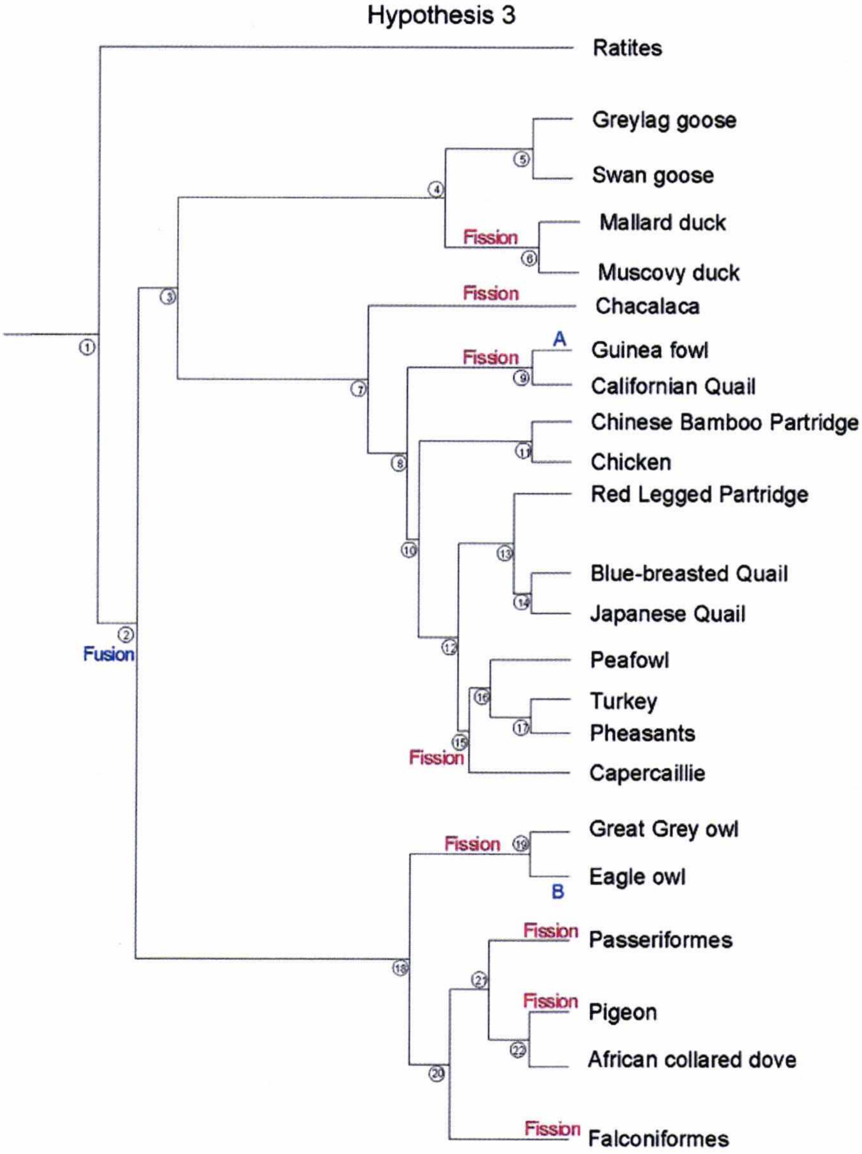
Telomeres and avian evolution

As in mammals, avian telomeres are composed of a repeat sequence, 5′-$(TTAGGG)_n$-3′, a pattern conserved throughout vertebrate evolution over 400 million years



(Meyne et al., 1989). While the avian genome is only one third the size of the average mammalian genome, the telomeric sequences comprise 4% of it, making them ten times more prevalent than in mammals, (c.f. the prevalence in humans is 0.3%) and with a length range of 0.5–2 Mb (at least in chicken; Delany, 2000). Telomere array organisation studies by Delany (2000) in chicken divided them into three classes based on telomere size, chromosome location and stability. Class I telomeres are interstitial, 0.5–10 kb, and show no evidence of telomere shortening. Class II are terminal, 10–40 kb, and show age related shortening. Class III are terminal, 40 kb–2 Mb, and do not show shortening.

Nanda et al. (2002) used FISH to study the distribution of telomeric sequences in 16 different bird species, and showed an enrichment of telomeric DNA on microchromosomes compared with the macrochromosomes. This pattern of centric and interstitial sequence in addition to chromosome ends has been found in chicken and turkey (Galliformes), Bell's vireo (*Vireo bellii*; Passeriformes), red tailed hawk (*Buteo jamaicensis*; Falconiformes) and Inca dove (*Columbina inca*; Columbiformes) (Meyne et al., 1990; Nanda and Schmid, 1994). The Californian condor, studied by Raudsepp et al. (2002), in contrast, showed no interstitial hybridisation sites, similar to the house sparrow (*Passer domesticus*; Passeriformes), and lesser adjutant stork (*Leptoptilos javanicus*; Ciconiiformes) (Meyne et al., 1990); signals were confined to chromosome ends. This is also found in two vultures studied by Nanda et al. (2006), *Gyps fulvus* and *Gyps barbatrus*, as well as in the black-winged kite *(Elanus caeruleus)* studied by Bed'Hom et al. (2003). When compared to the macrochromosomes, telomere signals were stronger on the microchromosomes in all of the studied bird species, with the strongest



signals on the smallest chromosomes. This signifies higher numbers of telomeric repeats, and one suggestion is that these serve as caps to protect the gene dense microchromosomes from telomere erosion (Delany, 2000).

Among the outstanding questions in telomere research is the paradox that, although there is a reduced proportion of repeat sequences in the avian genome overall, the abundance of telomeric sequences does not follow that rule (quite the opposite in fact). A second question is the determination of whether the interstitial arrays on the larger chromosomes reflect an ancient fusion point of smaller microchromosomes during evolution. Nanda et al. (2006) did not find any interstitial telomeres in the Old World Vultures *(G. fulvus, G. rueppelli, G. barbatus)* and we have further examined whether there is any evidence to suggest that interstitial telomeres represent ancient fusion points; we can find none.

Genome organisation from a different perspective (nuclear positioning of chromosomes)

Assessment of the spatial and temporal arrangement of chromosomes in the interphase nucleus is the best known assay for levels of genome organisation in interphase nuclei. Perturbations in either the gene density arrangement and/or the size-related arrangement have been associated with different cell types, states and with disease (Foster and Bridger, 2005). Habermann et al. (2001) conducted the first detailed two dimensional study and three dimensional reconstruction of chromosome territories in chicken fibroblasts and neurons. They used whole chromosome paints for chicken group A 1–10 and Z, and 19 pairs of smaller chromosomes (from 14 to 4 Mb). In both cell types, the largest chromosomes 1–5 and Z, plus the smaller group A chromosomes 6–10 were predominantly found at the periphery of the nucleus, while the microchromosomes formed clusters, mainly towards the centre of the nucleus (though some microchromosomes formed small clusters at the surface of the nucleus, between the macrochromosome territories). Of course, given that the smaller chromosomes are also the more gene-rich, this arrangement fits both the size-related and the gene density related models. In total 21 neuronal nuclei and 28 fibroblast nuclei were analysed. In neurons, chromosomes 1–5 and Z were peripheral, while 6–10 shifted slightly towards the centre. The microchromosome territories were central in both cell types, although they were more peripheral in the neurons than in the fibroblasts. From this it was suggested that this radial arrangement may be a common motif in all chicken cell types.

Zoo-FISH offers a method of using whole chromosome paints as a means of investigating the genome organisation of other avian species, for example the turkey, in which studies are in progress on the position of ancestral chromosomes 4 and 10 (turkey 4 and 9), and may help to explain why these two chromosomes are particularly prone to fusion.

Concluding remarks

The avian genome is fascinating and, as with many studies, the more it is examined, the more complex it seems. Many questions remain that are fundamental to our understanding of not only the avian genome but to our understanding of genome dynamics in general. Of course, performing more zoo-FISH experiments on more species is an obvious way to go. To this end we have available a set of chromosome paints or BACs for all/most of the avian karyotype including a set of ancestral chromosome paints (where 1–3, 5–9 + Z were derived from the chicken and 4 + 10 (chicken 4p + 4q) were derived from turkey chromosomes 4 and 9 (www.farmachrom.net). At present, for obvious reasons, the Galloanserae are over-represented compared to the rest of the orders and this is a situation that will, no doubt, change in the future.

More fundamental questions lie ahead of us however. For instance why, when this unusual genome organisation appeared, did it remain relatively 'untouched' for millions of years? It might be speculated that having so many chromosomes was sufficient, through both random segregation and crossing over (at least one chiasma per bivalent) to drive evolutionary change without the need for extensive chromosome rearrangement as a means of speciation. That is, while, in mammals, chromosome rearrangement is a consistent feature of species divergence, it is clearly less so in birds despite them having many more chromosomes. Put simply, birds may have, on the whole, not facilitated their evolution through chromosomal changes because there was less pressure for them to do so. What is the reason behind the very strange behaviour of chromosome 4? Examination of a rapidly evolving chromosome in a background of a genome where change is rare may shed light on the reasons as to why, and under what circumstances, chromosome change occurs. How did the required structures (e.g. telomeres and centromeres) arise in newly formed microchromosomes? The formation of de-novo centromeres is not unknown and perhaps arose through endoreduplication of pre-existing non-coding DNA. What, if anything, is the role of telomeres in avian evolution and why are telomeres so big in birds? Why are ancestral telomeres not detectable following fusion e.g. in the Accipitridae (are they eliminated completely or simply reduced to sub-detectable levels?) What is the role of nuclear organisation in genome evolution? Finally, what can we deduce about the genome organisation of species that are long since extinct (e.g. the dinosaurs); detailed comparative analysis of extant species may now make this possible.

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Note added in proof

While making the final alterations to the proof of this manuscript, it came to our attention that Nanda et al. have submitted a manuscript for publication in this volume describing zoo-FISH experiments of chicken chromosome paints on Psittacidae species. Readers are encouraged to refer to this manuscript also when updating their knowledge on avian chromosome evolution.

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Cytogenetic and Genome Research

Practicable approaches to facilitate rapid and accurate molecular cytogenetic mapping in birds and mammals

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Abstract. Molecular cytogenetic mapping by FISH is a common feature of most genome projects as it provides a global, low-resolution overview of the genome and facilitates comparative genomics. An essential prerequisite for cytogenetic mapping is the ability to identify accurately the chromosome on which the clone (e.g. BAC) resides. This is not usually a barrier to human mapping as knowledge of the human karyotype is commonplace. For other species however accurate assignment can be problematic either because, as in birds, the karyotype is too complex to analyze by standard means or because of the paucity of individuals skilled to perform the karyotyping. Using chicken as a model we have developed a reproducible approach for accurate cytogenetic mapping that involves: a single colour FISH, measurement of the ratio of the size of the signal bearing chromosome to that of chromosome 8, and final assignment through a small series of dual colour experiments. Reference values for size ratios were established using base pair estimate information from the Ensembl browser. By this method cytogenetic mapping to highly complex karyotypes can be achieved in a small number of simple steps. We have also developed and tested a karyotyping tutorial programme adapted from one previously reported in this journal. That is, we have used pig as an example of a model species with a relatively tractable karyotype and demonstrated that scientists and students, even after only one hour using our tutorial, can readily identify pig chromosomes and thus make appropriate assignments using FISH. Simple, practicable means often provide preferable solutions than complex alternatives (e.g. m-FISH) to the solution of scientific problems. Such is the case for the approaches described here.

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Cytogenetic mapping involving FISH experiments on metaphase chromosomes (or interphase nuclei) is the lowest resolution but most global of the many iterations of the term 'gene mapping'; it has thus been a common feature of all genome projects to date (e.g. Matsuda and Chapman, 1995; Bray-Ward et al., 1996; Schmid et al., 2000, 2005). Although the method of isolation theoretically ensures that clones (more commonly BACs in recent years but also including YACs, PACs and cosmids) are linked to a particular locus in the developing sequence assembly (and therefore already assigned to a particular chromosome region) experience suggests that this is an imperfect process as BACs can often be clearly shown by FISH to map to a different chromosome than the molecular evidence would suggest (Aerts et al., 2005). Moreover, BACs are frequently generated that are not assigned to the sequence assembly (so-called anonymous clones) but nevertheless can be easily assigned by FISH (Aerts et al., 2005). FISH assignment, either of anonymous clones or to confirm the location of 'known' sequences, is the most robust means of mapping a clone by virtue of the fact that the signal is physically visible on a particular chro-

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mosome. Finding the chromosomal location of a large series of BACs also allows the transfer of genetic and physical information directly from one species to another (comparative genomics) (O'Brien et al., 1999). These comparative maps can then be used to prognosticate quantitative trait locus expression in a range of species, identify candidate disease genes, characterise the genetic basis for complex traits, find modulators of disease susceptibility and address fundamental questions of genome organisation and evolution (O'Brien et al., 1999).

An essential prerequisite of cytogenetic mapping is the ability to identify unequivocally the chromosome on which the FISH signal appears. This is achieved either by experienced recognition of a pseudo G-banding (reverse DAPI) pattern or co-hybridisation of a known clone in a different fluorescent colour to that chromosome. In humans this is rarely a barrier as a working knowledge of human chromosome banding patterns is commonplace in most institutions that perform FISH. In other animals however there is a paucity of cytogenetic expertise and often labs will rely on combinatorial multicolour FISH (m-FISH) experiments, which can be time consuming and expensive (Jentsch et al., 2001). It could be argued however that, in the majority of mammals, karyotype analysis is not more difficult than in humans. In species for which there is a significant genome mapping effort therefore (e.g. chimpanzee, macaque, pig, opossum, horse etc.) chromosome banding patterns could be analyzed (and therefore BACs assigned) relatively easily following a single colour FISH experiment (Macgregor, 1993). In other animals however (e.g. mouse, rat, cattle, dog, sheep, tilapia; also all birds) karyotyping is much more difficult as the chromosomes are more similar and sometimes more numerous (Macgregor, 1993). Of these species, birds are a special case because the chromosomes are so numerous, so small and banding patterns are less distinct than in mammals; thus karyotyping by standard means is not only difficult but literally impossible (Griffin et al., 1999; Masabanda et al., 2004). In chicken for example (the most genomically characterised of birds), of the 39 pairs of chromosomes, only the largest nine pairs (including the sex chromosomes) can be distinguished by banding; the others are simply too small (Griffin et al., 1999; Masabanda et al., 2004).

As the genomes of a rapidly increasing number of species are being mapped and sequenced (and key species such as human, mouse and chicken provide a reference point for comparative genomics) it seems likely that the robust and reproducible cytogenetic identification of chromosomes in a range of animals will become more important. Two major obstacles to high throughput cytogenetic mapping in non-human species however are: 1) when chromosome analysis is beyond the skill of even a very experienced cytogeneticist (e.g. in birds) and 2) when the operator lacks the confidence or skill to analyse the chromosomes. The purpose of this study was therefore to make a significant contribution towards alleviating both these problems by the development of simple and practicable approaches that can be used in most laboratories.

We have therefore developed a rapid FISH-based approach for BAC mapping for species in which chromosome assignment is either impossible or very difficult by standard means. Here we measured the ratio of the area of the smallest easily identifiable chromosome (chromosome 8) in the chicken (*Gallus domesticus* 2n = 78) relative to the area of the chromosome containing the BAC. This provided the basis for a small number of dual FISH experiments designed to make an accurate assignment.

It is our contention that a partially non-lab based approach i.e. incorporating prior training via a computer based tutorial is a preferable means to an m-FISH approach for the accurate mapping of BACs in species where chromosome analysis is not more difficult than in humans. We have thus developed and tested such a tutorial for this purpose in the domestic pig *(Sus scrofa)* which has a relatively easy karyotype (2n = 38) to analyse (Ducos et al., 1998a, b; Pinton et al., 2000).

Both animals were chosen as 'proof of principle' species as they have extensive genome mapping efforts associated with them partly because of their agricultural importance and partly because of their utility as model organisms. That is, the chicken is a key model for developmental biology, genome organization and mapping (Schmid et al., 2005) and the pig is a primary model for human nutritional, reproduction and xenotransplantation studies (Cooper et al., 2002; Rothschild, 2003).

Materials and methods

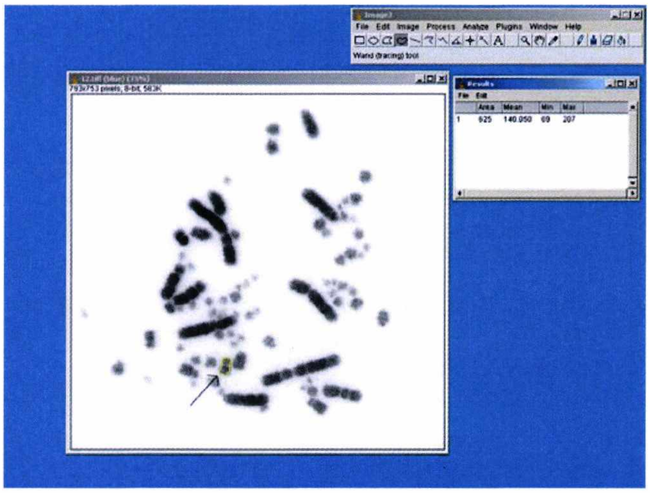
Chicken metaphases were prepared from cultured fibroblasts taken from 5–6 day old fertilised eggs. Pig metaphases were prepared by standard protocols from peripheral blood lymphocytes. Briefly whole blood was cultured in the presence of phytohaemagglutinin for 72 h. In both cases, cells were arrested in metaphase using colchicine, then hypotonic swelling in 75 mM KCl preceded fixation to a glass slide with 3:1 methanol:acetic acid.

FISH was performed by standard protocols (Griffin et al., 1999). Chicken BACs were obtained from Richard Crooijmans (Wageningen University), pig BACs were obtained from the pigE BAC library (Anderson et al., 2000) and both held at our own resource centre (www.farmachrom.net). Probes were labelled by nick translation with biotin (and/or digoxigenin for the dual colour experiments), ethanol precipitated and re-suspended in hybridisation buffer (containing 50% formamide and 10% dextran sulphate). Slides were aged overnight at 37 °C and chromosomal DNA was denatured using 70% formamide at 70 °C then quenched in ice-cold 70% ethanol before dehydration (ethanol series) and air-drying. Concurrently probes were denatured for 5 min at 75 °C then cooled to 37 °C for preannealing. Overnight hybridisation under a sealed coverslip at 37 °C preceded stringency washes (3 × 50% formamide at 42 °C and 2 × 2× SSC). Slides were incubated in blocking buffer (2–3% BSA in 4× SSC, 0.5% Tween-20) and hapten labels detected using Cy3 avidin (for biotinylated probes; Amersham Biosciences) and/or FITC anti-digoxigenin (for digoxigenin labelled probes; Roche). Incubations with fluorochrome conjugates diluted according to manufacturer's guidelines were performed in a buffer of 1.5% BSA in 4× SSC, 0.5% Tween-20. Slides were then rinsed in distilled water, air dried and mounted in antifade medium containing DAPI (Vector labs). For images used in the porcine karyotyping tutorial, slides were taken directly to the antifade/DAPI stage without performing FISH.

Images were captured on an Olympus epifluorescence (BX-61) microscope equipped with cooled CCD camera and SmartCapture soft-

Table 1. Relative size ratios in base pairs of chromosomes 9–24, 26, 27, 28 and 32 as measured against chromosome 8 (data taken from www.ensembl.org/Gallus_gallus)

Chromosome number	Chromosome length in base pairs	Size ratio cf. chromosome 8 (3dp)
8	30,024,636	1.000
9	23,409,228	0.779
10	20,909,726	0.696
14	20,603,938	0.686
12	19,821,895	0.660
11	19,020,054	0.633
13	17,279,963	0.576
20	13,506,680	0.450
15	12,438,626	0.414
17	10,632, 206	0.354
19	9,463,882	0.315
18	8,919,268	0.297
21	6,202,554	0.207
24	5,910,111	0.197
23	5,666,127	0.189
28	4,731,479	0.158
26	4,255,270	0.142
27	2,668,888	0.089
22	2,228,820	0.074
32	1,018,878	0.034
16	239,457	0.008



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     Area  Mean     Min  Max
1    625   140.050  69   207
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Fig. 1. Screenshot of IMageJ programme measuring the area of chromosome 8.

ware (Digital Scientific, UK). DAPI banded images were enhanced using the '5×5 moderate enhance' function and inverted to resemble G-bands, then imported into Adobe Photoshop (for development of the karyotyping tutorial) or ImageJ – a freely available Java-based scientific image processing software (Abramoff et al., 2004) enabling measurement of the size ratios of the chicken chromosomes.

In order to facilitate accurate assignment to chicken microchromosomes, a ratio measurement approach was used to narrow down identification of the chromosome of interest. Chicken chromosomes are, in general, numbered by size, however recent base pair estimates (www.ensembl.org/Gallus_gallus) reveal variations to this rule (Table 1). We adopted an approach that involved measurement of the area of the chromosome containing the signal compared to the smallest identifiable chromosome – chromosome 8 (GGA8). GGA8 is easily identifiable as it is metacentric, unlike the others immediately smaller and larger than it, which are telocentric. For area measurements enhanced images were exported as TIFF files from SmartCapture and then analysed using the programme ImageJ. Each image was split into individual RBG channels. Using the blue (DAPI) channel the area of both GGA8 and the chromosome with the BAC signals were measured using the freehand selection tool and the analysis function (Fig. 1). From this, the ratio of the area of the chromosome of interest to the area of GGA8 was calculated. A minimum of three metaphases (six chromosomes) were measured for all images of each BAC. The mean value was compared with predetermined ratios for chromosome size of each chromosome against GGA8 derived by comparison of base pair length published in the Ensembl browser (www.ensembl.org/Gallus_gallus) (Table 1). The initial approach involved selecting the two chromosomes corresponding to the reference ratio measurements immediately greater than, and the two immediately less than our derived value, which allowed us to narrow down the chromosome of interest to four candidates (see Results). With experience however it became clear that more accurate assignments were obtained if the four values immediately smaller than the ratio measurement were taken. As also described in the results occasionally it was necessary, for the smaller chromosomes, to take the six values immediately below. This then prompted four (or six) parallel dual colour experiments with previously assigned digoxigenin labelled 'marker' BACs to facilitate a final assignment. Accurate mapping (which would be virtually impossible by classical means) was therefore achieved in three simple steps: one single colour FISH, one ratio measurement and one set of dual colour experiments.

In order to develop the porcine karyotyping tutorial programme, individual chromosomes were cut out using the lasso tool in Adobe Photoshop for importation into the programme. The programme itself, which we dubbed 'KaryoLabPorc' was written in a virtual lecture interface designed in Macromedia Authorware 6.5, an icon based multimedia development application. The main menu consists of four topics ('Background', 'Tutorial', 'Practice' and 'Assessment'), with each of these leading to further sub-topics. Further sub-topics give (with representative images) specific instructions on how to identify pig chromosomes and distinguish 'problem' chromosomes. The 'Practice' section allows the user to practice the skill of chromosomal analysis in an interactive way through five different interactive karyotyping scenarios. Operators use a drag and drop function to manipulate individual chromosome images into 'bins' that represent the appropriate place in the karyotype (Fig. 2). Formative feedback is given in the three 'Practise' karyotypes; that is, if the chromosome is dragged to the incorrect place, the chromosome will automatically return to its original position. A summative assessment was enforced in the remaining practise karyotypes; that is, the chromosome stays where the user puts it and, when they have finished a 'mark' button is pressed that instructs the computer to give feedback on whether the chromosomes were placed correctly. If help is required at any point during this section the user can return to the tutorial section. The 'Assessment' section is similar to the 'Practice' section in that it includes five different karyotyping scenarios; however, the computer does not provide formative feedback, merely a mark on the number of correctly place chromosomes at the end. On completion of each karyotype, the user then proceeds to a FISH image (Fig. 3) where he is asked to identify the chromosome onto which a BAC has been hybridised.

'KaryoLabPorc' was evaluated on a total of 14 postgraduate students in the Department of Biosciences, University of Kent with no previous experience of pig karyotyping. They were asked to attempt a version of the 'Assessment' section of the KaryoLabPorc before doing the tutorial/practice section. They were then given one hour to use the

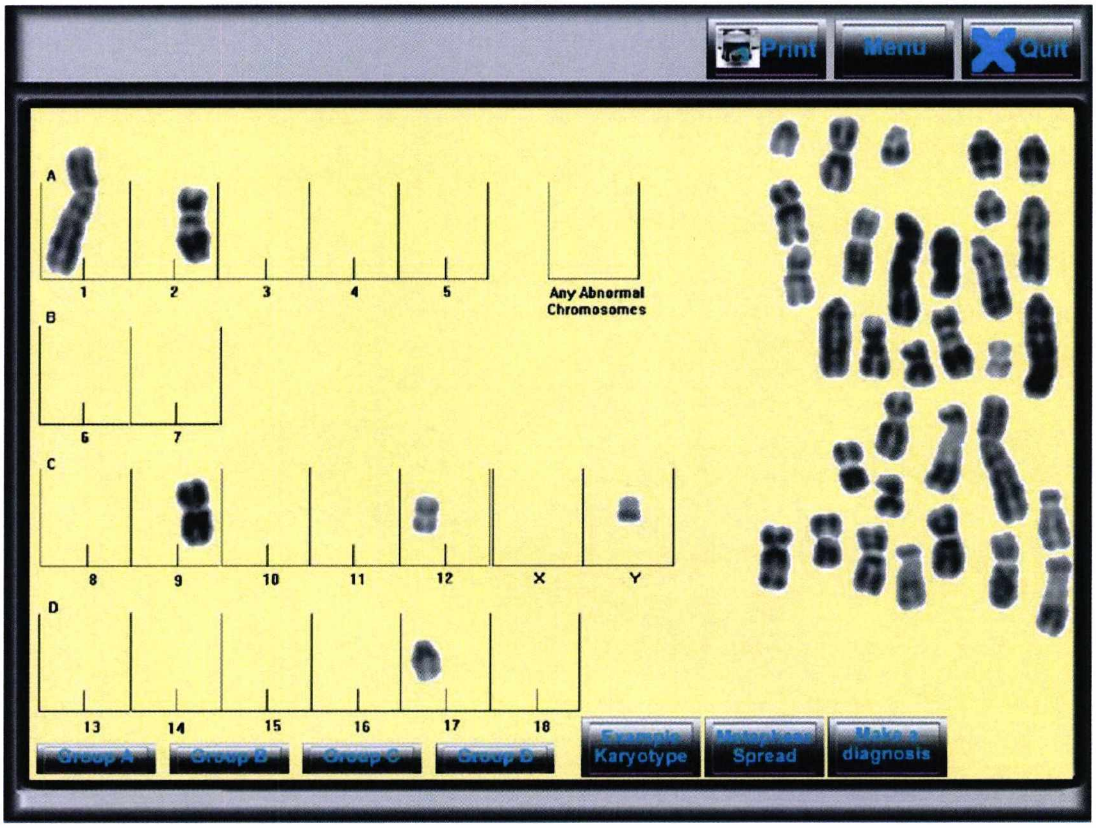
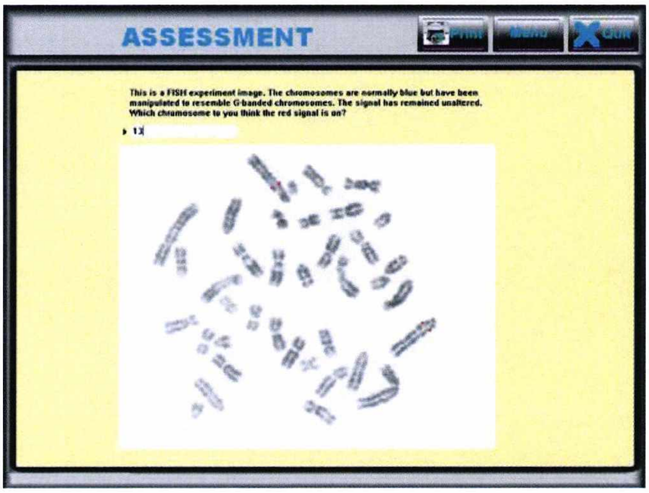


Fig. 2. Screenshot of the practice section of the KaryoLabPorc tutorial programme. Chromosomes placed in the correct bin stay put, those assigned incorrectly return to their original position.

tutorial/practice section and, after this time, tested again with equivalent karyotypes. One mark was assigned for each correctly placed chromosome (before and after the tutorial) and they were also given two individual images of FISH experiments before and after the tutorial/practice and asked to identify the chromosome on which the FISH signal resided. A total mark of up to 40 was therefore awarded for the whole exercise with matched marks pre- and post-tutorial, a mean of the whole group was taken and we tested for any significant differences between the pre- and post-tutorial mark using Student's t test.

Results

For the chicken chromosomes, 28 anonymous BACs were mapped using the approach described. Details are given in Table 2. Likely ratios derived by comparison of base pair length published in Ensembl (www.ensembl.org/Gallus_gallus) (Table 1) proved to be reliable in narrowing down the ratio to, in most cases, four chromosomes. According to our results, ratio measurements could narrow down the chromosome of interest to four possible candidates for all but five of the BACs (82%). If we considered six possible candidates then all but one were narrowed down (96%) but of course estimates became less reliable with de-



This is a FISH experiment image. The chromosomes are normally blue but have been manipulated to resemble G-banded chromosomes. The signal has remained unaltered. Which chromosome to you think the red signal is on?

13

Fig. 3. Screenshot of assessment section of the KaryoLabPorc programme showing a FISH image and the prompt to ask the user to assign a chromosome on which the BAC signal appears.

Table 2. Assignment of 28 BACs based on ratio measurements. Note that all but five could be narrowed down to four chromosomes and all but one were narrowed down to six chromosomes using this approach.

BAC name	Putative assignment to four chromosomes by 'ratio to 8' measurement	Final assignment confirmed by dual FISH
LEI0110	10, 14, 12 or 11	11
ABR0070	15, 17, 19 or 18	15
MCW0213	13, 15, 17 or 19	13
LEI0099	12, 11, 13 or 15	12
CRY3A4	20, 15, 17 or 19	15
HSPSA	17, 19, 18 or 21	17
LEI0090	21, 24, 23 or 22	23
MCW0244[a]	10, 14, 12 or 11	13
ADL0034	11, 13, 20 or 15	20
ROS0113A[a]	17, 19, 18 or 21	24
MCW0151	20, 15, 17 or 19	17
ADL0231	9, 10, 14 or 12	10
B2M	10, 14, 12 or 11	10
ADL0272	10, 14, 12 or 11	10
MCW0132	10, 14, 12 or 11	10
ADL0149	13, 20, 15 or 17	17
ADL0184	13, 20, 15 or 17	18
APOA1sts	19, 18, 21 or 24	24
CTG1704	17, 19, 18 or 21	19
HUJ0010	15, 17, 19 or 18	18
LEI0069[a]	15, 17, 19 or 18	24
MCW0045	15, 17, 19 or 18	18
MCW0198	10, 14, 12 or 11	12
MCW0219	15, 17, 19 or 18	18
MCW0286[b]	13, 20, 15 or 17	26
ROS0027[a]	13, 20, 15 or 17	18
SCW0024	20, 15, 17 or 19	19
CTG0107	20, 15, 17 or 19	19

All assignments made to within four chromosomes apart from:

[a] correct within six chromosomes.

[b] incorrect assignment.

creasing chromosome size. We also noted that chromosome 16 reproducibly adopts an 'isosceles triangle' shape (Fig. 4), whereas the others are roughly circular in appearance and that the published base pair number does not correspond to the size of the chromosome, probably due to rRNA gene repeat elements on this, the NOR chromosome (Masabanda et al., 2004). For chromosomes 27–38 we did not map any BACs in this study but noted, from our previous experiments, that we could merely identify that the chromosome of interest was one of these, thus potentially prompting a larger number of dual FISH experiments. It was also noteworthy that there was considerable size variation of the smallest chromosomes, even among homologues, and that chromosome 25 does not have either markers assigned to it nor an estimated base pair number and we have previously provided evidence that this chromosome may contain a large proportion of repeat elements (Masabanda et al., 2004). Mapping assignments were confirmed by dual FISH experiments (Fig. 5; Table 2) in all cases.

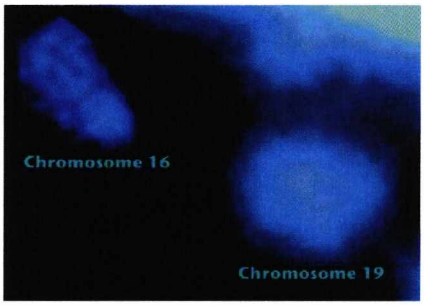


Fig. 4. Chicken chromosomes 16 and 19 (assignments confirmed by FISH – signal not shown). Chromosome 16 is smaller that chromosome 19, chromosome 16 adopts an 'isosceles triangle' shape whereas chromosome 19, like most other microchromosomes, appears roughly circular.

Of the 14 students tested using KaryoLabPorc, mean marks pre-tutorial (out of 40) were 7.8 (19.5%), reflecting the student's relative lack of karyotyping experience. After one hour with the tutorial however, the mean mark was 28.2 (70.5%) indicating a highly significant improvement in karyotyping skills ($P < 0.01$ by Student's t test) among the group as a whole and suggesting that accurate BAC mapping could be achieved relatively easily through use of this tutorial.

Discussion

Simple approaches often (in science and elsewhere) offer more practicable solutions than more high-tech alternatives to particular biological problems. In the case of cytogenetic mapping of non-human species, although m-FISH experiments can theoretically be used to resolve complex karyotypes and facilitate BAC mapping in a single step, in practice for most laboratories such approaches are precluded by cost, time and resources. For high throughput strategies therefore, a series of single or dual colour experiments is the only realistic option for the majority of laboratories. For the chicken, we are hard-pressed to imagine a simpler approach to achieve cytogenetic mapping with such speed and reproducibility. In the rare occasions that the choice of candidate chromosomes is incorrectly estimated then it is relatively easy to establish whether the chromosome of interest is larger or smaller than the measurements indicated. This is an approach that is, potentially, transferable to all birds. It should be noted, however, that the metacentric 8 is unique to chicken as it arose from a pericentric inversion from the telocentric ancestor (Schmid et al., 2000; Shibusawa et al., 2004). If this approach were to be adapted to other avian species it might be appropriate to perform a dual rather than a single first experiment using marker chromosome identifier probe(s) to narrow down the assignment. Obviously, difficulty of assignment is inversely proportional to chromosome size and therefore our approach becomes less accurate for the smallest chromosomes. It is noteworthy how-

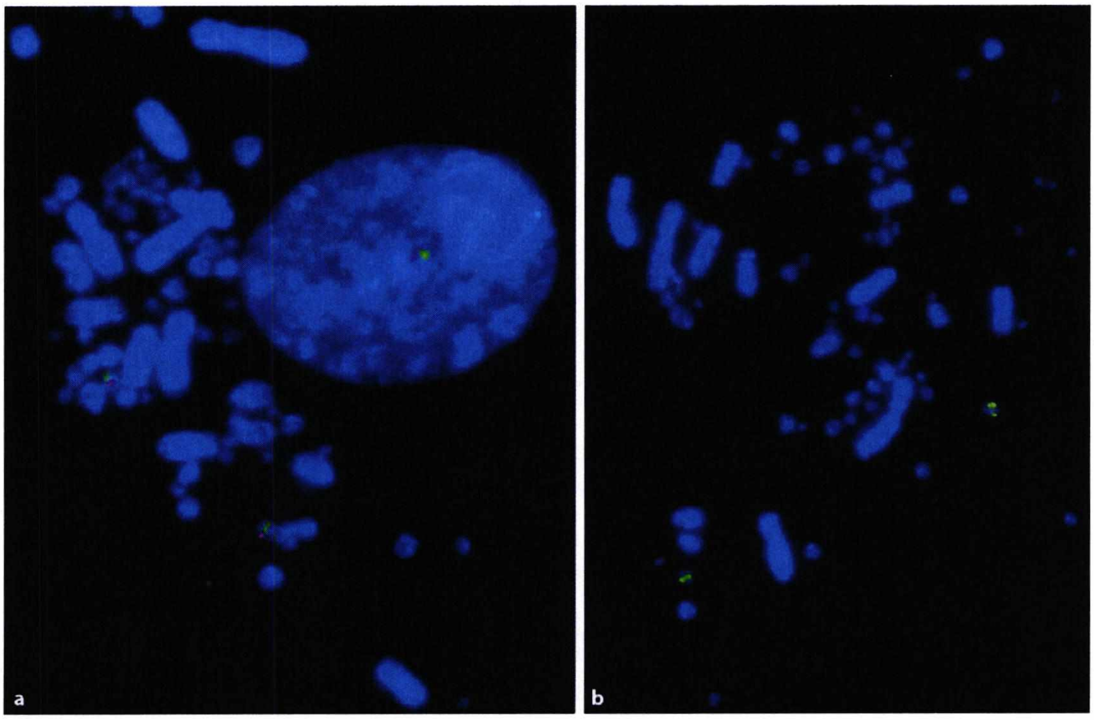


Fig. 5. Dual FISH experiments (**a**) showing confirmation of a chromosome assignment (red and green signals on the same chromosome); (**b**) where the experiment revealed that the unknown BAC was not on the chromosome being tested (red and green signals on different chromosomes).

ever that the only probes thus far assigned to the chicken genome for chromosomes 29 onwards are single cosmids for chromosomes 29, 30 and 31 and chromosome specific paints isolated by chromosome microdissection for chromosomes 33–38 (the 'D group' as described in Masabanda et al., 2004). Thus cytogenetic mapping is unlikely to become commonplace for these chromosomes. The only occasion in which we could not make an accurate assignment was for a BAC on chromosome 26. It is possible that this chromosome is larger than the base pair estimate would suggest (as is chromosome 16) and it may be the case that we will alter some of our reference ratios on the basis of our experience.

We have previously reported (in this Journal) a tutorial programme for the teaching of human karyotyping in universities designed to encourage more students to engage in cytogenetics (Gibbons et al., 2003). Here we demonstrate that this technology is transferable to non-human species and has a particular application for cytogenetic mapping studies. We estimate that for the majority of species for which karyotypes can be analysed by G-banding, adaptation of the programming would take 1–2 days per species. We contend therefore that our approach is a transferable one and a highly practicable solution to the problem of accurate cytogenetic mapping in non-humans. Of course the steepness of the learning curve for learning any karyotype is directly proportional to the complexity of the karyotype – factors such as chromosome number, proportion of (sub-) metacentric chromosomes and distinctiveness of banding patterns all play a part in this. Moreover, the decision of which approach to choose for which species depends on the complexity of the karyotype. Mouse and dog are examples of species that are potentially applicable for both approaches in that both species have a relatively large ($2n = 40$ in mouse; $2n = 78$ in dog) (Cowell, 1984; Switonski et al., 1996; Graphodatsky et al., 2000) number of acrocentric chromosomes that are numbered roughly according to size but nevertheless, with training, are analysable by G-banding. In all cases of course, cytogenetic assignment can be confirmed by dual colour FISH as described for the chicken experiments. It is also noteworthy that syntenic information (e.g. Rettenberger et al., 1995; International Chicken Genome Sequencing Consortium, 2004) can provide a clue to cytogenetic assignment thereby helping to narrow down the chromosome on which the BAC resides.

Karyotyping has been of considerable interest to the artificial intelligence community for more than 25 years. Various authors have published algorithms, heuristics, and technological improvements in artificial imaging, classifying, sorting, and representation, and the manifestation of such research can now be seen in commercial systems for karyotyping such as SmartCapture Quips and Cytovision. The state of the art involves automated measurement of p- and q-arms of metaphase chromosomes and associated ratios as well as overall length relative to other chromosomes, identification of centromere position, identification of secondary constrictions, resolution of overlapping chromosomes, densitometry profiles of banding patterns, probabilities of mismatches, coincidence intervals for decisions, suggestions for pulling out individual chromosomes for re-investigations, contour or perimeter features, pseudocoloring, identifications of deletions, insertions, translocations, aneuploids and polyploids and the ability to 'learn' classifications as more are entered into the memory (Piper et al., 1980; Nickolls et al., 1981; Groen et al., 1989; Carothers and Piper, 1994; Graham and Piper, 1994; Charters and Graham, 1999; Popescu et al., 1999; Ritter and Schreib, 2001). The majority of operators of these systems however will attest that, while they are excellent at providing a 'first-pass' classification of chromosomes, human intervention by someone skilled in karyotyping is almost always required. Thus, while there are highly sophisticated programmes available for automated karyotyping, there are very few, technically simpler ones (Gibbons et al., 2003, 2004) designed to train individuals to be sufficiently confident to interact with these programmes in order to make accurate chromosomal assignments. It is not unreasonable therefore to envisage both types of programmes being used in tandem, one to cut out the chromosomes and make initial classifications, the next (e.g. as described in this paper) to train operators to confirm those classifications and make final assignments.

In conclusion we contend that the approaches developed here represent a significant advance in cytogenetic gene mapping which can, in combination with pre-existing commercial karyotyping programmes, be used to map BACs, cosmids, YACs and PACs in most or all species.

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Quantum Dots as new-generation fluorochromes for FISH: An appraisal

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Abreviations

BAC(s) – Bacterial Artificial Chromosome(s)
BSA – Bovine Serum Albumin
DAPI - 4',6-diamidino-2-phenylindole
ddH_2O – double distilled water
DS - Dextran Sulphate
DOP – Degenerate Oligo Primed
DTT – Dithiothreitol
dUTP – 2' deoxyuridine 5' triphosphate
FA – Formamide
FISH – Fluorescent *in-situ* hybridisation
FITC - Fluorescein IsoThioCyanate
HFEA - Human Fertilisation and Embryology Authority
MAA - MercAptoacetic Acid
NIR – Near Infra Red
PBS - Phosphate Buffered Saline
QDs – Quantum Dots
QD-FISH – Quantum Dot – Fluorescent *in-situ* hybridisation
RT – Room Temperature
PCR – Polymerase Chain Reaction
SERT - Serotonin Transporter Protein
SSC – Saline Sodium Citrate
UV – UltraViolet

Abstract

In the field of nanotechnology, Quantum Dots (QDs) are a novel class of inorganic fluorochromes composed of nanometer scale crystals made of a semiconductor material. Given the remarkable optical properties that they possess, they have been proposed as an ideal material for use in Fluorescent in-situ Hybridisation (FISH). That is, they are resistant to photobleaching, they excite at a wide range of wavelengths but emit light in a very narrow band that can be controlled by particle size and thus have the potential for multiplexing experiments. The principal aim of this study was to compare the potential of QDs against traditional organic fluorochromes in both indirect (i.e. QD conjugated streptavidin) and direct (i.e. synthesis of QD labelled FISH probes) detection methods. In general, the indirect experiments met with a degree of success, with FISH applications demonstrated for chromosome painting, BAC mapping and use of oligonucleotide probes on human and avian chromosomes/nuclei. Many of the reported properties of QDs (e.g. brightness, "blinking," and resistance to photobleaching) were observed. On the other hand, signals were more frequently observed where the chromatin was less condensed (e.g. around the periphery of the chromosome or in the interphase nucleus) and significant bleed-through to other filters was apparent (despite the reported narrow emission spectra). Most importantly experimental success was intermittent (sometimes even in identical, parallel experiments) making attempts to improve reliability difficult. Experimentation with direct labelling showed evidence of the generation of QD-DNA constructs but no successful FISH experiments. We conclude that QDs are not, in their current form, suitable materials for FISH because of the lack of reproducibility of the experiments; we speculate on reasons as to why this might be the case and look forward to the possibility of nanotechnology forming the basis of future molecular cytogenetic applications.

Introduction

Traditionally associated with engineering and physical science (e.g. in computer chips) "nanotechnology" is a research field that manipulates and creates structures of particles with dimensions smaller than 100nm (Chan 2006). Within the last decade however there has been a growing interaction between nanotechnology and biology (Parak et al. 2003), particularly in fluorescent microscopy. One novel class of inorganic fluorophores arising from nanotechnology and useful in fluorescent microscopy are "Quantum Dots" (QDs) (Miller et al. 1986; Reed et al. 1986). QDs are composed of nanocrystals of a semiconductor material (e.g. either Cadmium Sulphide (CdS), Cadmium Selenide (CdSe), Indium Phosphate (InP) or Lead Selenide (PbSe)) at the core (Lipovskii et al. 1997). This is coated with a (usually zinc sulphide, ZnS) shell that improves the optical properties (Michalet et al. 2005; Invitrogen 2006); plus an extra polymer coating that serves as a site for conjugation with biomolecule moieties. This brings the total size of the nanocrystal to 10 – 20nm. The core material is chosen dependent on the emission wavelength range that is targeted (e.g. CdS for Ultraviolet-blue, CdSe for the visible spectrum and CdTe for the far red and near infrared (QuantumDotCorporation 2006)), thus fluorophore colour is size- dependent and controlled during synthesis (Chan et al. 2002).

A unique property of QDs is their broad excitation and narrow symmetric emission spectra. The full spectral width of QDs at half maximum is 12nm and leads to less overlap between absorption and emission spectra (Chan and Nie 1998). Thus different QDs can be excited by a single wavelength shorter than their emission wavelength (Green 2004; Alivisatos et al. 2005; Arya et al. 2005). Such an approach cannot be achieved with classical organic fluorophores because they have narrow excitation and broad emission that often results in spectrum overlap or red tailing (Dabbousi et al. 1997). QDs produce significantly brighter fluorescence (2-11 times) (Larson et al. 2003) because of the large molar extinction coefficients (10-50 times larger than organic fluorophores) (Gao et al. 2005). Due to their inorganic composition they are more resistant to photobleaching than organic fluorophores (Alivisatos 1996; Bruchez et al. 1998; Michalet et al. 2001; Jaiswal et al. 2003; Parak et al. 2005) and have a longer fluorescence half-life than typical organic dyes (Lounis et al. 2000).

There are many *in-vitro* applications using QDs reported in the literature. For instance: detection of the cancer marker Her2 on the surface of fixed and live cancer cells (Wu et al. 2003) targeting the serotonin transporter protein (SERT) in transfected HeLa cells and oocytes (Rosenthal et al. 2002) and identifying the erbB/HER family of transmembrane receptor tyrosine kinases that mediate cellular responses to epidermal growth factor (Lidke et al. 2004). QDs have been used as cellular markers because they can be internalised by cells using a receptor (Chan and Nie 1998; Zheng et al. 2006) or by non-specific endocytosis (Parak et al. 2002). QD cell markers have been used in cell–cell interaction studies by creating unique colour tags for individual cell lines (Mattheakis et al. 2004). In addition, QD resistance to photobleaching has enabled 3D optical sectioning studies of the vascular endothelium (Ferrara et al. 2006), applications in cell motility assays for studying actomyosin function (Mansson et al. 2004) and phagokinetic tracking of small epithelial cells responsible for 90% of cancers (Parak et al. 2002).

The optical properties of QDs have also been exploited for *in-vivo* uses. For instance, as a means to deliver drugs to target molecule sites after injection (Akerman et al. 2002) and to study the behaviour of specific cells during early stage embryogenesis in Xenopus and Zebrafish embryos by microinjection of micelle encapsulated QDs (Dubertret et al. 2002; Rieger et al. 2005). Gao et al. (2004) reported *in-vivo* cancer targeting and imaging using antibody-conjugated QDs for human prostate cancer and QDs have been used as contrast agents during surgery to map sentinel lymph nodes in the pig and mouse (Kim et al. 2004).

Given the potentially much-vaunted properties of QDs, they seem a ideal candidates for the study of chromosomes through adaptations of FISH protocols. Since its inception, FISH has continuously evolved but, as with all experiments involved in fluorescent microscopy, faces limitations imposed from the use of organic fluorophores. The number of available fluorochromes and their broad emission spectra make multicolour experiments difficult to resolve due to overlapping and the rapid photobleaching of organic fluorochromes. Published work related to QD-FISH is currently limited. Xiao and Barker (2004b) utilised biotinylated total genomic DNA on human metaphase chromosomes detected using streptavidin conjugated QDs. Comparisons between detection with QDs and organic fluorochromes (Texas Red–streptavidin and FITC-streptavidin) showed that QD probes were significantly more photostable and 2-11x brighter than organic flourochromes. Furthermore, they applied this technique to detect the Her2 locus in low copy human breast cancer cells demonstrating that QD-FISH has the potential to become a medical diagnostic tool. A similar indirect labelling approach has been used on plant chromosomes (Muller et al. 2006) with limited success. Chan et al. (2005) developed a direct labelling approach to target specific mRNAs in mouse brain sections. Biotinylated labelled oligonucleotides were conjugated with QD-streptavidin in the presence of biocytin to block excess streptavidin sides that could result in oligonucleotide cross-linking. Bentolila and Weiss (2006) using a biotin–streptavidin strategy labelled oligonucleotide probes with QDs; in this case complexes were analysed using gel electrophoresis and the optimum molar ratio of QD-DNA was used against the major (γ) family of mouse satellite DNA in both interphase and metaphase preparations. In addition they also used oligonucleotides labelled with different coloured QDs to target two classes of repetitive DNA in the centromeric region. Their results showed that QD–based probes are more efficient at hybridisation than organic fluorochromes and have great potential in multicolour assays. Furthermore, Jiang et al. (2007) generated QD-genomic DNA probes to visualise gene amplification in lung cancer cells, while most recent study involving direct labelling of maize chromosomes was published by Ma et al. (2008) in which QDs were solubilised with an MAA (mercaptoacetic acid) monolayer and then a thiol-DNA to create probes. Apparently, with this method, the probes were small enough to hybridise with the DNA sequences. This study also highlights the problem of steric hindrance regarding QDs and that pH (Xiao et al. 2005), ionic strength and formamide (FA) could affect the affinity of QD-probes to chromosomal targets (Ma et al. 2008)

Given the potential of QD-FISH, it is puzzling how few studies (notwithstanding the above) there are in this area. Clearly more studies are required to explore the use of QD–FISH. For instance we are aware of no published data using QD–labelled probes to target whole chromosomes (chromosome painting) either in two dimensions or in 3D nuclear organisation studies. The overall aim of this study was to therefore to explore the use QDs in the place of organic fluorochromes, specifically with a view to using QDs in multiplex experiments (i.e. to target multiple regions simultaneously).

The specific aims of the current study were thus as follows: a) to ask whether streptavidin-QD conjugates could be used for the detection of biotinylated (or digoxigenin) labelled probes in "indirect" FISH labelling experiments under a range of conditions; and b) to develop strategies for the direct coupling of QDs to biotinylated probes (including oligonucleotides and chromosome paints) for use in "direct" FISH experiments (with the ultimate goal of performing multiplex experiments).

Materials and Methods

Biological material

Lymphocytes from peripheral blood cultures and sperm from freshly ejaculated semen samples formed the basis of target material for most of the experiments. Both cell types were obtained after written consent from a chromosomally normal male donor. Research was approved by the Research Ethics Committees of the University of Kent and carried out under the auspices of the treatment licence awarded the Human Fertilisation and Embryology Authority (HFEA). Whole blood was cultured in "Karyomax" medium (BRL) arrested in metaphase using colcemid (D1925 – Sigma) then swelled and fixed to glass slides using 75mM KCl and three changes of 3:1 methanol/acetic acid. Fresh ejaculate was washed in 10mM NaCl/10mM Tris pH 7.0 sperm wash buffer and then centrifuged for 7 minutes at 1900rpm. The supernatant was removed and resuspended up to 5 times depending on the pellet size and colour. The sample was then fixed in a drop-wise fashion using 3:1 methanol acetic acid to final volume of 5ml. The process was repeated up to 5 times (pellet dependent) and 5 to 20µl of the sample was spread on a Poly-L-lysine coated slide (631-0107 -VWR) (for better fixation of cells) and air dried at room temperature (RT). In addition cultured embryonic fibroblasts from chicken and turkey were utilised, cells were suspended in metaphase using colcemid, trypsinised, swelled and fixed for cytogenetic analysis by standard protocols. For all experiments performed with avian samples or human lymphocytes superfrost glass slides (AG00008232E - Menzel Glaser) were utilised.

QD-streptavidin conjugates

Two suppliers were used for these experiments, Invitrogen (QD525 and QD585) and Evident (QD520, QD600 and QD620).

Source of probes

In early experiments, a commercially available pancentromeric probe (Cambio - 1695-B-02) was utilised, as were bacterial artificial chromosomes (BACs) from chicken labelled with biotin by nick translation. Also, in-house chromosome paints were generated from flow-sorted human and chicken chromosomes (a kind gift from Department of Pathology, University of Cambridge). The degenerate primer 6MW (5'→3' CCG ACT CGA G NNN NNN ATG TGG) was used in a standard DOP-PCR experiment to generate sufficient material which was then labelled with biotin or digoxigenin via nick translation and used in indirect FISH experiments. A custom made DOP-PCR primer labelled with biotin (through a C6 linker – Invitrogen, personal communication.) was used to generate DOP-PCR products with a single biotin on each length of DNA for direct QD conjugation experiments (Invitrogen). In addition, for direct labelling experiments (and for indirect FISH), an oligonucleotide probe specific for a region on chromosome 12 with a single biotin molecule attached to the 5' end was used. The biotin was incorporated during synthesis through biotin phoshoramidite by linking the 5' OH to the phosphorus atom (Sigma Genosys, personal communication)

The following protocol (Bentolila and Weiss 2006) was used to couple streptavidin conjugated QDs to biotinylated oligonucleotides and chromosome paints labelled with a single biotin molecule. Direct coupling requires probes to have a single biotin (per primer binding site) to prevent QD aggregation and therefore unspecific signals. PCR products were purified using a QIAquick spin column (Qiagen) following the manufacturer's instructions. QD:DNA constructs (i.e. FISH probes labelled with QDs) were made by mixing 1µl of 500nM QD with 1µl of 50ng/µl biotinylated probe. These were gently vortexed for 5 seconds, allowed to incubate at room temperature for a minimum of 30 minutes and stored on ice until ready for use. The QD:DNA construct was purified (from unbound probe) using S300 columns (Amersham Microspin S-300 HR) following the manufacturer's instructions. In order to establish that the QD-DNA complex still had fluorescent activity, the tube was checked for fluorescence under a UV transilluminator. To test for QD:DNA construct formation standard 2% agarose gel electrophoresis was used under the premise that "naked" DNA has greater mobility that QD conjugated DNA, and than QD alone.

For all experiments, 100-200ng/µl of probe was dissolved in standard hybridisation buffer (50% formamide (20% for oligonulceotide probe), 2 X SSC, 10% dextran sulphate, 60-200µg of salmon sperm DNA). For direct FISH experiments, formamide was reduced to 25%, dextran sulphate was removed, and 5x Denhardt's solution together with 50mM Phosphate Buffer, 1mM EDTA were included). For the commercial pancentromeric probe, the manufacturer's standard hybridisation buffer was used and the probe denatured at 85°C prior to use as per the manufacturer's guidelines.

FISH

Slides containing metaphase preparations were dehydrated in an ethanol series, air dried and treated with 100µg/ml RNase under a coverslip (Menzel – Glaser) at 37°C for 1 hour, then washed twice in 2xSSC for 5 minutes each, before a second ethanol series and air drying. Slides bearing sperm preparations were washed in 0.1%DTT 0.1% Tris-HCl (pH 8.0) at room temperature for 20-30 minutes to swell the sperm heads and then rinsed in 2xSSC. This was followed by pepsin treatment in a pre-warmed at 39°C coplin jar with 49ml of ddH2O, 0.5ml of 1N HCl, 0.5ml of 1% pepsin for 20minutes. Slides were subsequently washed in ddH2O followed by rinsing in 1xPBS before incubation in 4% paraformaldehyde/PBS (pH 7.0) at 4°C for 10minutes slides were then rinsed with 1xPBS followed by ddH2O at room temperature and another ethanol series was carried out at RT for 2 minutes each and slides were air dried.

The cells were then denatured at 70°C in 70% formamide/2 X SSC (pH 7.0) for 2 minutes (8-10 minutes for sperm) before washing with 70% ice cold ethanol for 2 minutes followed by 80 and 100% ethanol for 2 minutes each prior to air drying.

Labelled probe in hybridisation buffer (10μl) was denatured at 65-85°C for 1-10 minutes then added to a specified marked area under a 18x18mm coverslip which was sealed with rubber cement and hybridized at 37°C overnight. For direct labelling experiments the slides were heated at 80°C for 3 minutes to prevent any reannealing of the DNA strand after denaturation. The rubber cement was removed and slides washed in 2xSSC to remove the coverslips. Slides were then washed in 37°C 50% formamide/2xSSC solution for 20 minutes (2x5 minutes in 20% formamide/2xSSC solution at 37°C for oligonulceotide probes), then for 1 minute in 2xSSC 0.1% Igepal (v/v) at RT. For indirect FISH, slides were incubated in storage buffer [4XSSC 0.05% Igepal (v/v)] for 15 minutes, then in blocking buffer [4xSSC 0.05% Igepal (v/v), 3% BSA (w/v)] for 25 minutes at RT. The detection mix (QD conjugated streptavidin for experiments and Cy3 conjugated streptavidin for controls) was prepared at 4°C for 20-25 minutes before use, centrifuged at 1300rpm for 5 minutes then applied to the slide under coverslip and incubated for 35 minutes at 37°C. For QD conjugates the detection mix consisted of 1μl of QD in 99μl of TNB buffer (pH: 7.5), [0.1M Tris-HCl, 0.15M NaCl, 0.5% BSA (w/v)] per slide; for controls, the detection mix was Cy3 streptavidin in blocking buffer diluted 1:200. The coverslip was then removed and slides washed in fresh storage buffer (in the dark) for 10 minutes, followed by a brief rinse with ddH2O. Slides were then air-dried and counterstained using Vectashield with DAPI (Vector labs). Direct FISH experiments had post-hybridisation washes of 2x10 minutes in TST buffer [0.1M Tris, 0.15M NaCl, 0.05% Tween 20 (v/v), 2xSSC pH 7] at 37°C then proceeded straight to the ddH2O stage following post-hybridisation washes.

Variations to protocol

In order to improve the efficacy and reliability of the QD experiments various FISH conditions were altered, including removal of the block buffer step, changing the temperature and time of the post-hybridisation washes.

In order to test the hypothesis that the presence or absence of dextran sulphate in the hybridisation mix affected subsequent binding of QD conjugates in indirect FISH experiments (the direct QD FISH hybridisation mix did not contain dextran sulphate) controlled experiments with and without dextran sulphate in the hybridisation mix were performed.

To minimise steric hindrance of the biotin, biotin-21-dUTP was used in place of biotin-16-dUTP in both direct and indirect experiments. Also the effect of different ratios of biotin labelled and unlabelled probes were assessed to minimise steric hindrance.

To determine whether there was a hapten-specific effect (i.e. whether biotin *per se*, was the best hapten to use) we attempted to detect digoxigenin labelled probes with mouse anti-digoxigenin antibody followed by a layer of QD-conjugated goat anti-mouse antibody.

In order to test the hypothesis that QD conjugates were aggregating and adhering to the sides of the tube, we performed controlled experiments sonicating the conjugates before use and using siliconised tubes and pipette tips.

To test the hypothesis that use of DAPI as a counterstain could affect visualisation of the QDs, experiments were performed with and without DAPI.

Results

Indirect labelling

Use of streptavidin conjugated QD525 and QD585 produced a degree of success in generating analysable preparations for FISH experiments. Figures 1-6 demonstrate successful experiments (some compared to Cy3 controls). We were successful in hybridising chromosome paints from both human and birds to metaphases and interphases of the same species (Figures 1-4), BAC clones for chicken chromosomes successfully hybridised (Figure 5) and the oligonucleotide sequence specific for chromosome 12 gave a reproducible signal (Figure 6).

By and large, when results were successful, the properties of QDs were apparent. Most notably the preparations were significantly brighter by visual inspection than Cy3 preparations and were resistant to photo-bleaching. That is, when Cy3 labelled preparations were exposed continually to the light, photo-bleaching occurred after about 5 minutes. On the other hand, when QD preparations were exposed to the light, no appreciable loss of signal was seen after one hour of exposure.

We also observed that preparations displayed the phenomenon known as "blinking," that is, when samples were visualised the fluorescent signal repeatedly appeared to switch "on and off". In general terms QD preparations in these experiments had more background than was observed for Cy3 preparations. Also there was a notable difference in the appearance in the fluorescent signal from QD compared to Cy3 which is perhaps best explained with an analogy: That is Cy3 signals gave the impression of examining fluorescent "dust" compared the fluorescent "rocks" impression given by the QDs. It was noticeable that, in many chromosome painting experiments, the QD signal was brighter around the periphery of the chromosome giving the impression of a fluorescent "sheath" (Figure 3), moreover, in selected cases, a bright signal was visible in the interphases of the cell but not the metaphases. Another point of note was that the emission spectra of the QDs did not appear to be as narrow as the manufacturer's claimed. That is, despite the use of narrow band-pass filters, both QD525 and QD585 showed a significant "bleed-through" into the channel of the other. Most importantly however it was noticeable that, while the Cy3 controls worked successfully with rare exceptions, success from equivalent QD experiments was notably intermittent. In particular identical QD experiments could often be perfectly successful on one day but unsuccessful on the next or, even more confusingly, identical experiments run in parallel would work for one slide but not the other on a regular basis. As an overall estimate, indirect QD experiments were successful 25-35% of the time when controls gave an acceptable result (>95%).

In general terms, amidst this background of intermittent success, we were unable to identify any particular factor that would improve the success of the experiments. Controlled studies varying hybridisation times and temperatures did not especially favour QD experiments on any occasion. There was no appreciable difference whether or not the blocking buffer and/or dextran sulphate in the hybridisation mix and/or DAPI in the mountant was utilised. We did observe good signals through the use of biotin-21-dUTP however, this was, at least by visual inspection by a number of observers, not noticeably different from the use of biotin-16-dUTP, nor did our efforts to vary the relative concentrations of labelled versus unlabelled probes lead us to draw firm conclusions. The only intervention that we observed to demonstrate a degree of success was the use of silicon coated eppendorf tubes and sonication of the conjugate prior to use. In both scenarios we observed an (albeit temporary) improvement in the reliability of the results.

Direct FISH

Efforts to conjugate streptavidin-QDs to biotinylated DNA were initially encouraging. Figure 7 demonstrates a noticeable shift in the mobility of the DNA-QD construct compared to either biotinylated DNA alone or streptavidin QD alone. These results were reproduced on approximately 20 occasions for both the oligonucleotide chromosome 12 probe and the chromosome paints however repeated attempts at subsequent FISH experiments (employing a range of different conditions of stringency, hybridisation buffer etc.) without exception ended in failure (despite known Cy3 conjugate controls working reliably).

Finally it is worth noting that records from all QDs purchased were kept and results were only obtained through the use of Invitrogen samples (Lot:48184A – for QD585). In contrast there were no results through the use of Evident samples.

Discussion

To the best of our knowledge, this is the first study to demonstrate a comprehensive appraisal of the utility of QDs for FISH experimentation. That is, while several studies have demonstrated the use of QDs in FISH, as with the majority of studies in the literature, there may be a tendency to present only the positive data. QD-based FISH studies are conspicuous mostly by their absence (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008), that is, if QDs had fulfilled their promise they would have, at least in part, replaced organic fluorochromes; one would expect orders of magnitude more QD-FISH papers in the literature and several companies marketing QD labelled probes which, at the time of writing, is simply not the case.

While we would not claim that we have explored every possible avenue with respect to QD-FISH, we have extensive experience in FISH over many years and have, for the last three or four of them, been running parallel QD-based experiments, mostly in avian and human cells. Put simply, lack of reproducibility appears to be the hallmark of QD-FISH in contrast to the more robust applications with antibody conjugates for cell labelling. This is possibly because of incomplete technical knowledge of the factors associated with QD probe penetration into a complex structure such as a chromosome or nucleus. Furthermore, in commercially available QD-streptavidin conjugates we are yet to understand many chemical and physical factors are well-understood for organic fluorophore (e.g. FITC, Texas Red and the Cy dyes) conjugates. For these reasons we conclude that, for indirect FISH, QD conjugated streptavidin (at least in its current form) is an unsuitable material compared to equivalent Cy3 conjugates. For direct labelling, despite recruiting the services of leading proponents involved in QD conjugation (Bentolila, personal communication), we were unsuccessful in generating a single successful FISH preparation by this means. It seems reasonable to suggest that, had we continued our attempts, we would eventually have met with a degree of success however, given the intermittent success of the simpler indirect approach, we are not confident that the experiments would have been reliable. In addition, we have gone to the lengths of canvassing like-minded groups who would benefit from the use of QDs and organised symposia to share knowledge and experience. Without exception, the message we have received from our colleagues is a similar experience to our own. In addition, recent studies (Bruchez 2007) also hint at the unreproducible nature of QDs for FISH and stress the need for tailored protocols established by empirical means. If this were achieved then the reliability may well improve and the benefits of QDs observed in this and other studies (e.g. increased brightness, resistance to photobleaching) may be properly realised.

It is of course appropriate to speculate as to why QDs lack reproducibility in for FISH applications. One possible explanation is their size. QDs vary in size (this is the basis of the fluorescent colour that they emit) from 2 to 10nm. A Cy3 molecule on the other hand is <2nm in size (Bailey et al. 2004). This may explain in part, why our successful FISH experiments gave the impression of larger fluorescent particles and why there was a greater degree of background for most experiments. It might also explain the fluorescent "sheath" effect seen on some metaphases (Figure 3) and why certain preparations were successful at interphase but not metaphase (Figure 4). That is steric hindrance may have led to signals being brighter in areas where the chromatin is more compact (e.g. at the edge of the chromosomes and/or in the interphase nucleus). If this were the case however, we might have expected to see an improvement when we reduced the ratio of labelled to unlabelled dUTPs and/or when we made use of a "longer-arm" biotin dUTP; however we did not. Again a general background of intermittent success may have masked any appreciable difference seen in any given experiment. The steric hindrance problem was reported also by Muller et al. (2006) in their attempts to use streptavidin conjugated QDs to target plant chromosomes.

It is not entirely clear how streptavidin is bound to on the polymer site of the QD, the number of free streptavidin sites per QD varies from 10 to 15 and are prone to de-conjugation for reasons not completely understood (Bentolila, personal communication). We are also aware that QD streptavidin conjugates can be prone to degradation (a batch-specific attribute) and this can correlate with even subtle changes in temperature during storage. Additionally we are given to understand that QDs are prone to adhere to tubes sides and tips (Chan, personal communication). Our attempts to reduce this problem using siliconised tubes and regular sonication met with a degree of success (confirming this theory in part) however, did not completely eliminate our technical problems.

A further complicating factor was that the emission spectrum of the QDs used appeared to be not as narrow as the manufacturers claimed, in that, we observed "bleed-through" from red to green channels and *vice-versa*, despite using narrow band-pass filters. Anecdotal evidence suggests that this phenomenon is not uncommon (Bentolila, personal communication) and could vary from batch to batch. As we understand it controlling the size of the core during synthesis (that will determine the colour that the QD will emit) is an imperfect process and can lead to QDs being smaller or larger than expected. Moreover abnormalities in QD shape (failure of quality control) could result in the same effect (Bentolila L, personal communication). Such a phenomenon can potentially lead to a mixed population of QDs in any given batch. These findings are consistent with Bawendi and colleagues who have tried to address monodispersity of QD preparations (Murray et al. 2000). Supplementary figure S1 illustrates this phenomenon in that the different colours seen represent individual QDs that emit at longer (towards the red – large QDs) or shorter (towards the blue – small QDs) wavelengths. All these technical features that were attributed to the chemical synthesis of the QDs possibly require more experimental attention in order to improve QD synthesis.

Another observed QD feature that was observed was "blinking" which is not seen in conventional FISH (as shown in supplementary movie, S2). Blinking is a phenomenon where the QD alternates between an emitting (on) and non-emitting (off) state (Michler et al. 2000; Pinaud et al. 2006). This behaviour has been interpreted according to an Auger ionization model (Efros and Rosen 1997). Blinking affects single molecule detection applications by saturation of the signal, however one study suggests that this behaviour of the QD can be suppressed by passivating the QD surface with thiol groups (Hohng and Ha 2004). Photobrightening, where QD fluorescence intensity increases at the first stage of illumination and then stabilises, can impose limitations on quantitative studies (Gerion et al. 2001). Both of these properties are associated with mobile charges on the surface of the QDs (Fu et al. 2005). It is also noteworthy that, although preparations often displayed blinking, they could go to an irreversible photo-darkened state without easy explanation.

One possible explanation for the success of the groups that have published in this area therefore (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008) is that they possessed the facility to synthesise and batch-test their own streptavidin QD conjugates (something that we, in common with most groups, do not currently have). In other words they did not use commercially available streptavidin QDs. Ma et al. (2008) specifies that the QDs used were smaller than commercial ones and that could help avoid steric hindrance and confer hybridisation ability. Several authors (Xiao and Barker 2004a; Bentolila and Weiss 2006; Ma et al. 2008) used oligonucleotides to generate QD-DNA conjugates and highlight that, during the time of annealing of the QD-DNA probe to their target, steric hindrance has little effect but it may limit the QDs access to the target at the time of detection (Ma et al. 2008). This could also explain our negative results during direct FISH. A further consideration is the fact that QDs behave not as molecules but nanocolloids complicates their application in biological environments (Resch-Genger et al. 2008).

Taking all of the above into consideration, the future of QD-FISH requires further research and interaction within the interested groups. Advances in nanomaterial synthesis (regarding uniformity and size control) and solubility will assist conjugation to biomolecules. Yao et al. (2006) described a new generation of nanocrystals called "FloDots". These are dye-doped silica nanoparticles that possess all QD optical properties but, due to the silica matrix that encompassed the dots, it is easier to make them water soluble and, according to the authors, the silica surface could be modified to contain functional groups for bio-conjugation. In addition, a study by Choi et al. (2007) introduces a novel class of nanocrystals, "C-dots" that could be 2-3 times brighter than QDs, less toxic and an ideal material for *in-vivo* applications and cancer studies. Time will tell whether these or novel nanocrystals will be used robustly in FISH applications.

Nanotechnology has the potential to revolutionise the use of FISH in a wide range of molecular cytogenetic applications including gene mapping, clinical diagnostics, comparative genomics and microarray. The ability to multiplex much more effectively with a single excitation wavelength with bright, narrowly emitting fluorochromes that do not fade is highly desirable. QD-FISH will, in time, probably be seen as a significant stepping-stone towards this goal. Nanotechnology quite possibly holds the key to future of molecular cytogenetics. That future however, is not yet with us.

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Figures

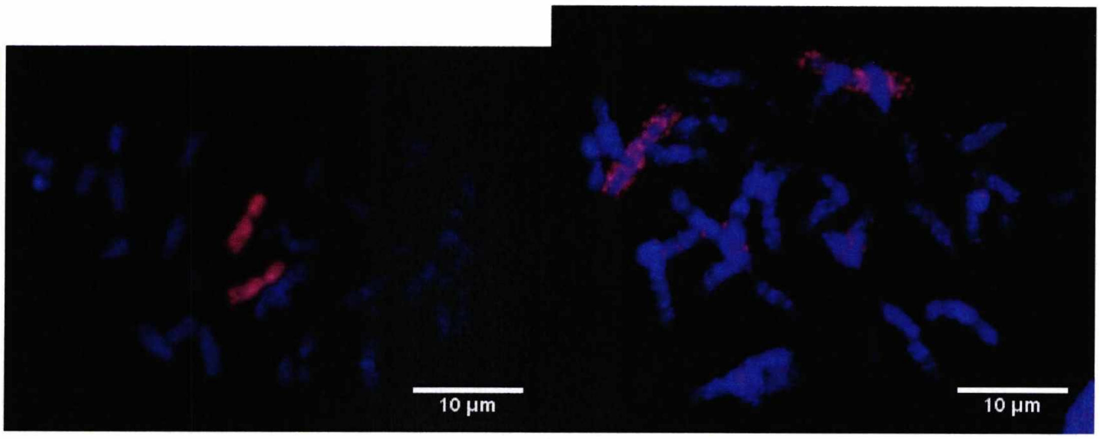

Figure 1: Detection of biotinylated human chromosome paint 2 with a) Cy3 conjugated streptavidin; b) QD585 conjugated streptavidin. The Cy3 labelled probe gives a more specific signal with less background.

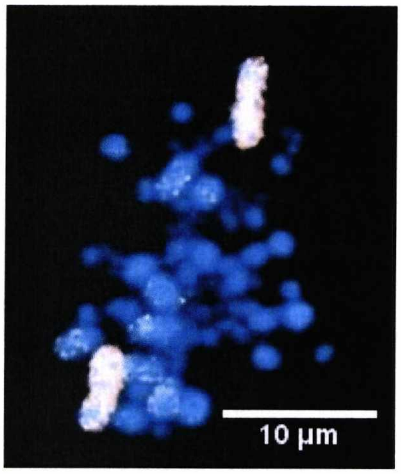

Figure 2: FISH of turkey chromosome 1 paint to turkey chromosomes using QD525 conjugated streptavidin

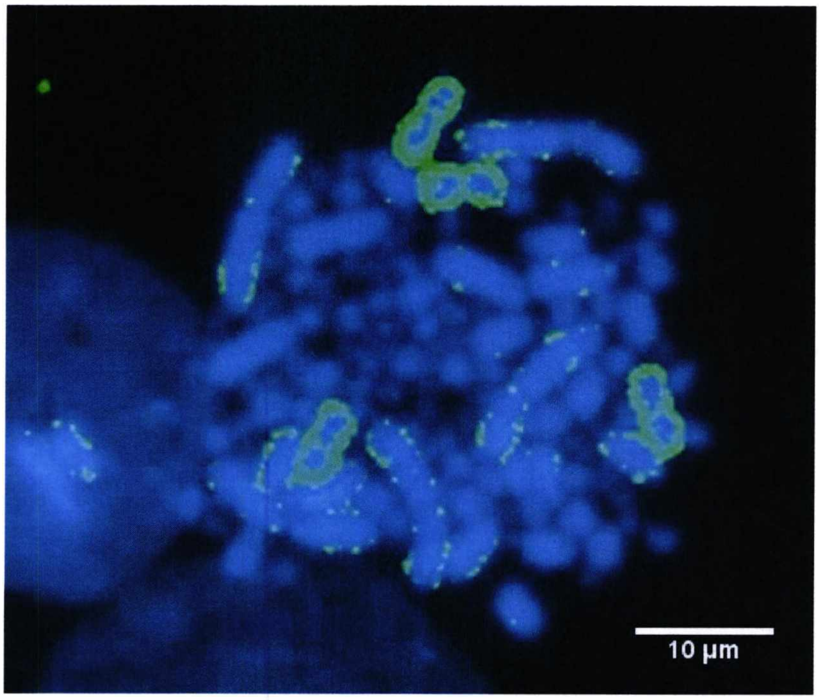

Figure 3: FISH of chicken chromosome 2 paint to a chicken tetraploid chicken metaphase using QD525 conjugated streptavidin. Hybridisation signals are brighter at the periphery of chicken chromosome 2 where the chromatin is less condensed.

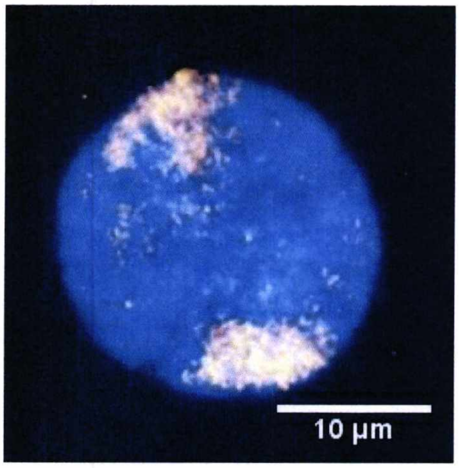

Figure 4: Turkey nucleus showing hybridisation of turkey chromosome 4 paint detected by QD525 conjugated streptavidin.

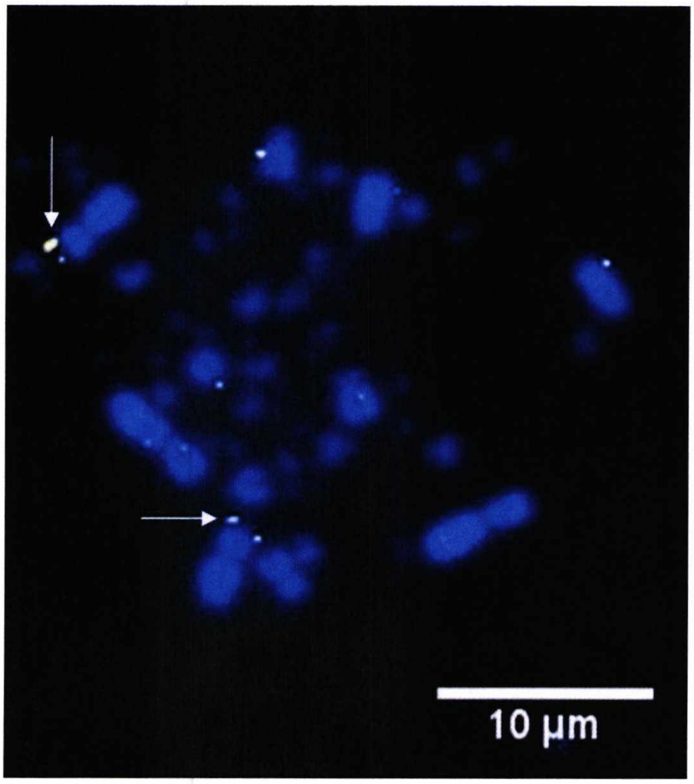

Figure 5: Hybridisation of a BAC probe to terminal chromosome 2p in chicken using QD525 conjugated streptavidin. Arrowheads indicate the specific hybridisation sites (2p).

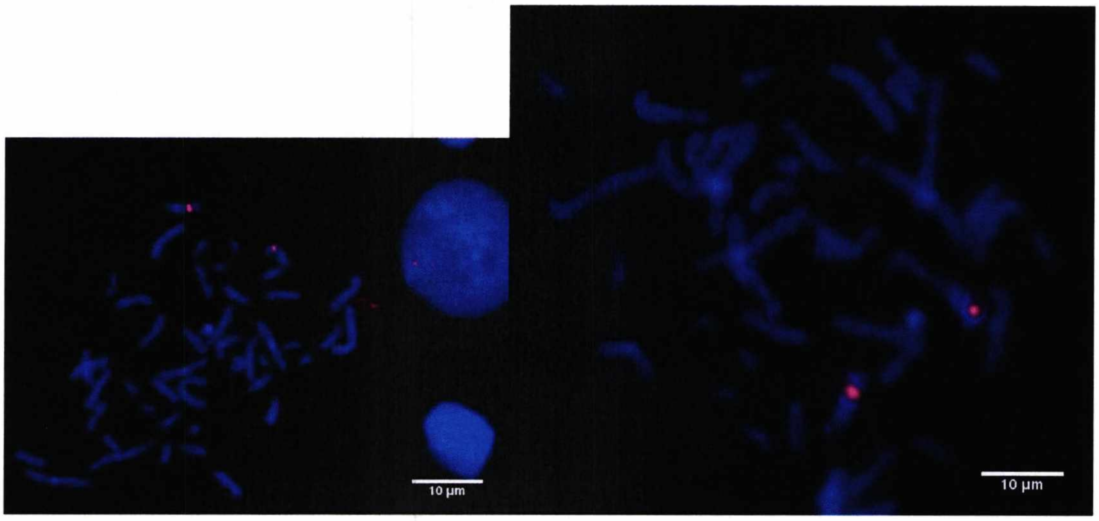

Figure 6: FISH hybridisation of an oligonucleotide probe for the centromere of human chromosome 12 on human metaphases detected by a) QD585 conjugated streptavidin; b) Cy3 conjugated streptavidin

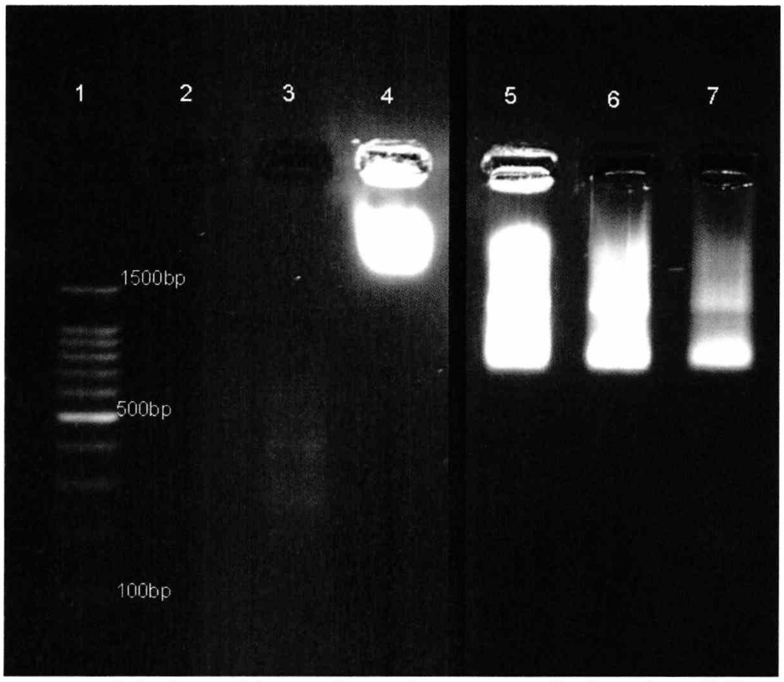

Figure 7: Agarose gel (selected lanes from the same gel) showing differential motility of amplified biotinylated DNA (lane 3), QD alone (lane 4), and QD:DNA construct at varying concentrations (lanes 5-7). The differential motility seen in lanes 5-7 indicates that the construct was successfully generated. Lane 1 is a 100bp ladder and lane 2 is blank.

S1: QD585 dissolved in hybridisation mix and viewed directly under the microscope using 4 barrier filters i.e. 525nm (blue), 565nm (green), 585nm (red) and 605nm (far red but pseudo-coloured purple for the purposes of this figure). The image is a merge of all four filters, the QDs are predominantly red (as would be expected) however a smaller number of green, blue and purple QDs are seen. The discrete appearance of QDs of one or other of the colours indicates there is a mixed population of QDs in each preparation.

S2: Movie of a) QD585 and b) QD605 dissolved in hybridisation mix and viewed directly under the microscope using 585nm and 605nm barrier filters respectively. The phenomenon of "blinking" is clearly seen.

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An appraisal of nuclear organisation in interphase embryonic fibroblasts of chicken, turkey and duck

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Key words: chromosome positioning, genome, chicken, turkey, duck

Abstract

Determining the nuclear "addresses" of chromosome territories is a well-documented means of assaying for nuclear organisation in many cell types and species. Data in avian species are however limited at best, despite the pivotal role played by birds (particularly chickens) in agriculture, and as model organisms in developmental biology. That is studies have hitherto focussed mostly on mammals (especially humans) and have demonstrated the importance of chromosome territory positioning in embryology, disease and evolution. Thus a detailed study of nuclear organisation in many species, many cell types and many developmental stages in birds is warranted, however, before this is achieved, "baseline" needs to be established to determine precisely the relative locations of chromosome territories in at least one cell type of at least one bird. With this in mind we hybridised FISH probes from chicken chromosomes 1-28 to embryonic fibroblast nuclei, determining nuclear addresses using a newly developed Plugin to the image analysis package ImageJ. In our experience, evenly spaced representative BAC clones yielded more consistent results than hybridisation of chromosome paints. Results suggested that chromosome territory distribution best fitted a chromosome size-based (rather than gene density-based) pattern. Identical BAC clones were then hybridised to turkey and duck in a comparative genomic strategy. Observations were consistent with those seen in chicken (although, less well-defined in duck) providing preliminary evidence of conservation throughout evolution.

Introduction

The organisation of the interphase nucleus in three-dimensional space is crucial for cellular function. Variation in organisational patterns and changes over time have been correlated with specific phenotypes, diseases and developmental stages (Foster and Bridger, 2005; Cremer, 2006). Whole chromosomes have long been demonstrated to occupy distinct addresses in the interphase nucleus, termed chromosome territories (reviewed by Cremer et al., 2006). Indeed it has been suggested that determination of the relative nuclear positions of chromosome territories is a useful assay for determining nuclear organisation (Foster and Bridger, 2005). There are several models proposed to describe how chromosomes are organised in the interphase nucleus, however two predominate: First, in a "gene density based" organisation, the gene poor chromosomes are found towards the periphery and the gene rich chromosomes are more interior. Human lymphocytes for example follow a gene density related organisational pattern (Croft et al., 1999). In a second model, a chromosome size based distribution, the largest chromosomes are found toward the periphery of the nucleus and the smaller chromosomes are more interior. Such organisation has been reported for quiescent human fibroblasts (Bolzer et al., 2005). Beyond these global models of nuclear organisation, considerable variation is known to exist between cell types and between healthy and diseased cells of the same cell type. There are also several examples of chromosome position changing during cell differentiation and/or disease. For instance, repositioning of the X chromosome has been seen in neurons in epilepsy sufferers (Borden and Manuelidis, 1988), the repositioning of the sex chromosomes and the centromeres of the autosomes towards the nuclear centre has been reported in mammalian spermatogenesis (Foster et al., 2005; Turner et al., 2006; Zalensky and Zalenskaya, 2007), and rearrangement from a "chromocentric" (i.e. centromeres in the middle) to a "telocentric" (i.e. telomeres in the middle) organisation has been reported in murine eye development (Solovei et al., 2007).

To date, most studies of nuclear organisation in vertebrates have focused on humans and other mammals e.g. primates (Neusser et al., 2007, Tanabe et al., 2002; 2005, Mora et al., 2006; Zalensky and Zalenskaya, 2007), pigs (Foster et al., 2005) and mice (Mayer et al., 2005; Turner et al., 2006). Far less is known in comparison about nuclear organisation in birds and information is restricted to a small number of studies in the chicken. These studies demonstrate that, in embryonic fibroblasts and neurons, the larger chromosomes tend to be more peripheral while the microchromosomes are more internal, but suggest that some hitherto unidentified microchromosomes exist at the nuclear periphery (Habermann et al., 2001). Studies of chicken proerythroblasts, myeloblasts and macrophages have shown that chromosome position is cell type specific, and that gene loci may change position during differentiation (Stadler et al., 2004). Studies of chicken sperm heads indicate that most chromosomes do not appear to adopt a non-random position as they do in somatic cells (Solovei et al., 1998; Tsend-Ayush et al., 2008). This is in stark contrast with the situation in the sperm of monotremes, marsupials and placental mammals, where nuclear organisation seems to be more ordered (e.g. Greaves et al., 2001, 2003; Zalensky and Zalenskaya, 2004).

Taking all available studies into account therefore, our knowledge of individual chromosome territory positions in somatic cells of birds is very limited, and, at best, restricted to chicken chromosomes 1-5 and Z. That is, the positions of the remaining chromosome territories have thus far been studied using only pooled chromosome paints revealing merely a general trend, and not the individual nuclear positions of all or most of the chromosomes (Habermann et al., 2001). In some ways, this paucity of knowledge is surprising given that chicken is a crucial and well-established model for medically important traits, and in developmental biology. Based on current evidence it seems reasonable to suggest that changes in nuclear organisation might well correlate with specific developmental stages, and that this may reflect a general relationship between nuclear organisation and embryology. The well-described embryonic phenotypes and "fragmented" genome of chicken make it ideal to study this phenomenon. It is feasible that differences in nuclear organisation may correlate to specific phenotypes or breeds; given the economic importance of chickens (and other birds such as turkey, duck, quail, pheasant, goose etc.) as a source of meat and eggs, this information may ultimately be applicable in agriculture. Finally, because of the small genome size and prevalence of microchromosomes, birds are a fascinating class of organisms for the study of genome organisation and evolution in general.

For all the above reasons a series of studies of nuclear organisation in many cell types and developmental stages of chicken and other birds is warranted. Before any of this can be achieved however a detailed appraisal of the position of most or all individual chromosome territories in a least one cell type of at least one bird (i.e. a "baseline") needs to be established; this is the first aim of the current study. The obvious "baseline species" is the chicken (*Gallus gallus)* due to it being, at the time of writing, the only bird with both a fully sequenced genome and easily available BAC clones. As a "baseline cell type" we chose embryonic fibroblasts due to the ease in which material can be obtained and their frequent use as a material for cytogenetic investigations. A standardised method of analysis and a transferable set of reliable FISH probes are essential to allow inter-laboratory comparisons that will ultimately address many of the above questions; this is our second aim. Moreover, it is unclear whether the microchromosome nuclear positions correlate closely to their individual sizes, or whether they exist in a more flexible 'pool' towards the nuclear centre; in this paper we test this hypothesis. In addition, the chicken is often referred to as fitting "both models of nuclear organisation" (gene density related and size related) due to the correlation between chromosome size and gene density present in the avian genome. Therefore a detailed appraisal of chromosome territory position in comparison to physical size (which can be easily measured) and gene density (which can be inferred from size measurements and data from a genome browser) will, in theory, differentiate whether the chicken genome (at least in one cell type) best-fits one or other of the models. Here, we perform studies aimed to differentiate these two models. Finally, it is appropriate to initiate what could ultimately be a large number of studies aimed at establishing whether nuclear organisation patterns are conserved in avian species generally, as they appear to be in primates. The obvious first targets for such studies are the turkey (*Meleagris gallopavo*, MGA) and the duck (*Anas platyrhynchos*, APL) because of: a) their agricultural importance; b) the availability of information on detailed comparative cytogenetic maps (and thus comparative karyotypic differences) compared to chicken; and c) prior knowledge that cross hybridisation of chicken probes is technically feasible to the interphase nuclei of these birds (Griffin et al, 2008, Fillon et al, 2007, Skinner et al, in submission). With this in mind we tested the hypothesis that the global patterns of nuclear organisation seen in chicken are conserved in turkey and duck by making use of a comparative genomic (zoo-FISH) strategy.

Materials and Methods

Cell culture and nuclear preparation

All chromosome preparations were made from cultured cells derived from fertilised eggs. Chicken eggs were supplied by Hill Top Farm, Cambridgeshire, UK and Friday's Farm, Kent, UK. Duck eggs were provided by Cherry Valley Ltd, Market Rasen, UK. Turkey eggs were supplied by British United Turkeys, Chester, UK. Fibroblast cultures were established from 5- to 7-day-old embryos. Chromosome preparation using mitostatic treatment with colcemid for 40 minutes at 37ºC, hypotonic treatment with 75mM KCl for 15 minutes at 37ºC and fixation with 3:1 methanol:acetic acid followed standard protocols (Ahlroth et al., 2000, Griffin et al., 1999). Although experimental data was gained exclusively on interphase nuclei, metaphase preparations were essential to confirm the genomic location of the probes. For nuclear organisation studies, one cell suspension from one embryo per species was used.

Preparation of BAC clones

To achieve the aims set out in the introduction we identified and generated panels of chicken BAC clones, each representing individual chromosome territories for chromosomes 1-28. Clones were selected from the Wageningen chicken BAC library (Crooijmans et al., 2000), and isolated using a Qiagen plasmid midi kit (#12143) according to the manufacturer's instructions. BACs were selected that are known to hybridise to both turkey and duck from the comparative cytogenetic maps of Griffin et al. (2008) and Skinner et al. (in submission). At least one BAC was available for GGA1-28 (except 25) and several BACs were used in increasing number correlating with the size of the chromosome (Table S1). No BACs exist for the smallest microchromosomes and chromosome paints generated in our previous studies (Masabanda et al., 2004) have long degraded. Insufficient BACs were available for GGAZ and W and thus we used chromosome paints for these chromosomes. The BACs were labelled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) following standard protocols. These clones are available to the scientific community on a "cost recovery only" basis by contacting the BBSRC avian chromosome resource centre laboratory housed within our laboratory (www.farmachrom.net).

Fluorescent in-situ hybridisation (FISH)

In order to perform direct (chicken) and comparative genomic (turkey and duck) experiments, labelled BAC clones were hybridised to interphase nuclei and metaphase chromosomes of embryonic fibroblast cells. Slides were aged for 1 hour at 70°C on a hotplate then treated with 100µg/ml RNase A for 1 hour at 37°C. The chromosomes were denatured for 1 minute 30 seconds in 70% formamide in 2xSSC at 70°C. BACs were applied and sealed under coverslips. Hybridization was carried out in a humidified chamber for 72 hours at 37°C. Following post-hybridization washes (40% or 30% formamide in 2xSSC for 20 minutes; 1 minute in 2xSSC/0.1% Igepal at RT; 15 minutes in 4xSSC/0.05% Tween 20 at RT; 25 minutes in 4xSSC/0.05% Tween 20/2% BSA at RT), probes were detected with 1:200 streptavidin-Cy3 (Amersham), plus 1:200 FITC-anti-digoxigenin (Amersham) for dual colour experiments, in 4xSSC, 0.05% Igepal, 1.25% BSA for 35 minutes. Slides were washed in 4xSSC, 0.05% Igepal for 3x 5 minutes then counterstained using Vectashield with DAPI counterstain (Vector Labs).

Chromosome size measurements and gene density estimation

We measured relative individual chromosome sizes by comparison to the smallest easily identifiable chromosome in the karyotype using previously published approaches (Morris et al., 2007) in chicken, turkey and duck, by comparing the labelled chromosome of interest to an easily identified small 'marker' chromosome (GGA8, APL5 and MGA7). Images were analysed using ImageJ (Abramoff et al., 2004). Chromosome areas were measured on metaphase spreads using the freeform selection tool.

Chromosome specific gene densities for chicken alone were calculated by reference to the Ensembl genome browser. Reported gene numbers for chicken chromosomes were taken from Ensembl (http://www.ensembl.org/Gallus_gallus/index.html; August 2006 gene build, chicken genome release 2.1) and divided by the measured chicken chromosome areas to generate gene densities. No such data exists for turkey or duck; hence the analysis of chromosome position in these species was based on chromosome size information only.

Analysis of chromosome territory positions

Here we present a hitherto unpublished plugin to the image analysis package ImageJ (Abramoff et al., 2004) designed to assess relative 3D nuclear position of FISH signals in flattened nuclei. That is, for these studies, a tailor-made program or "macro" was written for ImageJ that was based on previously published approaches (Croft et al., 1999) but with added functionality and interactivity. The program splits each image of a nucleus to separate RGB planes and then converts the blue image (representing the DAPI counterstain) to a binary mask from which concentric regions of interest (rings) of equal area are created (Figure 1). The proportion of signal in each channel within each ring is measured relative to the total signal for that channel within the area covered by the binary mask. Results are logged and output to a Microsoft Excel spreadsheet for analysis (e.g. Figure 2).

At least 50 nuclei were analysed for each BAC, providing a minimum of 100 signals per BAC. The raw data for all BACs on the same chromosome were pooled. The overall chromosome position and associated standard error were calculated from this pooled data.

The data were normalised against the DAPI intensity to compensate for flattening of the nucleus (Boyle et al., 2001). The overall percentage of signal within each shell was calculated and a χ^2 test was performed to test for a significant difference to a random distribution. A random distribution was assumed to be an equal proportion of normalised signal in each ring i.e. 20% in each shell.

Spearman's rank (non-parametric) correlation coefficients were calculated to test for relationships between chromosome sizes, gene densities (available for chicken only) and chromosome positions.

Results and Discussion

Chromosome size and gene density in chicken

Physical chromosome sizes are not always accurately reflected by the amount of sequence assigned to chromosomes in published genome sequences due to an underrepresentation of repeats. For example, cytogenetic observations show that GGA8 and GGAW have approximately similar sizes (Ladjali-Mohammedi et al., 1999); however, according to Ensembl, GGA8 contains 30,671,729 bp, while GGA8 is reported to contain merely 259,642 bp. In order to obtain more accurate estimates of gene density, calculated gene densities for each chromosome based on measured chromosome size and reported gene content from the Ensembl database. A strong inverse correlation was seen between chromosome size and gene density (Spearman's rank, $\rho= -0.63$, $n=29$, $p=0.0006$), which agrees with previous findings based on the analysis of the chicken genome sequence (Hillier et al., 2004).

Two outlying chromosomes were identified, GGA16 and 22; the gene densities for these two chromosomes are much lower than the gene densities for other chromosomes of similar size (Table 1). The deviation of GGA16 from the general correlation may be partially explained by the fact that this chromosome contains the nucleolar organiser region (NOR) (Masabanda et al., 2004). The NOR has been shown to contain between 80 and 400 tandem repeats of rRNA genes depending on the chicken breed (Delany and Krupkin, 1999). These genes are not represented in the Ensembl gene-build for GGA16. GGA16 also contains the MHC region in chicken (Dominguez-Steglich et al., 1991), another rich source of repeats. Consequently the current Ensembl gene-build for GGA16 almost certainly underestimates the number of genes on this chromosome. GGA22 has a lower gene density than its size would suggest (or a smaller size than its gene density would suggest), and thus was a useful discriminator for studies aimed to differentiate size-based from gene density-based models. While we cannot rule out the possibility of incomplete sequence assignment in the current Ensembl gene-build, in the absence of other evidence, we worked on the assumption that GGA22 is a small, relatively gene-poor chromosome.

Nuclear organisation in chicken:

Initial experiments with chromosome paints were quickly superseded by the use of relatively evenly spaced representative BAC clones. That is, many of the chromosome territories for the larger chromosomes, while clearly located tight to the nuclear periphery, often, after analysis as described above, appeared predominantly in shell 2 (next to the periphery – results not shown). In our opinion and experience this was because of the extra "bulk" of the visualised signal as a direct result of the dextran-sulphate laden hybridised probe and the biotin-streptavidin-Cy3 complex meant that a large chromosome territory could never be consistently demonstrated to occupy the nuclear periphery by our analysis. A BAC-based strategy (pooling results from several BACs for each chromosome) on the other hand revealed much more reliable and reproducible results.

Eighty chicken BACs for chromosomes 1-28 (except 25 for which no working BACs could be isolated) were successfully hybridised to chicken fibroblast interphase nuclei. The overall nuclear addresses of each chicken chromosome was calculated by pooling the data from all the BACs for that chromosome (Figure 3A); the number of BACs per chromosome are shown in table S1 (supplementary material). Twenty-four of the 29 chromosomes examined showed a significant non-random distribution (χ^2 tests, df=4, $p<0.05$); those that did not were chromosomes GGA4 ($p=0.19$), GGA5 ($p=0.89$), GGA11 ($p=0.31$), GGA20 ($p=0.25$) and GGAW ($p=0.053$). Overall, smaller chromosomes tended to be located towards the nuclear centre, while larger chromosomes were located more towards the nuclear periphery (Figure 3A). This correlation between chromosome size and position was statistically significant (Spearman's rank, $\rho= -0.71$, n=24, $p=0.0006$).

These results confirm and extend findings of a previous study in proliferating chicken fibroblasts and neuronal cells (Habermann et al., 2001) that provided inferred chromosome positions by visual inspection alone for GGA1-5 and Z. Pooled chromosome paints for GGA1-5 and Z, for GGA6-10, and for 19 pairs of microchromosomes indicated, by visual inspection, that the largest chromosomes were found at the periphery of the nucleus, the intermediate sized chromosomes were slightly more internal, and the microchromosomes were predominantly internal, though with the possibility that some microchromosomes could adopt a peripheral position. However, there was no data to suggest whether the positions the microchromosomes adopt were correlated with their individual sizes. Here, we extend these analyses significantly by demonstrating that there is indeed a correlation between size and position across all the measured chromosomes that showed a non-random nuclear address (chromosomes GGA1-3, 6-10, 12-19, 21-24, 26-28, Z). Notably, we did not observe any microchromosomes towards the periphery, in contrast to Habermann et al. (2001). This may be explained by the larger number of nuclei examined; where we used 50 nuclei per BAC, their analysis looked at 21 neuronal nuclei and 28 fibroblast nuclei, and was hence more susceptible to artefacts. The present study therefore extends the available chromosome territory positioning information to chicken chromosomes 1-28 (save 25).

The strong inverse correlation between chromosome size and gene density makes it challenging to determine if nuclear organisation in the chicken follows a chromosome size-based or a gene density-based pattern. Our data however appear to fit a size-based distribution better than a gene-density based distribution; the correlation between chromosome size and position is statistically significant (Spearman's rank, $\rho= -0.71$, n=24, $p=0.0006$), while the correlation between gene-density and chromosome position is not (Spearman's rank, $\rho=0.34$, n=24, $p=0.097$) (Figure 3B). However, given that the reported number of genes for GGA16 is almost certainly an underestimate (see above), it seems reasonable to remove this chromosome from the analysis, which improves the correlation substantially (Spearman's rank, $\rho= 0.55$, n=23, $p=0.009$) but still not to the level of the size-based distribution (Spearman's rank, $\rho= -0.70$, n=23, $p=0.001$). For the sake of example, if GGA22 is also removed from the analysis the correlation between chromosome position and gene-density becomes even more statistically significant (Spearman's rank, $\rho= 0.69$, n=22, $p=0.0016$) and equivalent to that between chromosome position and size (Spearman's rank, $\rho= -0.69$, n=22, $p=0.0016$). Therefore, the presently available data provide evidence favouring a chromosome size-based model of chromosome territory position over a gene-density based model with the caveat that a single chromosome is somewhat of a discriminator. Despite not having any available BACs for GGAW, in a separate set of experiments, we hybridised a chromosome paint for this chromosome to chicken embryonic fibroblast nuclei and results displayed a pattern not significantly different from a random distribution ($p=0.053$). Given that this chromosome is largely heterochromatic and very gene-poor, in a gene density-based distribution we would have expected a clearly observable location close to the nuclear periphery. As our results show however, apparently random distributions are not uncommon, even when the general trend is towards a size-based distribution. On the balance of probabilities therefore, we propose that, in chicken embryonic fibroblasts, a size-based nuclear organisation is favoured over one based on gene-density.

A size-based organisation is consistent with previous studies on human fibroblasts (Bolzer et al., 2005, Sun et al., 2000, Cremer and Cremer, 2001). Fibroblasts have elliptical nuclei; other cell types, such as lymphocytes, in which a gene density based organisation has been seen (Croft et al., 1999), have spherical nuclei. It is still unclear precisely what the link is between nuclear shape and chromosome positioning. One suggestion is that a size based organisation in elliptical nuclei may be a functional consequence of mitotic spindle formation; that is, space constraints force the arms of the larger chromosomes towards the nuclear periphery (Bolzer et al., 2005). This then posits a gene density based organisation as the default, disrupted in elliptical nuclei. In future studies we will examine the position of chromosome territories in lymphocytes and other cell types.

Nuclear organisation in turkey and duck:

Nuclear positions in turkey and duck were assayed as for chicken, though effects of background and non-specific hybridisation meant that fewer BACs provided reliable signals for interphase analysis. A total of 59 BACs were successful in turkey, covering 28 chromosomes (MGA1-19, 21-26, 28-30); 43 BACs were successful in duck, covering 22 chromosomes (APL1-12, 14, 15, 17, 22-28). The overall pattern of nuclear organisation observed in turkey and duck was similar to that seen in chicken, with smaller chromosomes located towards the nuclear centre and larger chromosomes oriented towards the nuclear periphery (Figures 3C, 3D). The correlation between chromosome size and position observed in chicken was also found to be statistically significant in turkey (Spearman's rank, $\rho = -0.88$, $n=21$, $p=0.0002$) and duck (Spearman's rank, $\rho = -0.50$, $n=20$, $p=0.023$). The observed decrease in p-values in turkey and particularly in duck compared to chicken was likely, in part, due to an increased level of non-specific background. This problem is expected to be more severe in distantly related species, which agrees with our data given that chicken and turkey diverged approximately 30 million years ago (Pereira and Baker, 2006), while chicken and duck diverged approximately 90 million years ago (van Tuinen and Hedges, 2001). This is also consistent with the fact that fewer chicken BACs gave analysable signals in duck than in turkey. Taken together, our findings suggest that nuclear organisation in birds follows a general pattern that is also similar to patterns observed in other organismic groups, providing further evidence for the hypothesis that the principles underlying nuclear organisation are evolutionarily highly conserved (Neusser et al., 2007).

Conclusions:

This study has determined chromosome sizes and nuclear organisation in three avian species in considerably more detail than previously reported. It provides some evidence for a size based chromosome organisation in elliptical fibroblast nuclei in birds, which has previously been described in mammalian nuclei only, and adds to the body of data suggesting that the principles governing nuclear organisation are conserved among vertebrate species. Moreover, the approaches described here provide the tools and technologies for future nuclear organisation studies in addressing this fascinating area of genomics, specifically for avian species. Such studies will eventually determine whether or not nuclear organisation is a major player in embryology, disease phenotype, agriculture and genome evolution.

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Figures

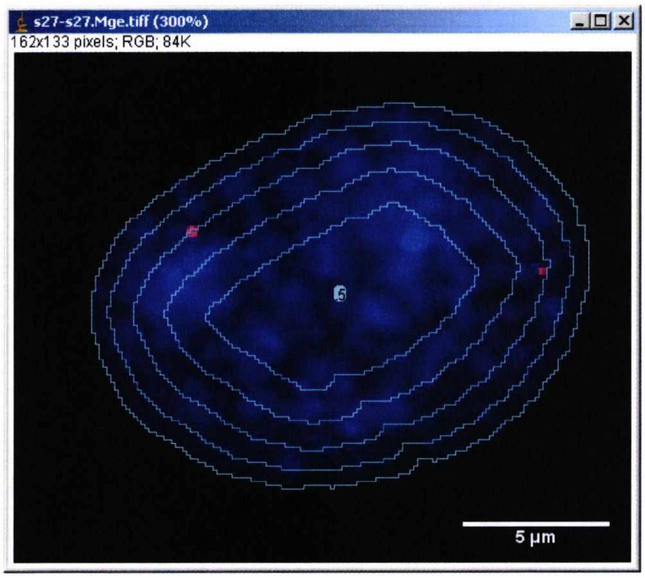

Figure 1: Chicken embryonic fibroblast nucleus with a GGA1 BAC hybridised, and the ImageJ plugin applied, dividing the nucleus into five rings of equal area.

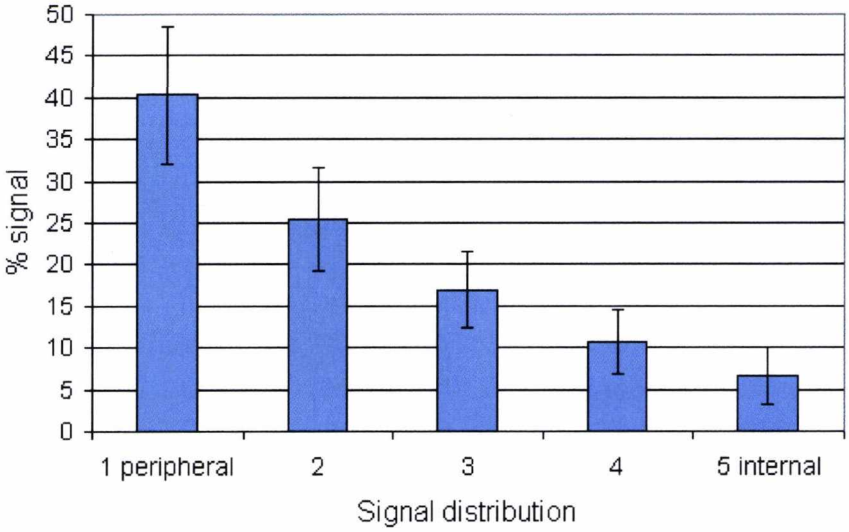

Figure 2: Example of the distribution of signal in each of the five rings after measuring 50 nuclei for one BAC. In this case, the signal was predominantly at the periphery of the nucleus.

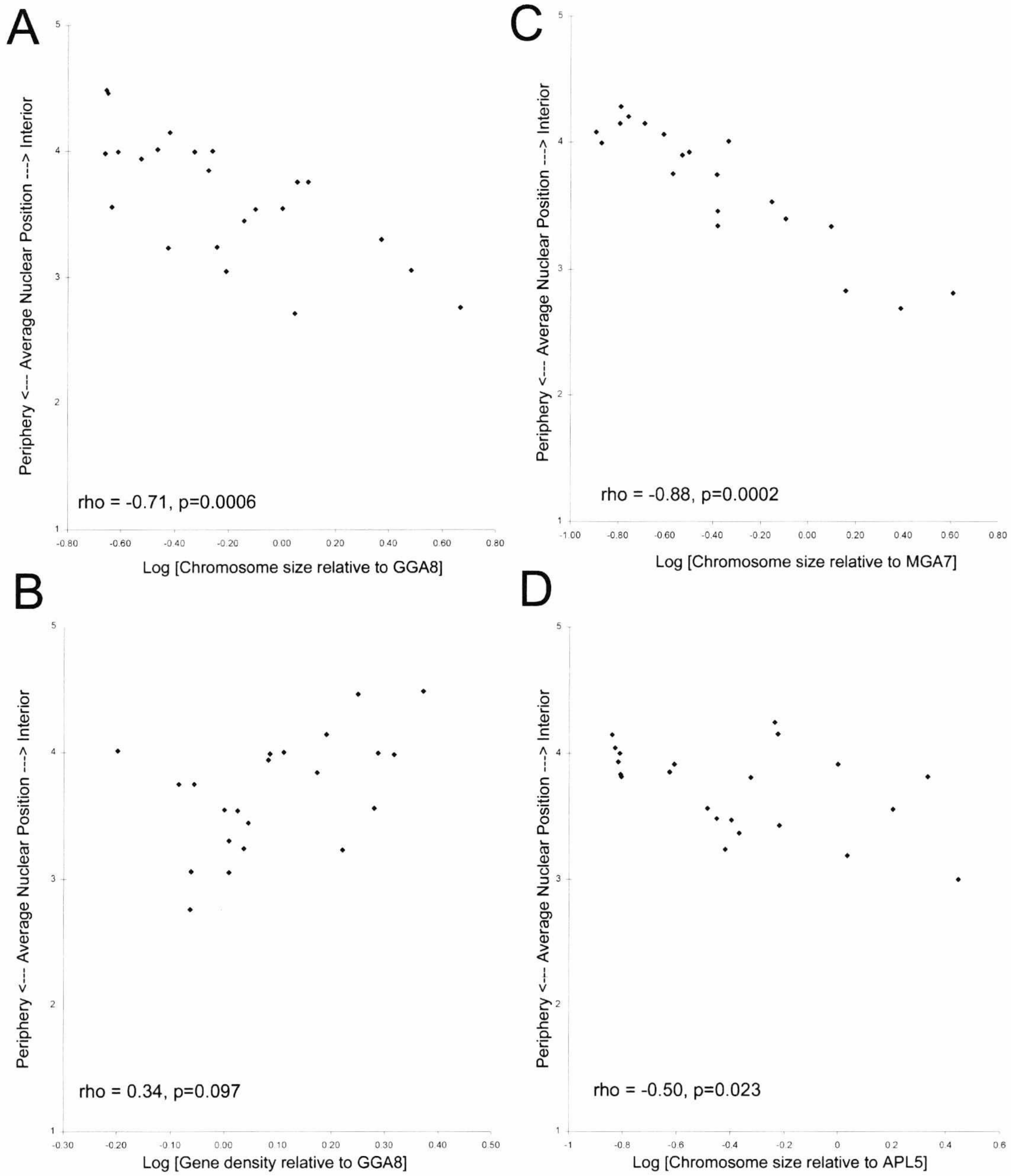

Figure 3: Plots of chromosome positions against A) chicken chromosome size; B) chicken gene density; C) turkey chromosome size; D) duck chromosome size. Log values of chromosome size and gene density are shown to improve clarity of the charts over the wide size range; correlations are unaffected. Though the non-parametric test does not permit a best-fit line, the trends are clearly visible (Spearman's rank correlations are shown in the graphs).

Chicken						Turkey					Duck				
GGA	Median Position	IQR	Area c.f. GGA8	Density c.f. GGA8	SE	MGA	Median Position	IQR	Area c.f. MGA7	SE	APL	Median Position	IQR	Area c.f. APL5	SE
1	2.76	1.58	4.66	0.86	0.01	1	2.81	1.49	4.05	0.01	1	*3.00*	*1.85*	2.78	0.05
2	3.06	1.38	3.04	0.87	0.01	6	3.34	1.96	1.25	0.01	2	3.82	1.53	2.14	0.04
						3	*2.91*	*1.62*	1.90	0.01					
3	3.30	2.06	2.35	1.02	0.02	2	2.69	1.38	2.45	0.01	3	3.56	2.03	1.59	0.03
4	*2.80*	*1.54*	1.84	1.13	0.02	9	*3.00*	*1.91*	0.60	0.01	10	3.37	1.88	0.43	0.02
						4	*3.10*	*1.82*	1.70	0.01	4	*3.19*	*1.75*	1.08	0.02
5	*3.03*	*1.81*	1.71	1.02	0.01	5	2.82	1.55	1.44	0.01	5	3.91	1.50	1.00	-
6	3.75	1.81	1.25	0.82	0.01	8	*3.00*	*1.70*	0.92	0.01	6	4.15	1.33	0.59	0.03
7	3.75	1.73	1.14	0.88	0.01	7	*3.00*	*1.79*	1.00	0.01	7	3.43	1.63	0.61	0.01
8	3.55	1.52	1.00	1.00	-	10	3.39	1.51	0.80	0.01	8	4.25	1.31	0.58	0.02
9	3.54	1.17	0.8	1.06	0.01	11	3.53	1.15	0.70	0.01	9	3.81	1.50	0.47	0.02
10	3.44	1.59	0.72	1.11	0.01	12	*3.00*	*1.94*	0.53	0.01	11	3.47	1.52	0.40	0.01
11	*2.59*	*1.00*	0.58	1.24	0.01	13	*2.49*	*2.05*	0.43	0.02	12	3.24	1.63	0.38	0.02
12	3.24	1.16	0.57	1.08	0.03	14	4.01	0.79	0.46	0.01	13	-	-	0.42	0.05
13	3.05	1.74	0.62	1.02	0.02	15	3.75	1.50	0.27	0.01	14	3.48	1.16	0.35	0.02
14	3.84	1.47	0.53	1.49	0.02	16	3.46	1.43	0.42	0.01	15	3.56	1.36	0.33	0.01
15	4.00	1.78	0.55	1.29	0.02	17	3.74	1.73	0.41	0.02	16	-	-	0.37	0.03
16	4.21	1.00	0.31	0.41	0.03	18	4.15	1.11	0.16	0.02	17	4.00	1.45	0.15	0.01
17	3.99	1.26	0.47	1.22	0.02	19	3.90	1.58	0.30	0.02	18	3.85	1.09	0.24	0.01
18	3.23	2.03	0.38	1.66	0.02	20	-	-	-	-	19	-	-	0.23	0.01
19	4.14	1.25	0.38	1.55	0.02	21	3.92	1.68	0.31	0.01	20	-	-	0.23	0.01
20	*3.39*	*1.88*	0.43	1.53	0.02	22	3.34	1.69	0.42	0.02	21	-	-	0.26	0.01
21	3.98	1.11	0.22	2.08	0.01	23	4.15	1.34	0.21	0.02	22	3.92	1.10	0.25	0.03
22	4.01	1.34	0.34	0.63	0.02	24	4.10	1.04	0.16	0.02	23	3.93	1.27	0.15	0.01

Chicken						Turkey					Duck				
GGA	**Median Position**	**IQR**	**Area c.f. GGA8**	**Density c.f. GGA8**	**SE**	**MGA**	**Median Position**	**IQR**	**Area c.f. MGA7**	**SE**	**APL**	**Median Position**	**IQR**	**Area c.f. APL5**	**SE**
23	3.56	1.71	0.23	1.91	0.04	25	4.06	1.06	0.25	0.01	24	3.83	1.74	0.16	0.01
24	3.94	1.62	0.3	1.21	0.03	26	4.20	1.06	0.18	0.02	25	3.82	1.47	0.16	0.01
26	3.99	1.31	0.24	1.93	0.03	28	4.33	1.22	-	-	27	4.15	1.35	0.14	0.01
27	4.49	1.09	0.22	2.35	0.01	29	4.00	1.28	0.13	0.03	28	4.04	1.04	0.15	0.01
28	4.46	0.88	0.22	1.77	0.02	30	4.08	1.38	0.13	0.02	29	-	-	0.13	0.01
Z	2.71	1.67	2.17	0.65	0.01	Z	-	-	2.06	0.01	Z	-	-	1.20	0.01
W	*3.04*	*1.57*	1.12	0.00	0.01	W	-	-	1.28	0.01	W	-	-	0.53	0.04

Table 1: Median positions of chromosomes (from periphery, 1, to interior, 5) in chicken, turkey and duck, with relative chromosome sizes and, for chicken, relative gene densities. Orthologous chromosomes are aligned. Positions that could not be distinguished from a random distribution are indicated in *italics*. IQR indicates the interquartile range of calculated positions for each BAC. SE indicates standard error of the mean; SE for chicken chromosome area and gene density are equivalent.

Table S1: Markers within BACs hybridised to chicken, turkey and duck nuclei, indicating those which produced images suitable for ring analysis (i.e. appeared free from non-specific background hybridisation)

Chicken Chromosome	Marker	Analysable images produced?		
		Chicken	Turkey	Duck
1	LEI0101	✓	✓	✓
1	LEI0134	✓	✓	✓
1	LEI0169	✓	✓	✓
1	LEI0194	✓	✓	
1	MCW0112		✓	
1	MCW0188	✓	✓	
1	MCW0200	✓		✓
1	LEI0068	✓		
1	LEI0146	✓		
1	MCW0248	✓		
1	LEI0091	✓		
1	ADL0101	✓		
2	LEI0129	✓	✓	✓
2	MCW0082	✓	✓	
2	MCW0157	✓		✓
2	MCW0131	✓	✓	✓
2	ABR0008	✓		✓
2	LEI0147		✓	
2	MCW0239	✓		
2	MCW0358	✓		
2	ADL0176	✓		
2	MCW0274	✓		
2	ADL0120	✓		
3	ADL0370	✓	✓	✓
3	GCT0083	✓	✓	✓
3	MCW0127	✓	✓	✓
3	MCW0261	✓		✓
3	MCW0162	✓		
3	LEI0115	✓		
3	MCW0224	✓		
3	MCW0040	✓		
4	LEI0063	✓	✓	✓
4	MCW0295		✓	
4	ADL0203	✓	✓	✓
4	MCW0180	✓	✓	
4	ADL0317	✓		✓
4	ADL0246	✓		
4	COM0085	✓		
5	HUJ003	✓	✓	✓
5	MCW0193	✓	✓	
5	MCW0210	✓	✓	✓
5	ROS0013	✓	✓	✓
5	MCW0113	✓	✓	✓
6	ADL0040	✓	✓	

Chicken Chromosome	Marker	Analysable images produced?		
		Chicken	Turkey	Duck
6	LEI0192	✓	✓	
6	ROS0003	✓	✓	✓
7	LEI0064	✓	✓	✓
7	MCW0120	✓	✓	✓
7	MCW0201	✓	✓	✓
8	ADL0301	✓	✓	✓
8	ADL0302	✓	✓	✓
8	MCW0160		✓	
8	MCW0275	✓	✓	✓
9	ADL0191	✓	✓	✓
9	MCW0134	✓	✓	✓
9	ROS0078	✓	✓	✓
10	ADL0272	✓	✓	✓
10	MCW0003	✓	✓	✓
10	MCW0035		✓	✓
11	ADL0308	✓	✓	✓
12	SCW0019	✓	✓	
13	MCW0213	✓	✓	
13	MSX2	✓	✓	✓
14	ADL0200	✓		
14	MCW0296	✓	✓	✓
15	CRY3A4	✓	✓	
15	MCW0211	✓	✓	
16	LEI0258	✓	✓	✓
16	MCW0371	✓	✓	
17	MCW0151	✓	✓	
18	HUJ0010	✓		
19	CTG1740		✓	
19	CTG7040	✓		
19	SCW0024	✓		
20	ADL0193	✓	✓	
20	GCT20050	✓		
21	AGRIN	✓	✓	✓
21	PLOD	✓	✓	
22	ROS0073	✓		✓
22	TVSB3	✓		
22	ROS0022		✓	
23	LEI0339	✓	✓	✓
24	ROS0113A	✓	✓	✓
26	MCW0286	✓	✓	✓
27	GCT0022	✓	✓	✓
27	MCW0328	✓	✓	✓
28	ST28BE07	✓	✓	
Z	Paint	✓		
W	Paint	✓		

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Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis

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Abstract

Background

The availability of the complete chicken (*Gallus gallus*) genome sequence as well as a large number of chicken probes for fluorescent *in-situ* hybridization (FISH) and microarray resources facilitate comparative genomic studies between chicken and other bird species. In a previous study, we have provided a comprehensive cytogenetic map for the turkey (*Meleagris gallopavo*) and the first analysis of copy number variants (CNVs) in birds. Here, we extend this approach to the Pekin duck (*Anas platyrhynchos*), an obvious target for comparative genomic studies due to its agricultural importance and resistance to avian flu.

Results

We provide a detailed molecular cytogenetic map of the duck genome through FISH assignment of 155 chicken clones. We identified one inter- and six intrachromosomal rearrangements between chicken and duck macrochromosomes and demonstrated conserved synteny among all microchromosomes analysed. Array comparative genomic hybridisation revealed 32 CNVs, of which 5 overlap previously designated "hotspot" regions between chicken and turkey.

Conclusion

Our results suggest extensive conservation of avian genomes across 90 million years of evolution in both macro- and microchromosomes. The data on CNVs between chicken and duck extends previous analyses in chicken and turkey and supports the hypotheses that avian genomes contain fewer CNVs than mammalian genomes and that genomes of evolutionarily distant species share regions of copy number variation ("CNV hotspots"). Our results will expedite duck genomics, assist marker development and highlight areas of interest for future evolutionary and functional studies.

Background

Comparative genomics allows the transfer of genomic information from a well-characterized species to another that is less well described. It can be applied at all levels from that of the chromosome to the genome sequence. However, despite the recent advances in sequencing technologies, the considerable effort involved in producing a genome sequence assembly is reflected by the small number of vertebrate genomes that have been sequenced to date. In birds, there is only one published genome sequence, that of the chicken [1], with the zebra finch genome due to be published soon.

Combining cross-species fluorescent in-situ hybridization (FISH) and microarray analysis using resources developed in the chicken provides a powerful tool for the identification of gross genomic rearrangements, gene gains/losses, copy number variants (CNVs) and gene order in other bird species. These techniques do not require sequence data for any species other than the reference (i.e. chicken). We have previously successfully used this approach for a genome wide comparison of chromosomal rearrangements and CNVs between chicken and turkey [2]. This revealed a strong conservation of genome structure over about 30 million years of evolution [3]. In particular, our results suggested that, when compared to mammalian genomes, bird genomes contain a low number of CNVs (i.e. polymorphisms in the number of copies of a DNA fragment 1 kb or larger [4], with the exception of insertions or deletions of transposable elements [5]). The latter finding indicates that patterns of CNVs in bird genomes mirror the low number of chromosomal rearrangements in this phylogenetic group [2, 6].

Following on from the turkey, the Pekin duck (*Anas platyrhynchos*, APL) is the next obvious target among domestic birds for detailed genomic studies due to its agricultural importance, with worldwide duck consumption being between 4 and 5% of the total poultry market [7]. Duck is also an important target for immunological studies because of its resistance to avian influenza [8]. Despite this, genomic information about the duck is limited to a few linkage and physical mapping studies. Huang *et al.* [9] produced a preliminary genetic map based on 240 microsatellite loci and assigned 11 out of 19 linkage groups to ten duck (APL) chromosomes by FISH mapping of 28 BACs. Cross-species chromosome painting and G-banding studies [10-12] have suggested one interchromosomal difference between the chicken and duck karyotypes - the ancestral chromosomes 4 and 10, fused in the chicken lineage to give GGA4q and GGA4p respectively, remain separate in duck [6]. This interchromosomal rearrangement presumably explains the difference in diploid chromosome number between the two species, which is 2n=78 in chicken and 2n=80 in duck. FISH mapping of 57 chicken BACs revealed small intrachromosomal rearrangements in APL2, 7, 8 and Z and confirmed synteny for GGA9, 11, 13-15, 18 and 28 in the duck genome [13]. However, no molecular markers are available for the remaining microchromosomes, which are indistinguishable by conventional cytogenetics. It is also unclear which duck chromosome corresponds to GGA4p (ancestral chromosome 10). Thus, from a molecular cytogenetic standpoint, the duck genome is at present only partially defined, and given the low number of physical markers mapped by FISH, it is possible that hitherto undetected intrachromosomal rearrangements exist.

No data are currently available concerning CNVs in duck or indeed any other bird species than chicken and turkey. CNVs have been found to contribute significantly to normal and disease-related genetic and phenotypic variation in humans and other primates [5, 14]. Studies of the evolutionary significance of CNVs have largely focused on primates and revealed numerous lineage-specific gene gains and losses and CNVs (e.g. [15-20]).
Our previous study of CNVs in chicken and turkey revealed a total of 16 CNVs [2]. Five of these CNVs appear to be shared in layer and broiler chickens, and in turkey, at regions dubbed "CNV hotspots" (i.e. genomic regions in which CNVs of approximately equal size were found in both chicken breeds and in turkey). Given that the contribution of CNVs to phenotypic variation is becoming increasingly clear, analyses of this kind of structural variation in organismic groups other than mammals are clearly needed.

Here, we present a detailed molecular cytogenetic map for the duck based on comparative FISH mapping of 155 chicken BACs, which revealed several hitherto undescribed intrachromosomal rearrangements. We also provide an analysis of CNVs in the duck genome by array comparative genomic hybridisation (array CGH) of duck DNA to a commercially available chicken whole-genome oligonucleotide tiling path microarray. The analysis of CNVs supports the hypotheses that bird genomes contain fewer CNVs than mammalian genomes and that some CNVs appear to be consistently shared across species, forming CNV hotspots.

Results

Comparative FISH mapping between chicken and duck: Of 400 BACs that successfully hybridized to chicken metaphases, 155 (39%) could be visualized with confidence on duck chromosomes. These covered the majority of the karyotype i.e. APL1-29 and Z (except 26). Figure 1 shows the G-banded ideograms for GGA and APL1-8 and Z [13, 21], with the positions of the BACs mapped to these chromosomes; the full data are presented in Table 1. Figure 2 shows example FISH results. Only one interchromosomal difference was detected among the macrochromosomes, the retention of the ancestral chromosomes 4 and 10 in duck (which are fused in chicken).

FISH mapping suggested intrachromosomal rearrangements on GGA and APL1, 2, 4, 7, 8 and Z. BACs WAG24H22, WAG30B21 and WAG36C14 clearly evidenced a rearrangement on GGA and APL1p. The order of BACs was not completely inverted, suggesting that the underlying rearrangement may be a translocation rather than a paracentric inversion. BAC WAG7C9 mapped to GGA1p and APL1q, indicating a small pericentric inversion. Besides, some BACs mapping to GGA and APL1q suggested possible rearrangements on these chromosome arms; however, substantial variation in the chromosomal position determined for these BACs in duck made it difficult to distinguish artefacts from real changes in marker order.

BACs WAG42G5 and WAG9L1 mapped to GGA2q and APL2p, evidencing a pericentric inversion. However, BAC WAG18G1, which mapped close to the centromere on GGA2p, also hybridized close to the centromere in APL2p. This demonstrated that the inversion involves only a small fraction of 2p.

BACs WAG13I5, WAG23K3, WAG21J21 AND WAG22J17 clearly demonstrated a paracentric inversion on GGA and APL4.

The morphological differences between GGA and APL7 were reflected in a change in marker order involving BACs WAG69P21, WAG59H11 and WAG21P13. However, like in the rearrangement on GGA and APL1p, marker order was not completely inverted, indicating that this rearrangement may be more complex than a simple pericentric inversion. Similarly, our FISH mapping results did not provide clear evidence for a pericentric inversion causing the morphological differences between GGA and APL8.

Marker order on GGA and APLZ chromosome was largely conserved, with the possible exception of a small inversion involving BACs WAG22P4 and WAG20L10. Thus, it seems that the morphological differences between the metacentric GGAZ and the subtelocentric APLZ are due to neocentromere formation rather than a pericentric inversion.

Dual-color FISH experiments with BACs mapping to either end of a microchromosome were used to check for synteny among the microchromosomes. This demonstrated synteny for APL9, 11, 14-16, 19, 21, 27-29. The BACs successfully hybridized to the microchromosomes are listed in Table 2.

FISH mapping of BACs WAG112C24 and WAG23I06 facilitated the identification of the duck orthologue of GGA4p. Chromosome area measurements suggested that this chromosome should be numbered as APL10. There were no major differences in size order between the remaining microchromosomes and their chicken orthologues.

Copy number variation between chicken and duck: Hybridisation of genomic DNA from two female ducks to the Nimblegen chicken whole genome tiling path microarray revealed 32 CNVs, of which ten were seen in both individuals (CNVs marked with ^{a} in Table 3). Both gains and losses relative to chicken were seen (8 gains and 24 losses). The mean and median lengths of the detected CNVs were 281kb and 50kb respectively, ranging from 2.8kb to 4.4Mb. The CNV locations were compared with those previously found in turkey and chicken broilers and layers [2]. Six CNVs overlapped with CNVs identified in turkey, and of these five CNVs matched the five potential CNV 'hotspots' described by Griffin *et al.* [2] (bolded in Table 3). Three of the 'hotspot' CNVs were seen in both ducks. Known or predicted genes were found to be associated with 22 of the 32 CNVs (68.75%). In the ten cases where no genes were associated, all were losses, and all except one were found either near to the beginning or end of the chromosome (between 2bp and 4Mb of the sequence start or end according to Ensembl, www.ensembl.org/Gallus_gallus). The exception, CNV#14, covered a sequence gap, likely centromeric. Two of the CNVs, #7 and #9, were in regions that are potentially involved in rearrangements on GGA7 and 8 respectively.

Discussion

Comparative cytogenetic map of the duck genome: Previous studies of avian genome evolution using cross-species FISH have suggested that gross genome structure is remarkably conserved among birds. Duck is no exception from this pattern; in fact it appears that the duck karyotype corresponds very closely to the putative ancestral avian karyotype (Griffin et al 2007). The conservation of ancestral chromosomes 4 and 10, as APL4 and APL10 are consistent with both the previous studies on duck and with broader patterns of avian genome evolution [6, 13]. These chromosomes appear intact in almost all birds, and ancestral chromosome 4 is seen intact in human chromosome 4 as well [1, 22].

In agreement with previous studies, no other interchromosomal rearrangements were detected between chicken and duck. However, our BAC mapping data are consistent with intrachromosomal rearrangements distinguishing chromosomes GGA and APL1, 2, 4, 7, 8 and Z, which confirms and expands on previous findings [13]. The detection of additional rearrangements on GGA and APL1 and GGA4q and APL4 was due to the much higher number of BACs hybridized in this study compared to previous studies. Likewise, higher-resolution mapping demonstrated that the morphological differences between GGA and APLZ are probably due to the formation of a neocentromere rather than a pericentric inversion. This type of chromosomal rearrangement was previously reported in birds only for the red-legged partridge [23] and the Japanese quail [24]. However, despite the good coverage of the duck cytogenetic map presented here, it was not possible to determine unequivocally the nature of all chromosomal rearrangements observed between chicken and duck. It seems likely however that, in addition to peri- and paracentric inversions and neocentromere formation, translocations contributed to avian genome evolution. This conclusion is based on the order of BACs associated with rearrangements on GGA and APL1, 7 and 8, which is not entirely consistent with the order expected if the rearrangements were inversions. Thus, it appears that while the available data from comparative FISH mapping suggest a relatively low frequency of intrachromosomal rearrangements in the evolution of bird genomes, the underlying processes may be more diverse than previously appreciated. Undoubtedly, the higher resolution afforded by genome sequencing projects such as that of the zebra finch will help to resolve this question.

The evolutionary direction of intrachromosomal changes could be determined for GGA and APL8 only. Comparison with the turkey map [2] suggested that APL8 likely represents the ancestral state; the order of BACs on turkey chromosome 10 (ancestral chromosome 8) and the morphology of this chromosome is the same as in duck, indicating that the rearrangement has occurred in the chicken lineage. Due to a lack of comparative data, it was not possible to determine the polarity of the remaining intrachromosomal rearrangements.

Among the rearrangements that we detected, the inversion observed in GGA4q and APL4 is of particular interest. Morphological differences in GGA4 have been described between different chicken breeds [25], and the ancestral bird chromosome 4 (corresponding to GGA4q and APL4, respectively) is also one of the chromosomes most prone to convergent independent fusions in birds (with ancestral chromosome 10) [6]. This contrasts with the conserved synteny of the ancestral bird chromosome 4 (corresponding to GGA4q and APL4, respectively) in humans. Together, the data suggest that rearrangements in chromosome 4 may be more common than has been suspected from previous comparative genomic studies, and analyzing them will prove valuable for understanding avian and other vertebrate genome evolution.

The present study extended the data previously available for synteny among the microchromosomes. Fillon *et al.* [13] and demonstrated synteny for seven microchromosome pairs (APL9, 12, 14-16, 19, 29); here we demonstrate synteny for ten microchromosome pairs (APL9, 11, 14-16, 19, 21, 27-29). The lack of detected rearrangements makes it reasonable to suggest that there are very few, if any, rearrangements among the remaining microchromosomes – including the as-yet unexamined smallest microchromosomes for which no markers exist. Indeed, no sequence data from the chicken genome has yet been assigned to these smallest chromosomes, and it is still unclear why, although there is a suggestion that there may be a cloning or sequencing bias against microchromosomal sequences [26]. Data on microchromosomal synteny in other bird species are restricted to the Japanese quail (*Coturnix japonica* [27]) and the turkey [2]. Despite the paucity of data, the emerging picture is one of remarkable conservation among most avian species, with the exception of a few groups where large-scale interchromosomal rearrangements are common (such as the Falconiformes or Psittaciformes [28, 29]).

The detailed cytogenetic map allowed for an improved definition of the duck karyotype. Chromosome banding and macrochromosome painting studies had previously shown orthology of APL1-9 and Z to GGA1-3, 4q, 5-8 and Z [10-12]. However, it was not known which duck chromosome corresponded to chicken chromosome 4p; Fillon *et al.* [13] suggested that this was approximately APL10-13. In this study we have used a combination of BAC mapping and chromosome area measurements which suggest that this chromosome should be numbered as APL10. Moreover we found no evidence for rearrangements among the microchromosomes. Accordingly, we propose that duck chromosomes be numbered as per chicken for 1-9; that APL10 corresponds to GGA4p; then GGA10 onwards correspond to APL11 onwards. The successful hybridization of at least one BAC from GGA1-28 (except 25) means markers are now available for APL1-29 (except 26) and Z. Taken together, these results enabled us to define unequivocally chromosomes APL1-29.

Copy number variation between chicken and duck: The purpose of our array CGH experiments was to test two of our earlier hypotheses: (i) that birds show a reduced number of CNVs compared to mammals, (ii) that genomic CNV hotspots described previously in chicken and turkey [2] are found in the duck as well (indicating conservation over a large evolutionary distance). The successful hybridizations that we observed, despite approximately 90 million years of divergence between chicken and duck [30], extend avian cross-species microarray experiments from the sole previous study in turkey [2]. The present study revealed a total of 32 CNVs in the duck when compared to the chicken, which is substantially fewer than the 58 CNVs discovered in a comparison of human and chimpanzee [31], which diverged only 6-7 million years ago [32]. While this result supports the hypothesis that bird genomes show fewer CNVs than mammalian ones, it should also be noted that we found twice as many CNVs in duck as were found in the comparison of turkey and chicken (32 versus 16) [2]. Only ten of these CNVs were found in both duck specimens examined, indicating substantial intraspecific variation. These findings highlight the need for further studies with larger sample sizes and call for some caution when comparing the frequency of CNVs in birds and mammals.

The comparison of CNVs between chicken breeds and turkey revealed five tentative CNV hotspots by virtue of the fact that they contained CNVs of similar size in different chicken lines and in turkey [2]. Of these five hotspot regions, three contained CNVs in both ducks and two contained CNVs in one duck. The hotspot regions contain a number of novel genes including most of the available sequence for GGA16, covering the MHC loci. Moreover these findings lend support to our avian "CNV hotspot hypothesis" but, of course, need to be confirmed by analysing a wider number of species.

All copy number gains (in duck compared to chicken) were located in coding regions. Genes in regions of copy number gain relative to chicken included transcription factors, neural proliferation control and neurotransmitter activity, and a predicted MHC class I gene. This is consistent with previously described duplication of the MHC class I locus in the mallard duck, followed by subsequent inactivation of some of the extra gene copies [33].

Where copy number losses relative to chicken were detected, two possible explanations exist: a true copy number loss, or sequence divergence leading to lack of hybridization on the microarray. If such sequence divergence had occurred, however, the loss might be expected in both individuals. This was only seen for 9 CNVs; the remainder are more likely to be true polymorphic copy number differences. However, it is important to note that about two thirds of all apparent copy number losses were observed in coding regions; hence, the observed loss in hybridization efficiency is likely associated with functional consequences, regardless of whether it is due to copy number change or sequence divergence. Thus these results highlight genomic regions that are of particular interest for further functional and evolutionary studies.

It has been suggested that segmental duplications are correlated with CNVs, and facilitate chromosomal rearrangements, the lack of segmental duplications in birds therefore explaining the relative paucity of CNVs [1]. Due to the vastly different levels of resolution afforded by cytogenetic mapping and microarray analysis, it is difficult to directly correlate the results of these two methods. Nevertheless, it is interesting to note that two of the CNVs revealed in this paper appear to coincide with rearrangements detected from the cytogenetic mapping. These are CNVs #7 and #9, on chromosomes GGA7 and 8 respectively. Further studies are necessary to examine this link between chromosomal rearrangements and CNVs in more detail.

Conclusions

The comparative cytogenetic map of the duck presented here highlights the extraordinary conservation seen among the genomes of many bird species, and how little structural genetic variation is readily apparent. The cytogenetic map will allow the transfer of genetic information directly from chicken to duck, expediting mapping studies in the duck and help to target marker development in duck through the prediction of new loci. The combination of area measurements and FISH mapping of chicken BACs allowed the identification of markers for chromosomes APL1-24 and 26-29 which will facilitate further mapping studies in the duck. Moreover, we have extended the analysis of CNVs in birds, providing further evidence that birds have low numbers of CNVs when compared to mammals and that bird genomes contain CNV hotspots. While overall we confirm the evolutionary conservation of bird genomes, the intrachromosomal differences and CNVs found highlight areas of future interest for evolutionary and functional studies.

Materials and Methods

Cell culture and chromosome preparation: All chromosome preparations were made from cultured cells derived from fertilized eggs. Chicken eggs were supplied by Hill Top Farm, Cambridgeshire, UK and Friday's Farm, Kent, UK. Duck eggs were provided by Cherry Valley Ltd, Market Rasen, UK. Fibroblast cultures were established from 5- to 7-day-old embryos. Chromosome preparation followed standard protocols [34, 35]: mitostatic treatment with colcemid at a final concentration of 0.1 µg/ml for 1 h at 37ºC, hypotonic treatment with 75mM KCl for 15 min at 37ºC and fixation with 3:1 methanol:acetic acid.

Selection and preparation of BAC clones: 400 BAC clones were selected from the Wageningen chicken BAC library [36] based on the position of markers on the chicken consensus linkage map [37]. The BACs were labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) following standard protocols. At least one BAC was available for GGA1-28 (except 25) and Z. The 155 BACs successfully hybridized to both chicken and duck are detailed in Tables 1 and 2.

Fluorescent *in-situ* hybridisation (FISH): Slides with metaphase preparations were aged for one hour at 70°C on a hotplate then treated with 4mg/ml RNase A for one hour at 37°C. The chromosomes were denatured for 1 minute 30 seconds in 70% formamide in 2xSSC at 70°C. BACs were applied and sealed under coverslips. Hybridization was carried out in a humidified chamber for 72 hours at 37°C. Following post-hybridization washes (40% or 30% formamide in 2xSSC for 20 minutes; 1 minute in 2xSSC/0.1% Igepal at RT; 15 minutes in 4xSSC/0.05% Tween 20 at RT; 25 minutes in 4xSSC/0.05% Tween 20/2% BSA at RT), probes were detected with 1:200 streptavidin-Cy3 (Amersham), plus 1:200 FITC-anti-digoxigenin (Amersham) for dual-color experiments, in 4xSSC, 0.05% Igepal, 1.25% BSA for 35 minutes. Slides were washed in 4xSSC, 0.05% Igepal for 3x 5 minutes then counterstained using Vectashield with DAPI (Vector Labs).

Dual-color FISH was used to determine whether there were any rearrangements among the microchromosomes, selecting BACs that were as close as possible to the ends of the chromosomes. For GGA11, only one hybridizing BAC in duck was available.

Image capturing and analysis: Slides were analyzed on an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera and appropriate filters. Images were captured using SmartCapture 3 (Digital Scientific UK). The signal positions were measured as the fractional length from the p-terminus (FLpter [38]). FLpter and area measurements were carried out in ImageJ [39]. The area of duck chromosomes was determined as a ratio of the easily recognized chromosome 5, as per [40]. For area measurements, 10 metaphases were measured per chromosome; the numbers of metaphases used for FLpter measurements are given in Table 1.

Array CGH: The NimbleGen chicken whole-genome tiling array (Catalogue Number/Design Name B3791001-00-01, galGal3 WG CGH – Roche NimbleGen, Milton

Keynes, UK) was used for the array CGH experiments. It contains 385,000 50-mer oligonucleotides with an average spacing of 2,586 base pairs (source – UCSC, build – galGal3) and was interrogated with duck whole genomic DNA extracted from blood from two female ducks using a DNeasy Animal Blood and Tissue kit (Qiagen, #69504); the reference (Red Jungle Fowl) DNA, from the same animal used in the chicken genome sequencing project, was kindly provided by Dr Hans Cheng (Michigan State University). Labeling of genomic DNA and hybridization to the Roche NimbleGen array were performed by the company (Roche NimbleGen) and used random priming to incorporate modified nucleotides by either amino-allyl or direct linkage to either of the two dyes used (Cy3 and Cy5). All of the hybridizations in this experiment used two dyes per slide (Cy3 and Cy5). Red Jungle Fowl reference DNA (Cy5) was co-hybridized with duck test DNA (Cy3).

CGH analysis proceeded in three stages, normalization, window averaging and segmentation. After combining the signal intensity and genomic coordinate information, the Cy3 and Cy5 signal intensities were normalized to one another using Qspline normalization [41]. Qspline is a robust non-linear method for normalization using array signal distribution analysis and cubic splines. Once normalized, the data was prepared for DNA segmentation analysis. This included a window averaging step, where the probes that fall into a defined base pair window size (25kb) are averaged, using the Tukey's biweight mean [42]. The Tukey's biweight method yields a robust weighted mean that is relatively insensitive to outliers, even when extreme. A new position was assigned to this average, which is the midpoint of the window. Segmentation was also performed on unaveraged data to permit smaller segments than the window size to be detected. The circular binary segmentation algorithm [43] was used to segment the averaged log2 ratio data. DNA segments were called by attempting to break the segments into sub-segments by looking at the t-statistic of the means. Permutations ($n = 1000$) were used to provide the reference distribution. If the resulting p-value was below the threshold (default of $p = 0.01$), then a breakpoint was called. A pruning step was used to remove spurious segments, rejecting segments where the standard deviation of the means was not sufficiently different. By default, a cut off of 1.5 standard deviations was used. CNVs were called for segments in which the log2 ratio was greater than $|\pm 0.5|$. Where overlapping CNVs were detected in window averaged and unaveraged data, the data were considered to represent a single CNV.

Authors' contributions

BMS performed probe preparation, hybridisations, microscopy and data analysis and prepared the manuscript. LBWR, HGT, EJL, DI and KEF assisted with probe preparation and microscopy. RPMAC provided the BAC clones. ADH and DKG conceived and designed the study. DKG was the PI on the BBSRC grant that funded the project and supervisor of BMS, LBWR, HGT, EJL, DI, KEF and MV. DKG critically revised the manuscript. MV performed hybridisations, data analysis and prepared the manuscript. All authors read and approved the final manuscript.

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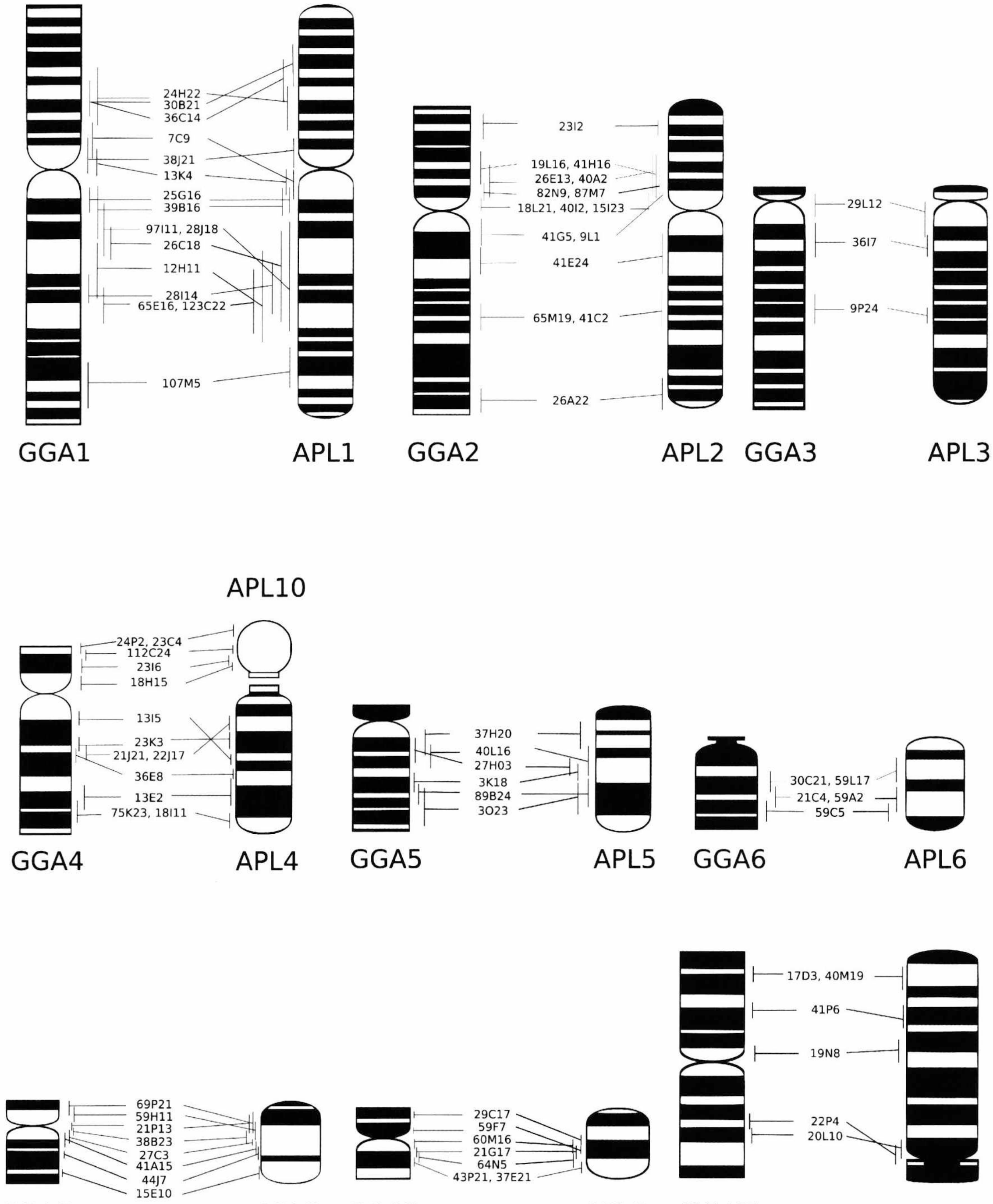

Figure 1: Comparative map of chicken and duck chromosomes 1-8 and Z. The G-banded ideograms of chicken (*Gallus gallus*; GGA) and duck (*Anas platyrhynchos;* APL) chromosomes 1-8 and Z are shown with the positions of all BACs successfully hybridized to both species as determined by FLpter measurements. Intrachromosomal rearrangements can be seen on GGA and APL1, 2, 4, 7, 8 and Z. GGA4p corresponds to APL10. Note the orientation of APLZ. Ideograms were prepared from [13, 21]. Error bars represent one standard deviation.

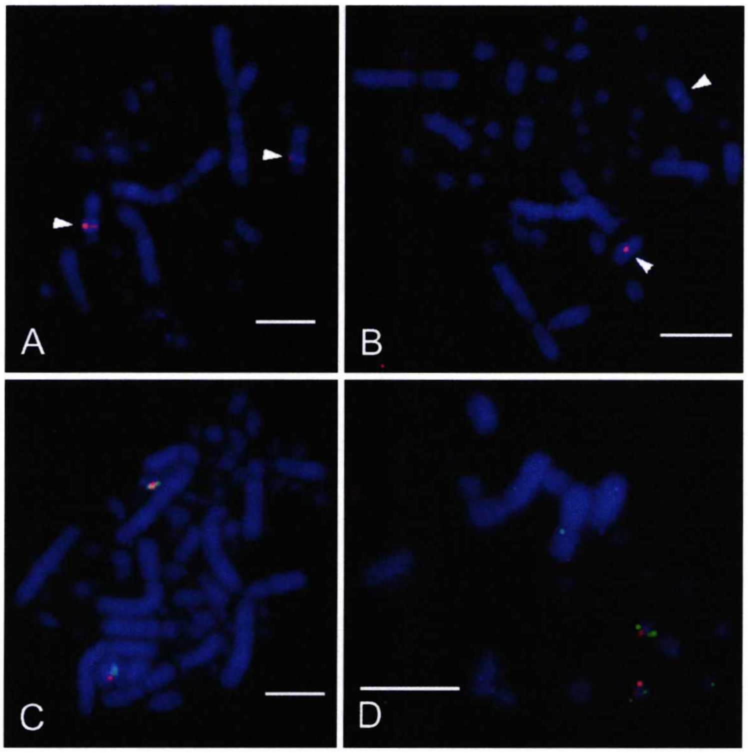

Figure 2: Example FISH results. BAC WAG13I5 on A) chicken (*Gallus gallus*; GGA) chromosome 4q and B) duck (*Anas platyrhynchos;* APL) chromosome 4 demonstrating part of the evidence that led us to deduce a paracentric inversion. Synteny among the microchromosomes was tested by dual color FISH. An example is shown for C) GGA15 and D) APL16 using BACs with markers CRYBA4sts1 and LEI0083. Scale bars represent five microns.

Tables

Table 1: Chicken (*Gallus gallus*; GGA) BACs successfully hybridized to duck (*Anas platyrhynchos*; APL) macrochromosomes. BACs with these markers successfully hybridized to duck chromosomes. FLpter represents Fractional Length from the p terminus [38]; SD represents standard deviation

GGA BAC clone	GGA chromosome	Marker	GGA FLpter	GGA FLpter SD	GGA metaphases	APL chromosome	APL FLpter	APL FLpter SD	APL metaphases
WAG24H22	GGA1p	LEI0068	0.23	0.03	9	APL1p	0.24	0.02	8
WAG30B21	GGA1p	LEI0194	0.24	0.03	8	APL1p	0.14	0.03	10
WAG36C14	GGA1p		0.24	0.03	6	APL1p	0.19	0.02	4
WAG7C9	GGA1p		0.32	0.03	6	APL1q	0.40	0.02	9
WAG38J21	GGA1p		0.38	0.03	7	APL1p	0.36	0.06	7
WAG13K4	GGA1p		0.42	0.03	9	APL1q	0.42	0.03	6
WAG25G16	GGA1q	LEI0101	0.47	0.03	4	APL1q	0.47	0.03	8
WAG39B16	GGA1q	MCW0068	0.48	0.03	10	APL1q	0.48	0.03	3
WAG26C18	GGA1q	MCW0200	0.58	0.02	9	APL1q	0.64	0.08	6
WAG12H11	GGA1q	LEI0091	0.63	0.04	8	APL1q	0.75	0.09	5
WAG28I14	GGA1q	LEI0169	0.69	0.02	6	APL1q	0.68	0.03	7
WAG65E16	GGA1q	LEI0107	0.73	0.02	8	APL1q	0.73	0.05	5
WAG107M5	GGA1q	ADL0101	0.90	0.04	8	APL1q	0.89	0.03	7
WAG23I2	GGA2p	MCW0082	0.06	0.02	8	APL2p	0.09	0.02	8
WAG19L16	GGA2p	ADL0309	0.21	0.03	3	APL2p	0.27	0.06	5
WAG40A2	GGA2p	ADL0176	0.25	0.03	6	APL2p	0.23	0.04	4
WAG41H16	GGA2p	MCW023	0.25	0.04	7	APL2p	0.24	0.04	11

GGA BAC clone	GGA chromosome	Marker	GGA FLpter	GGA FLpter SD	GGA metaphases	APL chromosome	APL FLpter	APL FLpter SD	APL metaphases
		9							
WAG26E13	GGA2p	MCW0131	0.25	0.03	5	APL2p	0.25	0.04	9
WAG85M7	GGA2p	ADL0120	0.27	0.02	3	APL2p	0.27	0.06	7
WAG82N9	GGA2p	MCW0274	0.29	0.03	3	APL2p	0.29	0.04	3
WAG15I23	GGA2p		0.31	0.04	11	APL2p	0.41	0.04	9
WAG40I2	GGA2p		0.34	0.03	10	APL2p	0.39	0.06	7
WAG18L21	GGA2q		0.36	0.03	10	APL2p	0.37	0.03	9
WAG9L1	GGA2q	LEI0129	0.42	0.03	8	APL2p	0.31	0.03	6
WAG41G5	GGA2q		0.42	0.03	11	APL2p	0.33	0.07	8
WAG41E24	GGA2q		0.51	0.04	10	APL2q	0.55	0.04	11
WAG41C2	GGA2q	LEI0147	0.67	0.03	2	APL2q	0.69	0.04	8
WAG65M19	GGA2q	ABR0008	0.68	0.02	7	APL2q	0.67	0.05	9
WAG26A22	GGA2q	MCW0157	0.96	0.02	7	APL2q	0.96	0.02	7
WAG29L12	GGA3	MCW0261	0.09	0.04	7	APL3	0.12	0.04	8
WAG36I7	GGA3	ADL0370	0.24	0.05	6	APL3	0.28	0.03	5
WAG9P24	GGA3	MCW0127	0.55	0.04	4	APL3	0.60	0.04	2
WAG112C24	GGA4p	ADL0317	0.05	0.02	8	APL10	0.45	0.05	6
WAG24P2	GGA4p		0.06	0.02	9	APL10	0.80	0.09	8
WAG23C4	GGA4p		0.06	0.03	9	APL10	0.90	0.06	5
WAG23I6	GGA4p	ADL0203	0.12	0.02	6	APL10	0.34	0.06	4
WAG18H15	GGA4p		0.15	0.04	10	APL10	0.24	0.08	6
WAG13I5	GGA4q		0.40	0.06	12	APL4	0.52	0.07	9
WAG22J17	GGA4q		0.47	0.05	15	APL4	0.17	0.07	8

GGA BAC clone	GGA chromosome	Marker	GGA FLpter	GGA FLpter SD	GGA metaphases	APL chromosome	APL FLpter	APL FLpter SD	APL metaphases
WAG23K3	GGA4q		0.53	0.04	9	APL4	0.34	0.05	7
WAG36E8	GGA4q		0.56	0.03	7	APL4	0.61	0.07	10
WAG21J21	GGA4q		0.58	0.03	7	APL4	0.25	0.08	10
WAG75K23	GGA4q		0.88	0.06	12	APL4	0.86	0.05	8
WAG13E2	GGA4q	LEI0063	0.89	0.04	6	APL4	0.92	0.04	5
WAG18I11	GGA4q		0.93	0.03	9	APL4	0.92	0.03	10
WAG37H20	GGA5	MCW0263	0.24	0.06	4	APL5	0.22	0.07	4
WAG40L16	GGA5	MCW0193	0.38	0.06	3	APL5	0.46	0.08	5
WAG27H3	GGA5	ROS0013	0.42	0.06	8	APL5	0.51	0.06	11
WAG03K18	GGA5	MCW0210	0.62	0.05	7	APL5	0.54	0.03	4
WAG89B24	GGA5	MCW0113	0.70	0.06	9	APL5	0.71	0.07	6
WAG03O23	GGA5	ADL0166	0.81	0.08	5	APL5	0.72	0.08	5
WAG59L17	GGA6		0.45	0.11	11	APL6	0.41	0.13	4
WAG30C21	GGA6	ADL0040	0.49	0.07	4	APL6	0.36	0.10	4
WAG21C4	GGA6		0.62	0.12	10	APL6	0.65	0.10	5
WAG59C5	GGA6		0.80	0.07	10	APL6	0.73	0.11	10
WAG69P21	GGA7	LEI0064	0.08	0.05	6	APL7	0.26	0.07	11
WAG59H11	GGA7		0.17	0.07	12	APL7	0.33	0.11	8
WAG38B23	GGA7		0.40	0.13	10	APL7	0.38	0.11	7
WAG27C3	GGA7	MCW0201	0.44	0.07	3	APL7	0.46	0.03	2
WAG41A15	GGA7		0.45	0.11	11	APL7	0.54	0.09	10
WAG44J7	GGA7		0.64	0.06	4	APL7	0.59	0.08	4
WAG15E10	GGA7		0.86	0.05	8	APL1q	0.84	0.07	9

GGA BAC clone	**GGA chromosome**	**Marker**	**GGA FLpter**	**GGA FLpter SD**	**GGA metaphases**	**APL chromosome**	**APL FLpter**	**APL FLpter SD**	**APL metaphases**
WAG29C17	GGA8	MCW0275	0.14	0.03	6	APL8	0.45	0.04	3
WAG59F7	GGA8		0.29	0.09	11	APL8	0.50	0.14	8
WAG60M16	GGA8	ADL0302	0.45	0.08	3	APL8	0.54	0.05	10
WAG21G17	GGA8		0.61	0.10	9	APL8	0.63	0.10	6
WAG64N5	GGA8		0.72	0.14	9	APL8	0.74	0.09	11
WAG43P21	GGA8	ADL0301	0.75	0.05	3	APL8	0.87	0.07	4
WAG37E21	GGA8		0.80	0.11	9	APL8	0.87	0.06	10
WAG40M19	GGAZ		0.08	0.04	14	APLZ	0.91	0.04	8
WAG17D3	GGAZ		0.11	0.04	19	APLZ	0.91	0.04	8
WAG41P6	GGAZ		0.27	0.04	10	APLZ	0.69	0.08	10
WAG19N8	GGAZ		0.48	0.05	10	APLZ	0.56	0.06	10
WAG20L10	GGAZ		0.74	0.05	10	APLZ	0.11	0.06	11
WAG22P4	GGAZ		0.80	0.06	8	APLZ	0.16	0.06	9

Table 2: Markers on duck (*Anas platyrhynchos*; APL) microchromosomes. BACs with these markers successfully hybridized to duck chromosomes. Duck chromosome number after APL10 was assigned as chicken (GGA) chromosome number plus one.

GGA chromosome	Marker / gene	APL chromosome
GGA9	ROS0078	APL9
GGA9	ADL0191	APL9
GGA9	MCW0134	APL9
GGA10	B2Msts1	APL 11
GGA10	ADL0272	APL11
GGA10	CYP19	APL11
GGA10	MCW0035	APL 11
GGA10	MCW0132	APL11
GGA10	MCW0003	APL11
GGA11	ADL0308	APL12
GGA12	SCW0019	APL13
GGA12	LEI0099	APL13
GGA13	MCW0244	APL14
GGA13	MCW0213	APL 14
GGA13	MSX2sts1	APL 14
GGA13	CAMLGsts1	APL14
GGA13	POU4F3sts1	APL14
GGA14	MCW0296	APL15
GGA14	GCT0908	APL15
GGA14	ADL0200	APL15
GGA15	LEI0083	APL16
GGA15	MCW0031	APL16
GGA15	LEI0120	APL16
GGA15	SFPQ	APL16
GGA15	ABR0070	APL16
GGA15	MCW0211	APL16
GGA15	MCW0080	APL16
GGA15	CRYBA4sts1	APL16
GGA16	MCW0371	APL17
GGA16	LEI0258	APL17
GGA17	ADL0149	APL18
GGA17	MCW0151	APL 18
GGA18	ADL0184	APL 19
GGA18	MCW0045	APL 19
GGA18	HUJ0010	APL19
GGA18	ROS0022	APL19
GGA18	ADL0290	APL19
GGA18	MCW0219	APL19
GGA18	ROS0027	APL19
GGA19	CTG1704	APL20

GGA chromosome	Marker / gene	APL chromosome
GGA19	CTG7040	APL20
GGA19	MCW0256	APL 20
GGA19	SCW0024	APL20
GGA20	GTC20050	APL21
GGA20	MCW0119	APL21
GGA20	ADL0193	APL21
GGA20	ADL0034	APL21
GGA20	FZFsts1	APL21
GGA21	AGRIN	APL22
GGA21	PLOD	APL22
GGA22	ROS0073	APL23
GGA22	TVSB3sts1	APL23
GGA23	CTG1080	APL24
GGA23	CTG1100	APL24
GGA23	ADL0262	APL24
GGA23	LEI0102	APL24
GGA23	MCW0165	APL24
GGA23	ADL0289	APL24
GGA23	LEI0339	APL24
GGA23	LEI0090	APL24
GGA24	ROS0113A	APL25
GGA24	ROS0123	APL25
GGA24	APOA1sts	APL25
GGA24	LEI0069	APL25
GGA26	CTG1120	APL27
GGA26	ABR0330	APL27
GGA26	MCW0286	APL27
GGA26	MCW0069	APL27
GGA26	LEI0074	APL27
GGA27	MCW0146	APL28
GGA27	MCW0328	APL28
GGA27	GCT0022	APL28
GGA28	CTG0070	APL29
GGA28	LEI0135	APL29
GGA28	ABR0341	APL29
GGA28	ABR0032	APL29
GGA28	ABR0054	APL29
GGA28	GCT0902	APL29
GGA28	ADL0299	APL29
GGA28	LEI0067A	APL29

Table 3: CNVs detected in duck (*Anas platyrhynchos*) relative to chicken reference DNA. The 5 CNVs overlapping the 5 potential hotspots from [2] are bolded.

CNV	Chicken chromosome	Start (bp)	Stop (bp)	Size (kb)	Fold change c.f. Red Junglefowl	Comments
1[ab]	**1**	**140580435**	**140655038**	**74.603**	**-4.23**	**Novel genes**
2	3	107012500	108262500	1250	-1.43	Mitochondrial oxidoreductase (β-oxidation cycle)
3[ab]	**3**	**113237500**	**113651334**	**413.834**	**-1.73**	**39S ribosomal protein L19 (ribosomal subunit)**
4	3	113605240	113652668	47.428	-3.88	39S ribosomal protein L19 (ribosomal subunit)
5[b]	**4**	**88710224**	**89072982**	**362.758**	**-1.61**	**Histone demethylation protein**
6	6	37387597	37400184	12.587	-1.86	
7	7	352	5048	4.696	-2.66	
8	7	38350336	38380092	29.756	-2.85	N-acetyltransferase 5 (acetylation of amino-terminal methionine residues)
9[a]	8	102	35016	34.914	-3.54	
10	10	252	27706	27.454	-1.54	Novel genes
11	10	22517883	22537510	19.627	-2.26	
12[a]	11	202	15241	15.039	-8.50	
13	11	12500	62500	50	-1.41	Novel genes
14[a]	11	2675046	3190227	515.181	-2.76	Centromere
15[a]	12	12500	62500	50	-2.86	Senescent cell antigen-like-containing domain protein (Plays a role in modulating cell spreading and migration)
16	13	4932529	4935329	2.8	3.75	Teneurin 2 (transcription factor activity)
17[b]	13	18865302	18907544	42.242	-2.28	
18	15	10240354	10295196	54.842	2.29	Novel genes
19[b]	**16**	**352**	**432851**	**432.499**	**-1.43**	**Covers all GGA16 sequence available; MHC**
20	16	262500	287500	25	1.50	Predicted similar to MHC Rfp-Y class I alpha chain
21[a]	17	802976	867945	64.969	1.99	Neural proliferation differentiation and control protein
22	18	1812500	1837500	25	-1.46	Novel genes
23	19	3665182	3845095	179.913	1.48	Myosin regulatory light chain, cardiac muscle isoform

CNV	Chicken chromosome	Start (bp)	Stop (bp)	Size (kb)	Fold change c.f. Red Junglefowl	Comments
24	22	3912586	3935246	22.66	1.43	Similar to 2-oxoglutarate dehydrogenase (TCA cycle)
25	26	481	5060	4.579	-1.92	
26	26	4577801	4750398	172.597	1.62	Forkhead box protein; MyoD family inhibitor (transcriptional regulators)
27[a]	26	2911	837508	834.597	-1.79	Novel proteins
28[a]	28	2	12526	12.524	-7.87	
29	28	12500	62500	50	-2.48	Major vault protein (ribonucleoprotein complex)
30	E64	402	45047	44.645	-1.43	Predicted similar to ligase (DNA replication)
31	Z	2090459	6530135	4439.676	1.59	Synaptotagmin-4 (neurotransmitter secretion)
32[ab]	**Z**	**71512500**	**71762500**	**250**	**-5.09**	**Novel genes**

[a] CNVs found in both individuals

[b] CNVs also seen in turkey [2]

Published and Accepted Abstracts

Genome organisation in the nuclei of avian cells: Implications for chromosome evolution

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Genome organisation is recognised as playing an important role in development, disease and evolution. As a key agricultural animal, the chicken (*Gallus gallus*) has the best studied avian genome organisation, with a distinction between the macro- and micro-chromosomes apparent during interphase. The macrochromosomes tend to reside at the periphery of the nucleus, while the microchromosomes are more internal. The precise positioning of the smaller macrochromosomes has not previously been determined.

Here, the macrochromosome territories in chicken are defined, and work is also presented extending the study to another Galliform (turkey; *Melegris gallopavo*) and an Anseriform (duck; *Anas playtyrhynchos*). This gives a clearer overview of the evolutionary basis of chromosome positioning at interphase, shedding light on the ancestral form.

Certain ancestral chromosomes have fused independently several times during avian evolution. One hypothesis to explain this phenomenon is that the ancestral chromosomes lie close to one another in interphase nuclei. This hypothesis was tested by examining the positions of relevant chromosomes in species where the chromosomes have remained separate, compared to those that have fused. Evidence suggests that ancestral chromosomes 4 and 10 lie together during interphase in turkey (which retains the ancestral form) thereby explaining why they are prone to fusion e.g. as they appear in chicken, goose and African collared dove. Spatial analysis of other avian chromosomes prone to fusion is presented.

Nuclear (genome) organisation and comparative genomics in birds

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Nuclear, or genome organisation – the position of chromosome territories within the interphase nucleus - is recognised as playing an important role in development, disease and evolution. Two models for genome organisation suggest that chromosomes can be organised either by chromosome size or by gene density. As a key model species and agricultural animal, the chicken (*Gallus gallus*) has the best studied avian genome organisation; the macrochromosomes tend to reside at the periphery of the nucleus, while the microchromosomes are more internal. The precise nuclear positioning of the smaller macrochromosomes and microchromosomes in chicken has not however been determined, nor has the model it fits more closely.

Here, nuclear addresses of macro- and microchromosome territories in chicken were determined by measuring the positions of BAC clones within fibroblast nuclei. The areas of metaphase chromosomes were measured, and gene densities were calculated. The chromosomes were ordered by size and by gene density to determine which model chicken follows more closely; results suggest no specific preference for either model. This study was also carried out in turkey (*Meleagris gallopovo*) and duck (*Anas platyrhynchos*), using the same BACs applied to chicken, to determine whether chromosomes involved in fusions or fissions occupy different positions in their ancestral and derived states.

Molecular cytogenetic maps of turkey, duck and zebra finch and their implications for genome evolution

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Comparative genomics allows the transfer of genomic information from a well characterised genome to one that is less well understood. In birds, most comparative genomics to date has been with reference to the chicken, an important model organism and the only bird with a published genome sequence. Comparative chromosome painting studies have demonstrated that inter-chromosomal rearrangements in birds are rare. Higher resolution studies involving the hybridisation of chicken BAC clones have allowed comparative physical maps to be created. These provide information on the intra-chromosomal rearrangements through evolution. Here, we report the current state of the art in comparative physical mapping in birds and the insights it provides on genome evolution. Comparative maps have been generated for the turkey (*Meleagris gallopavo*) and for duck (*Anas platyrhynchos*). We have also successfully mapped chicken clones to the Zebra Finch (*Taeniopygia guttata*) as a prelude to the generation of a comparative map. Breakpoint mapping using FISH and microarrays suggests that, in birds, fusions and fissions (but not inversions) have occurred at ancestral centromeres.

Manual vs. automated methods to assess nuclear organisation

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The position of individual chromosome territories and individual loci in the interphase nucleus has been the topic of much interest in recent years. Patterns of chromosome/locus position are widely accepted as indicators of nuclear (genome) organisation and indicators of "nuclear health." A number of approaches for generating 3D extrapolations from 2D flattened nuclei have been reported and the vast majority involve the overlaying of five "shells" of equal area. In order to generate data from previously captured images we have used a "manual" approach where the images were transferred to "Paint Shop Pro" and a five circle template was used. Here we introduce new software "Macro" that automatically divides nucleus into five concentric rings in order to determine the localisation of chromosome territories in interphase nucleus. We have applied this for the study different avian species as well as humans. The macro detects the nuclear periphery, and divides the nucleus into five rings of equal area using "ImageJ". The macro outputs the percentage of the total signal within each ring for the red, green and blue channels. The user can score chromosome position and perform statistical analyses in a spreadsheet thus reducing analysis time and increasing the accuracy of scoring for position of chromosome territories.

Evolution of the avian genome as revealed by molecular cytogenetics

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In avian genome evolution, the diploid number of $2n\approx80$ has remained remarkably constant with 63% of birds having $2n=74\text{-}86$. The most studied species is the chicken and molecular cytogenetic probes generated by us from this species have been used to understand further the evolution of the avian genome. The ancestral karyotype is very similar to that of the chicken, with chicken chromosomes 1, 2, 3, 4q, 5, 6, 7, 8, 9, 4p and Z representing the ancestral avian chromosomes 1-10+Z. Avian evolution occurred primarily in three stages: divergence of the Paleognathae (emu, ostrich etc.); divergence of the Galloanserae (chicken, goose etc.); and divergence of the higher "land" and higher "water" birds. Other than sex chromosome differentiation in the first divergence there are no specific changes associated with any of these evolutionary milestones although certain groups (e.g. the Falconiformes and the Psittaciformes) have undergone multiple fusions (and some fissions). Most changes seem to involve chromosomes 1, 2, 4, 6-9, 10 and Z and there are several convergent events. Of these, the most puzzling involves chromosomes 4 and 10, which appear to have undergone multiple fissions and fusions throughout evolution. The use of microarrays and detailed cytogenetic maps is shedding further light on avian genome organization and evolution.

How conserved are bird genomes? Insights from the chicken and zebra finch genome projects

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Comparative molecular cytogenetics has suggested that avian genomes are highly evolutionarily conserved. That is, few interchromosomal rearrangements have been detected among the macrochromosomes, and none among the microchromosomes. Few gross karyotypic rearrangements have been observed between chicken and zebra finch (these being the fission of ancestral chromosome 1 in the zebra finch, and the retention of ancestral chromosomes 4 and 10, as opposed to their fusion in chicken).

The availability of complete genome sequences for chicken and zebra finch now allows us to investigate the chromosomal rearrangements between the two species at a much higher resolution than previously possible for any avian species.

We have used bioinformatics to align the sequences of whole orthologous chromosomes, thereby identifying potential regions of chromosomal rearrangements. Based on this data, BAC clones covering regions of interest were selected in both species, and hybridised to metaphases from their respective species.

Our results indicate that there are more interchromosomal rearrangements than previously thought. In addition, such in silico approaches allow for the identification of different classes of rearrangements, such as translocations and inversions

Comparative genomics in chicken and Pekin duck using FISH mapping and microarray analysis

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The Pekin duck (*Anas platyrhynchos*) is an obvious target for comparative genomic studies due to its agricultural importance and resistance to avian flu. We have developed a comprehensive molecular cytogenetic map of the duck genome through FISH assignment of 155 chicken BAC clones which identified one inter- and six intrachromosomal rearrangements between chicken and duck macrochromosomes. Conserved synteny was demonstrated among all microchromosomes analysed.

We also used array comparative genomic hybridisation (array-CGH) to analyse copy number variants (CNVs) between chicken and duck. CNVs have been found to contribute significantly to normal and disease-related genetic and phenotypic variation in humans and other primates. Our array-CGH experiments revealed 32 CNVs, of which 5 overlap previously designated "hotspot" regions between chicken and turkey. The CNV data extend previous analyses in chicken and turkey and support the hypotheses that avian genomes contain fewer CNVs than mammalian genomes and that genomes of evolutionarily distant species share regions of copy number variation ("CNV hotspots"). Taken together, our results suggest extensive conservation of avian genomes across 90 million years of evolution, both in terms of large scale rearrangements and in CNVs.

An overview of copy number variation in birds

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One of the most exciting recent advances in genomics has been the realisation that copy number variants (CNVs) contribute substantially to normal and disease related phenotypic variation in humans. However studies of CNVs among vertebrates have been limited mainly to primates and mice. Birds have several contrasting genomic features when compared to mammals: a small, conserved genome size, a low repeat content and few chromosomal rearrangements (probably related to an evolutionary constraint on genome size). With respect to CNVs in birds this suggests that (1) there are fewer CNVs in avian genomes than in mammalian genomes; (2) only CNVs with functional effects have been retained through evolution; (3) CNVs should be on average smaller than those seen in mammals. Besides, previous studies of CNVs between chicken, turkey and duck have indicated the existence of CNV 'hotspots' (i.e genomic regions prone to independent recurrent copy number variation).

To test these hypotheses, we have conducted a broad survey of CNVs in 10 bird species using array-comparative genomic hybridisation on a chicken oligonucleotide microarray. This revealed 277 CNVs within 122 non-overlapping copy number variable regions (CNVRs). 43% of CNVRs were shared between two or more species. 80% of the CNVs were associated with Ensembl genes suggesting that they may have functional effects. Comparison with human CNV data gained with similar detection platforms suggest that, though preliminary, these data support the hypotheses outlined above, and that birds will make a useful model group for comparative studies of copy number variation

A detailed appraisal of nuclear organisation in embryonic fibroblasts of chicken, turkey and duck

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The organisation of the genome can be considered at a number of levels including the karyotype, gene order and organisation of chromosome territories within the interphase nucleus (nuclear organisation). Studies of nuclear organisation have focussed largely on mammals, and have demonstrated its importance in development, disease and evolution. Data on nuclear organisation in birds (and indeed other non-mammalian vertebrates) are limited to chicken, with information on individual chromosome territories available for macrochromosomes only. We have therefore performed a detailed study of individual chromosome territories by hybridisation of BAC clones from chicken chromosomes 1-28 to chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and duck (*Anas platyrhynchos*) embryonic fibroblast nuclei. Nuclear organisation was consistent with a chromosome size-based pattern in all three species. In chicken, where gene density information is available, we found the data fitted a chromosome size based organisation better than a gene-density based organisation, although we cannot strictly rule out that this may be partially due to inaccuracies in the current Ensembl chicken gene-build. Together with previously published data on nuclear organisation in mammals, our data provide evidence of conservation of the principles underlying nuclear organisation across 310 million years of evolution.