Did the evolution of multiple microchromosomes help save bird and other dinosaurs from extinction?

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Abstract: Birds currently number ~11,000 species and, despite enormous biodiversity, have numerous class-specific characteristics including feathers, flight, nesting, brooding, longevity and a very distinctive karyotype. Central to several academic fields including developmental biology, agriculture, virology, neurobiology, evolution, ecology and conservation, many birds have a fully-sequenced genome assemblies; aligning these to the karyotype (chromosome level assembly), is key to understanding avian biology and evolution. The "signature" avian karyotype (~2n=80) of these Therapod dinosaurs, many of which are threatened with extinction, is defined by ~30 microchromosomal pairs and ~10 larger macrochromosomes. Studies of phylogenetic relationships suggest that the domestic chicken (2n=78 - the most studied species) has close to an ancestral genome organization, from which all others are measured. Comparative genomics using whole chromosome painting and selected locus specific probes have been used to map chromosome rearrangements throughout evolution and indicate that the signature avian-karyotype first appeared before the dinosaurs emerged 240MYA. Some, if not many, extinct dinosaurs therefore probably had this unique karyotype. Why it appeared and persisted may be due to a) evolutionary advantages to retaining it, and/or b) a lack of opportunity for change. Dinosaurs survived several extinction events, mostly recently evolving as birds and, here, we review research suggesting that a contributory factor to dinosaur survival may have been this unique means of genome organization. Of note is the large number of microchromosomes, which provide the substrate for phenotypic variation, the driver of evolutionary change, through increased random segregation and genetic recombination compared to other groups or vertebrates.

1. The Biology, Diversity and Evolution of Birds: Where do the Microchromosomes Fit In?

Birds (currently as much as 11,000 living species) have developed a unique set of traits that are not found in other vertebrates: these include feathers, a lightweight skeletal structure, the ability to fly (with exceptions like penguins and ratites), oviparity, nesting behaviours, a beak without teeth, and a relatively elevated metabolic rate. Their core body temperature is notably high (between 39 and 41 °C), along with elevated blood glucose levels and energy expenditures that can be five times greater than typical mammalian rates. Interestingly, despite their higher energy requirements, birds of comparable size generally also have longer lifespans than mammals (Holmes and Ottinger, 2003). Another distinctive feature is their unique genome organization (karyotype) with a large number (around 30 pairs) of tiny microchromosomes. This "so many, so small" pattern (Fig. 1) is uniquely bird-like (reviewed in O'Connor et al., 2024) and this review considers how, when and why this pattern emerged and persisted.

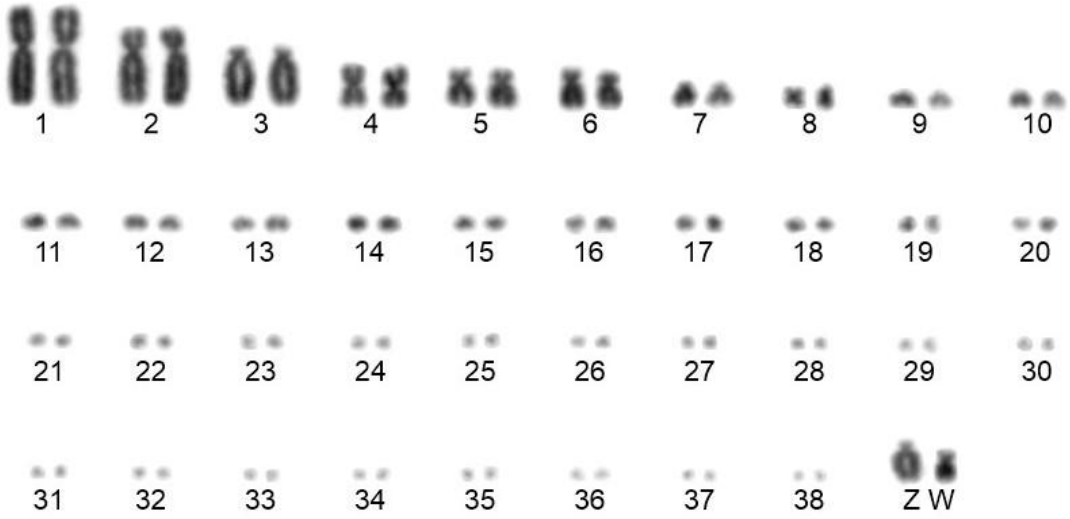

Fig. 1. A typical bird karyotype with 2n = 78. Data from Kretschmer et al. (2018). Karyotype constructed for this article by Victor Cruz Cuervo.

Birds serve as model organisms primarily in research fields such as developmental biology, neuroscience, immunology and virology (Dodgson and Romanov, 2004); they play a vital role in agriculture, contributing to both meat and egg production (chicken, duck, turkey, etc.) and some are valued as companion animals (Griffin et al., 2018). Birds are found in virtually all terrestrial environments and many aquatic ones, having adapted to extreme climates ranging from the middle of Antarctica to tropical regions, the greatest biodiversity occurring in tropical areas (Weir and Schluter, 2007). They exhibit remarkable phenotypic diversity; for instance, their sizes span from the tiny bee hummingbird (*Mellisuga helenae*), measuring about 5 cm, to the ostrich (*Struthio camelus*), which can exceed 2 metres in height (Weir and Schluter, 2007) (Fig. 2a). As we will explore in this article, however, their karyotype (the arrangement of their chromosomes, and thus their overall genomic structure) is remarkably similar from species to species, especially among the microchromosomes. The apparent paradox of karyotypic stability in a background of phenotypic diversity (Fig. 2b) and evolutionary survival is, herein, explored with evidence largely from studies that link karyotype to genome assembly.

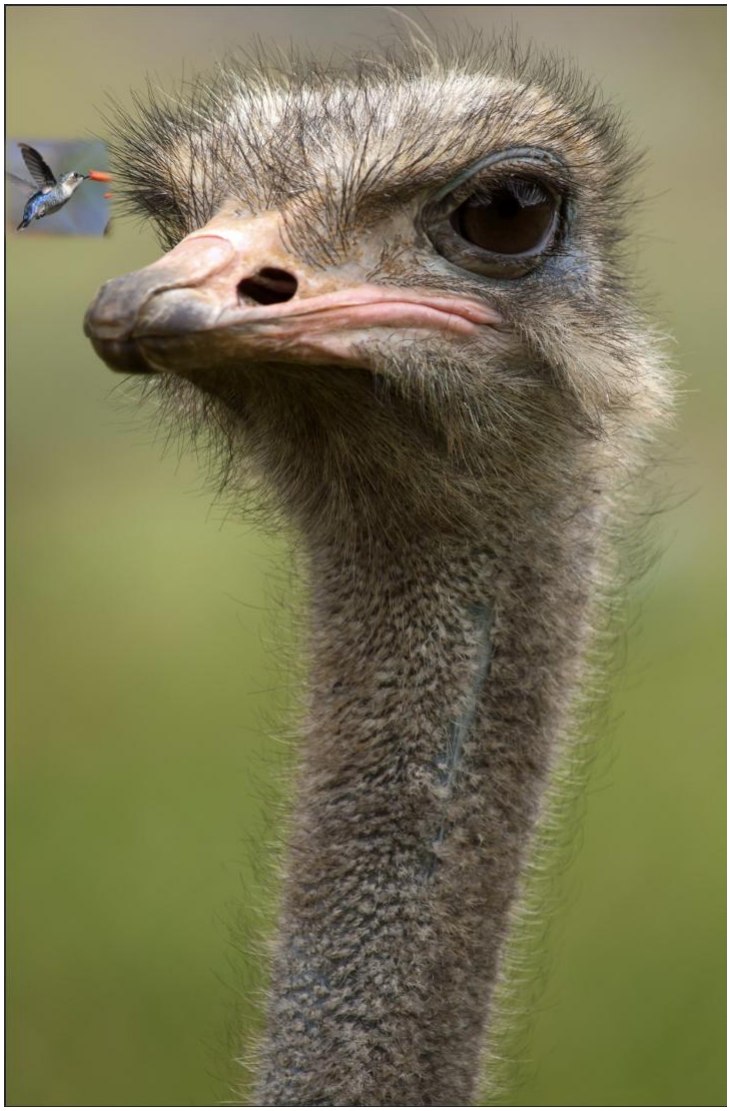

Fig. 2a. Images of the world's smallest and largest birds, approximately to scale (from Wikimedia Commons, CC-BY-2.0). The Bee Humming Bird *Mellisuga helenae* (https://commons.wikimedia.org/wiki/File:Mellisuga_helenae_(16626753658).jpg, by Ekaterina Chernetsova (Papchinskaya), 2015) – top left inset, at around 5 cm, is roughly the size of the eye of an ostrich *Struthio camelus* which stands at about 2 m tall (https://commons.wikimedia.org/wiki/File:Struthio_camelus_portrait_Whipsnade_Zoo.jpg, by William Warby, 2010). Smaller image cropped and scaled; larger image cropped.

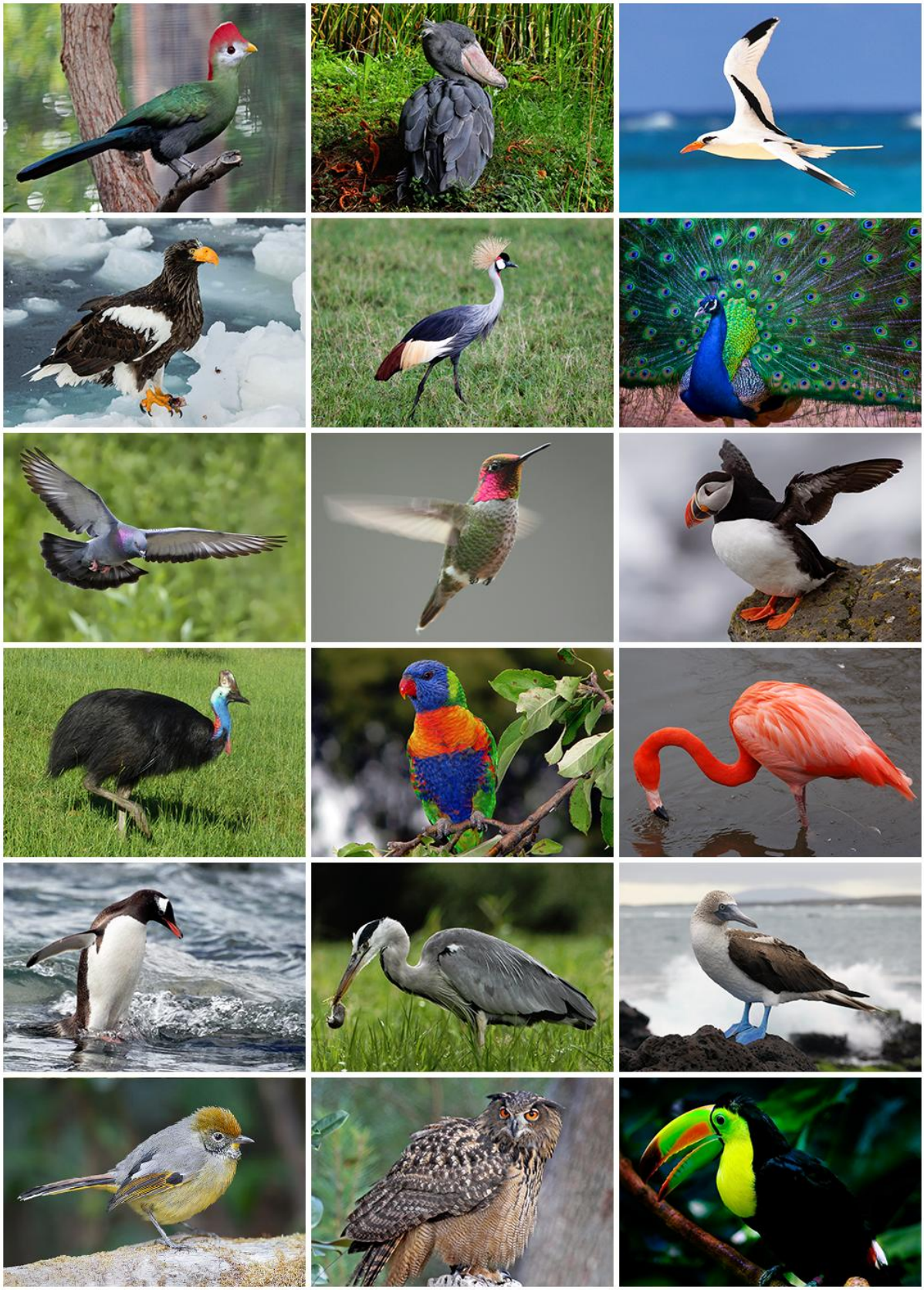

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Fig. 2b. The phenotypic diversity of birds. A composite image from Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Bird_Diversity_2013.png, by Concerto, 2013, CC-BY-SA-3.0; from top to bottom and from left to right):

- Row 1: Red-crested Turaco (*Tauraco erythrolophus*, Musophagiformes), Shoebill (*Balaeniceps rex*, Pelecaniformes), White-Tailed Tropicbird (*Phaethon lepturus*, Phaethontiformes);
- Row 2: Steller's Sea Eagle (*Haliaeetus pelagicus*, Accipitriformes), Grey Crowned Crane (*Balearica regulorum*, Gruiformes), Indian Peafowl (*Pavo cristatus*, Galliformes);
- Row 3: Rock Dove, or Pigeon (*Columba livia*, Columbiformes), Anna's Hummingbird (*Calypte anna*, Apodiformes), Atlantic Puffin (*Fratercula arctica*, Charadriiformes);
- Row 4: Southern Cassowary (*Casuarius casuarius*, Casuariiformes), Rainbow Lorikeet (*Trichoglossus haematodus moluccanus*, Psittaciformes), American Flamingo (*Phoenicopterus ruber*, Phoenicopteriformes);
- Row 5: Gentoo Penguin (*Pygoscelis papua*, Sphenisciformes), Grey Heron (*Ardea cinerea*, Pelecaniformes), Blue-footed Booby (*Sula nebouxii*, Suliformes);
- Row 6: Bar-throated Minla (*Minla strigula*, Passeriformes), Eurasian Eagle-Owl (*Bubo bubo*, Strigiformes), Keel-billed Toucan (*Ramphastos sulfuratus*, Piciformes).

An alarming number of birds (~1,400 species >10% of the total) risk extinction; >160 species becoming extinct in the past 500 years (for examples, see Fig. 3). Many are attributed to human-induced climate change and habitat loss (BirdLife International, 2022). Gaining a deeper insight into the developmental genomics of birds from an evolutionary perspective is a significant aspect of safeguarding existing species from further threats. Extinction is a pervading theme of this article,

which concludes by speculating whether large numbers of microchromosomes is related to evolutionary survival.

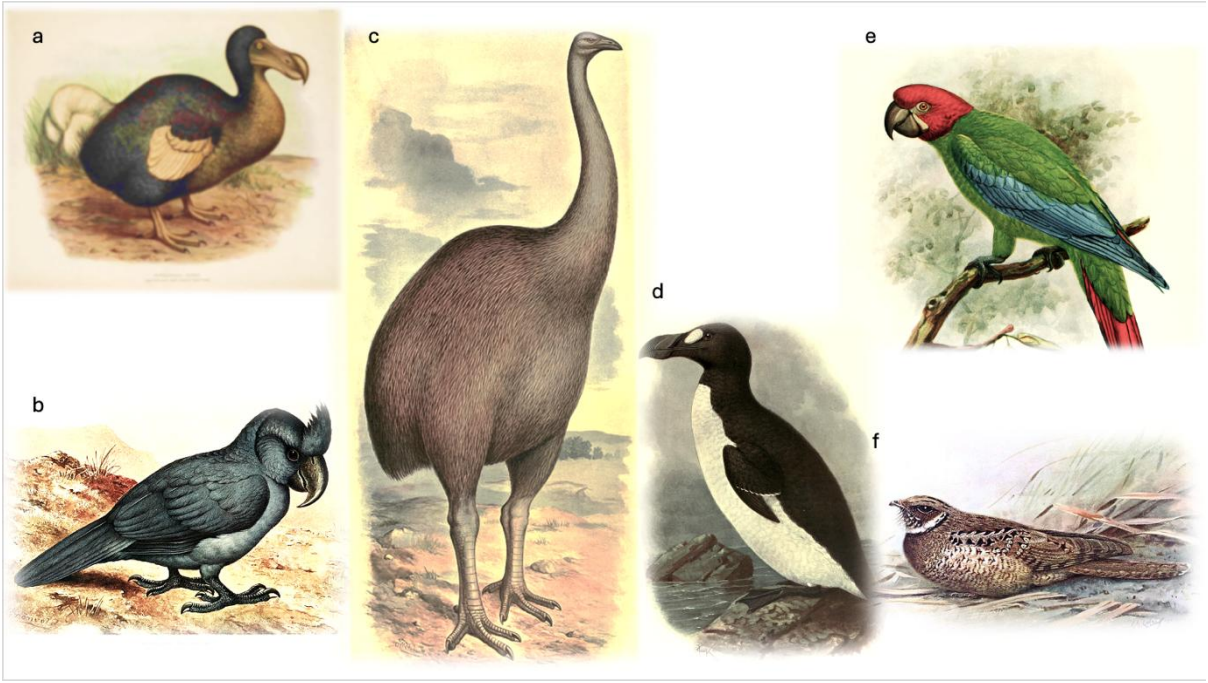

Fig. 3. Examples of extinct birds (from: Rothschild, L.W. (1907) Extinct Birds. London: Hutchinson & Co.; Wikimedia Commons, https://commons.wikimedia.org/wiki/Category:Extinct_Birds_(Rothschild_book), CC0 license; some images cropped): (a) Dodo (*Raphus cucullatus*), (b) Broad-billed Parrot (*Lophopsittacus mauritianus*), (c) Giant Moa (*Dinornis novaezealandiae*), (d) Great Auk (*Pinguinus impennis*), (e) Red-tailed Blue-and-Yellow Macaw, or Jamaican Red-tailed Macaw (*Ara erythrura*), (f) Jamaican Poorwill *(Siphonorhis americana)*.

Evolutionarily, birds are reptiles but constitute a distinct monophyletic group of the Class Aves and the Subclass Neornithes. Divergence of the synapsids (mammals and their extinct predecessors) plus the anapsids (turtles) along with the diapsids (other reptiles and birds) occurred approximately 310–350 million years ago (MYA), but birds trace their initial origins back to ~150 MYA during the late Jurassic period (Chiappe and Dyke, 2006). Birds represent the only surviving lineage of the dinosaur clade Theropoda and they are thus, themselves, dinosaurs. The fossil *Archaeopteryx lithographica*,

(Fig. 4a) which dates back approximately 150 MY serves as evidence of a transitional species bridging extinct dinosaurs and modern birds (Fig. 4, O'Connor et al., 2025). While initially thought to represent an early form of modern bird, characteristics such as a bony tail and teeth indicate that *A. lithographica* should not be classified as a true avian ancestor (Mayr et al., 2007). The oldest definitive fossil of Neornithes (modern birds) is a waterfowl bird, *Vegavis* (Fig. 4b), classified in the Anseriformes, and dating to around 67 MYA. This fossil supports the hypothesis that modern birds existed alongside non-avian dinosaurs before the Cretaceous–Paleogene (K–Pg) boundary and Chicxulub strike ~66 MYA (Clarke et al., 2005). Challenges in fossil dating, due to geographic and depositional sampling biases, have sparked considerable debate in palaeontology on this matter (Chiappe and Dyke, 2006), for instance, a water bird is more likely to fossilize to that living in an arid environment because of the increased frequency of mud deposition. Recently, the earliest short-tailed bird fossil *Baminornis zhenghensis* was discovered, originating from China in the Late Jurassic, demonstrating an earlier than previously appreciated appearance of avian features, and thus providing an earlier origin, and a radiation of, early birds during the Jurassic (Chen et al., 2025). In general terms, the ancestor of birds is generally believed to have been a bipedal, terrestrial relatively small dinosaur (Witmer, 2002), perhaps not dissimilar looking to a quail or primitive chicken (Fig. 4c, d). In this review, we consider what the karyotypes of these species might have looked like, as if we would have had the opportunity to make metaphase preparations and observe them down the microscope.

a **b** **c**

d

Fig. 4. Ancient proto-birds: a. *Archaeopteryx lithographica* (~150 MYA; reconstructed by DataBase Center for Life Science (DBCLS), 2020; https://commons.wikimedia.org/wiki/File:202010_Archaeopteryx_lithographica.png, CC-BY-4.0); b. *Vegavis,* the oldest fossil bird (~66MYA; AI rendition using DreamPhoto – composite of existing web-based images); c. a putative avian ancestor (terrestrial, feathered, small; Jurassic dinosaur, ~150MYA; AI rendition). Image created using AI (DreamPhoto) and based on lower image (d) of a feathered Therapod dinosaur: Shandong Tianyu Museum of Nature. Credit: https://commons.wikimedia.org/wiki/File:Feathered_theropod.jpg (by Bruce McAdam; CC-BY-SA-2.0).

Over the last century, a substantial amount of information has been accumulated regarding the karyotype, genetics, genome, transcriptome, proteome, physiology, biochemistry, developmental biology and evolution of multiple bird species. Perhaps the most significant breakthrough in this regard has been the chicken (*Gallus gallus*) genome project (Hillier et al., 2004). This, and

subsequent studies that arose from it, produced multiple genomic resources that can be applied in comparative studies of other birds and for illuminating key evolutionary, physiological and developmental processes. Domestic chickens and other key reference species play a crucial role in researching disease ecology of many avian species including the transmission of zoonotic diseases. For a full overview of genomic research and its relevance, the four reports on chicken genes and chromosomes provided a valuable resource (Schmid et al., 2000, 2005, 2015; Smith et al., 2022).

In this review, we largely consider one aspect of genomic analysis, that of "cytogenomics" (the interface of genomics and cytogenetics) and ask whether this provides any insight into the evolution, phenotypic variation and survival of all dinosaurs, birds included. To do this we first consider overall genome size of birds and other dinosaurs before moving to what molecular studies have told us about the taxonomy and evolution of birds. We then explore when and how large numbers of microchromosomes evolved, while apparently restrained by a small genome, their persistence of evolutionary time and speculate whether species most recently popularized by, for instance, Michael Critchton and Steven Spielberg, most likely had avian-like genomes.

2. Overall Genome Size

Birds are unique in their overall genome organization with features such as a small genome size which lead to a compact genomic structure, reduced numbers of repeats and gene duplications, and (as already mentioned) the presence of multiple microchromosomes. Among vertebrates, they have the most conserved genome size, with an average haploid genome size 1.45 picograms of DNA (1 picogram = 978 megabases) (Griffin et al., 2007). Genome sizes, typically represented as gametic nuclear DNA contents (known as 'C-values'), range from a low of 0.91 picograms in the black-chinned hummingbird (*Archilochus alexandri*) to a high of 2.16 picograms in the common ostrich (*Struthio camelus*). Generally, larger genomes are generally observed in flightless species. One hypothesis is that avian genomes evolved from an ancestral genome that underwent reduction due to the

physiological constraints associated with flying. This could, theoretically, drive selection for high metabolism and efficient flight capabilities. The relatively small avian genome sizes in birds is linked to the low abundance of repetitive sequences. Most avian genomes contain fewer repeat elements, averaging around 4% to 10%, in contrast to other tetrapod vertebrates such as mammals, where repeat content can range from 34% to 52%. However, there are notable exceptions, such as the downy woodpecker (*Picoides pubescens*) and the snowy owl (*Bubo scandiacus*). In the downy woodpecker (*Dryobates pubescens*), transposable elements (TEs) account for approximately 22% of the genome, primarily due to the expansion of chicken repeat 1 (CR1) transposons of the LINE (long interspersed elements) type specific to the species. For the snowy owl, repeat DNA makes up 28.34% of the genome, predominantly consisting of centromeric satellite DNA, which is believed to have originated from an endogenous retrovirus (ERV1) (Baalsrud et al., 2024). The larger genomes of the snowy owl and downy woodpecker thus argue against the correlation of small genomes with flight, large genomes and loss of flight and therefore this possible association is disputed. Research by Organ et al. (2007) suggested that bone cell size is closely correlated with genome size in both living vertebrates and extinct dinosaurs and birds. Reporting smaller cell sizes (and by extrapolation, genome sizes) in fossilized extinct dinosaurs, he suggests that small genome size may have pre-dated flight.

3. Comparative Avian Genomic Analysis and Phylogenomics

Beyond crude analyses of genome size, comparative genomics of individual chromosomes, blocks of synteny and genomic sequences can serve as a valuable complement to the fossil record, which may not fully represent avian ancestors (O'Connor et al., 2018; Duchêne et al., 2025). Indeed, avian genomics, initiated by the chicken genome project, significantly transformed the study of bird phylogenetics (Jarvis et al., 2014; Prum et al., 2015). Specifically, comparative genomics of examples of extant individuals can provide insight, by extrapolation, into the likely genomic characteristics of species and groups that are now extinct. Despite many years of research conducted by

222 developmental biologists, morphologists and molecular phylogeneticists, however, the evolutionary
223 relationships of modern birds are still under review. Disagreements in result interpretations can be
224 attributed, in part, to the variety of species sampled, the methodologies used in phylogenetic
225 analysis, and any specific genomic regions examined. Nonetheless, the paper of Jarvis et al. (2014)
226 and its subsequent updates (e.g., Jarvis et al., 2015) provide the most contemporary view of the
227 phylogenetic tree of birds. Several high-level clades are firmly established among modern birds
228 (Neornithes) namely: Palaeognathae (which includes tinamous and ratites), Galloanseres (such as
229 land and waterfowl), and Neoaves (which encompasses all other birds). . A more recent report by
230 Stiller et al. (2024) has refined avian phylogeny using >300 sequenced bird genomes and the
231 information from it led to the cladogram expressed in Fig. 5.

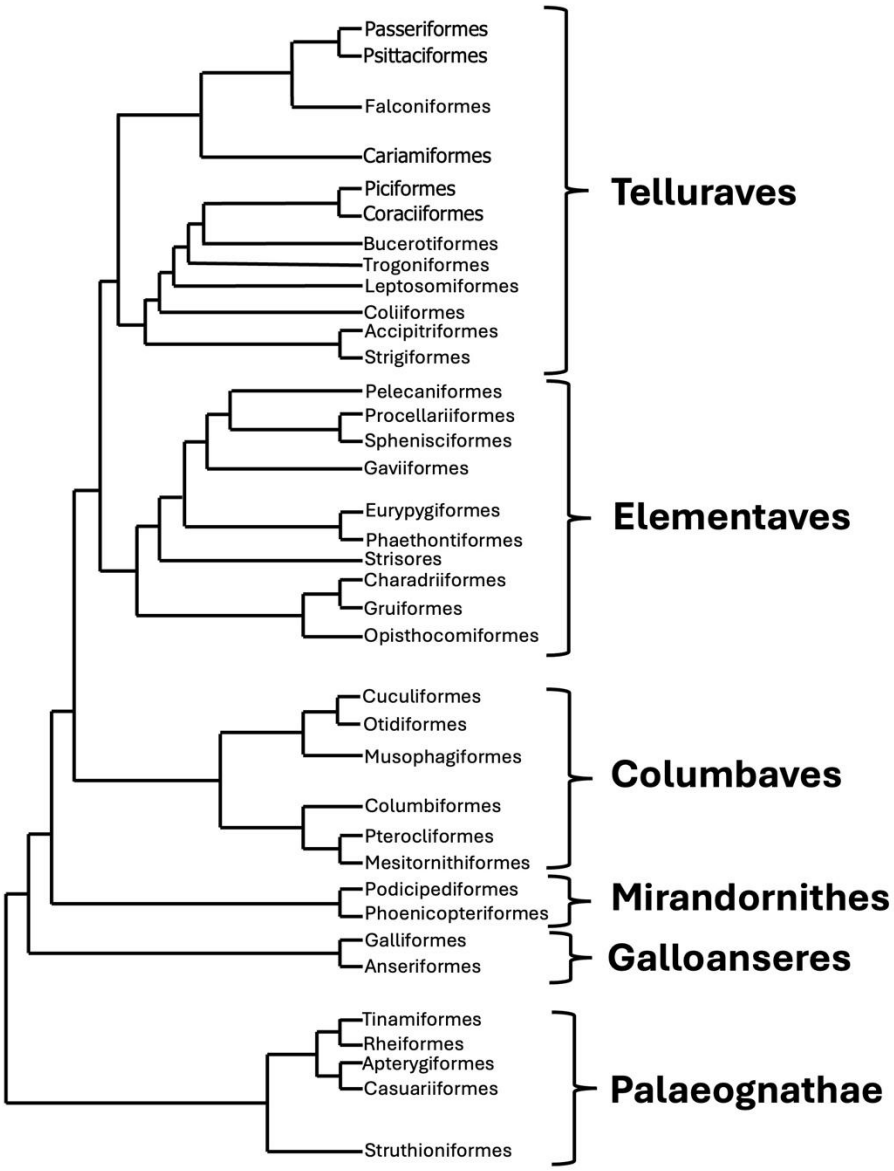

Fig. 5. The evolutionary tree of birds with latest groupings bracketed (adapted from Stiller et al., 2024).

Collectively, studies suggest that the Palaeognathae and Neognathae diverged ~100 MYA, the Galloanserae-Neoaves ~88 MYA, ratites and tinamous ~84 MYA. Ratites, however, appear to be paraphyletic – i.e., flightlessness has evolved convergently in this group (Cloutier et al., 2019). Galliformes (landfowl) and Anseriformes (waterfowl) diverged ~66 MYA and the primary divergence of Neoaves into Columbea and Passerea occurred before ~67–69 MYA. Recent fossil findings suggest that a significant radiation of advanced Ornithurae took place before the end of the Cretaceous

period, although this group experienced a sudden extinction during the K–Pg event, leading to their absence from the fossil record in the Paleogene (Longrich et al., 2011). According to genomic data from Jarvis et al. (2014) the K–Pg transition period was characterized by rapid speciation among Neornithines, with 36 lineages diverging over approximately 10-15 million years. They argue that these revised timelines question previous beliefs that Neornithine lineages experienced explosive diversification primarily after the K–Pg boundary. A core theme of this review is evolutionary studies that have aligned the karyotypes and genome assemblies, particularly the microchromosomes, of various species.

4. Aligning Physical Mapping, Genome Sequencing with Cytogenetics: Towards "Chromosome Level Assemblies"

A bacterial artificial chromosome (BAC)-based physical map of the entire genome of the chicken was originally merged with the genetic (linkage) map through the hybridization of probes containing molecular markers onto filter-spotted arrays (Lee et al., 2003; Ren et al., 2003; Romanov et al., 2003; Wallis et al., 2004). This methodology facilitated the alignment of the first- (Ren et al., 2003) and second-generation BAC-contig physical maps (Wallis et al., 2004) alongside the whole genome sequence to the linkage map, which enabled the physical assignment of BAC contigs to chicken chromosomes. The resulting integrated map encompasses approximately 91% of the chicken genome and has been instrumental in identifying chicken clones relative to positions in other genomes that have been fully sequenced. In addition, the chicken physical map was integrated with the chromosomal (cytogenetic) genome map. Numerous BACs corresponding to specific genes and markers have been hybridized using FISH across various chicken chromosomes (Sazanov et al., 2004a,b,c,d), with a detailed investigation of microchromosome 17 conducted through FISH (Romanov et al., 2005). These analyses revealed that the orientation of the chromosome 17 map is reversed compared to the orientation currently proposed for the linkage map and draft sequence. Confirmation of chromosome 17's reversed orientation and centromere location was achieved using

dual-colour fluorescence in-situ hybridization (FISH), employing terminal BACs and centromere-specific CNM oligonucleotides as probes (Romanov et al., 2005). A significant advantage of this cytogenomic approach (i.e. combining sequence-based information with FISH) is the enhanced alignment of sequence and linkage maps with chromosomal features, such as centromeres, telomeres, chromosome arms and staining patterns indicative of AT versus GC content. Incorporating these strategies helps to assess genomic changes efficiently within an evolutionary framework.

Indeed, the ultimate goal of any *de-novo* genome sequencing effort is a "chromosome-level assembly" with all the sequences correctly aligned and assigned on the correct chromosomes. This is a particular challenge in birds, because of all the microchromosomes (Damas et al., 2017; Deakin et al., 2023). Advancements in aligning the karyotypic structure of birds with the complete nucleotide sequence of whole genomes over the last 25 years are significantly enhancing our comprehension of population dynamics, evolutionary biology and genome function (reviewed in Griffin et al., 2024). By leveraging genomic databases, molecular cytogenetics ("cytogenomics" or "chromosomics" Deakin et al., 2019) can be advanced further, enabling deeper examination of chromosomal rearrangements and making them amenable to computational analysis (Volker et al., 2010; Griffin and Skinner, 2012; Damas et al., 2017). Such an approach is also beginning to offer new insights into the roles of repetitive sequences, transposable elements and the expansion of gene families as key evolutionary processes that promote diversification and adaptation (Deakin et al., 2019). There is still, however, a gap in our understanding of the functions of many genes, non-expressed sequences and unidentified regulatory components of the genome and this forms the basis of current research.

Avian genomes, being those of Therapod dinosaurs, are related to genomes of other reptiles such as crocodilians, turtles, lizard and snakes. They can be analysed by leveraging pivotal avian genomes like those of the chicken and zebra finch (the first two birds to be sequenced). Thereafter, by involving comparative genomic strategies, understanding of terrestrial vertebrate genome evolution, placing

294 extinct dinosaurs in their proper context, becomes possible (O'Connor et al., 2024). This review thus
295 examines the genomes of dinosaurs, both extinct and extant, through the lens of the chromosome
296 composition (karyotype). The karyotype serves as a low-resolution representation of the complete
297 genome of a eukaryotic organism (Griffin et al., 2007). Here, we present a thorough and up-to-date
298 synthesis of data obtained from a comparative analysis of the evolution of bird chromosomes,
299 utilizing techniques such as comparative chromosome painting (using chromosome paints of one
300 species, e.g., chicken, and applying it to the metaphases of others) and BAC mapping using FISH. The
301 insights discussed here thereafter serve as a valuable reference and information source, making
302 significant contributions to the field of avian comparative genomics and ultimately the composition
303 of the genomes of species that are no longer extant.
304

305 **5. Defining the Karyotype of Birds**

306 Fig. 1 shows a typical avian karyotype and illustrates that distinguishing the microchromosomes is
307 near-impossible because of their size, number and morphology. Therefore, although >1,000 partial
308 bird karyotypes have been published, most of these do not go beyond chromosome 10 or even
309 chromosome 5 (Degrandi et al., 2020). There is no clear dividing line in terms of size between macro
310 and microchromosomes and thus different studies report different number of macro vs. micro
311 chromosomes. Avian microchromosomes account for less than a quarter of the genome size, but are
312 gene-rich and, thus account for about half of the genes (McQueen et al., 1998; Smith et al., 2000;
313 Habermann et al., 2001; Burt, 2002). The karyotype of any eukaryotic species fundamentally defines
314 its overall genome structure. In chicken, molecular cytogenetic methods such as FISH have used to
315 define the whole karyotype (Masabanda et al., 2004) and thence it served as a reference for
316 comparative cytogenomics of many other birds. Initial banding studies gave way to comparative
317 chromosome painting (e.g., Fig. 6) (see also Griffin et al., 1999, 2007; Lithgow et al., 2014) and cross-
318 species analysis ("zoo-FISH") currently spans ~120 avian species from 22 different orders (reviewed in
319 O'Connor et al., 2024).

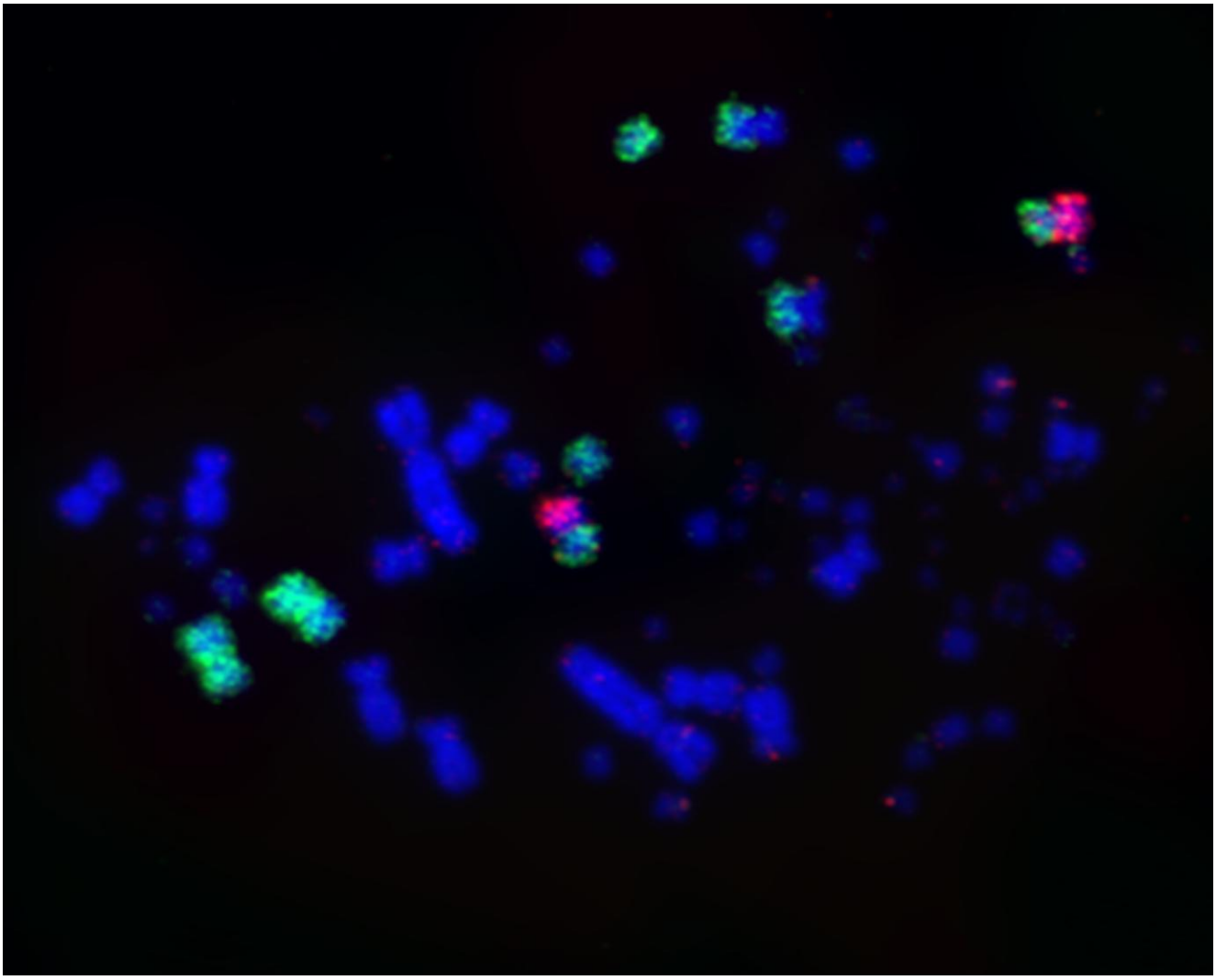

Fig. 6. Example of dual colour cross species chromosome painting (zoo-FISH). In this case to *Jacana jacana* (wattled jacana) metaphases, probes derived from *Zenaida auriculata* (eared dove) and *Gallus gallus* (chicken).

In 2004, the whole genome sequence of the chicken and the full definition of its karyotype was published concurrently (Hilier et al., 2004; Masabanda et al., 2004). Chromosome paints were created from microdissected chromosome preparations to identify all microchromosomes individually. However, subsequent attempts to sequence DNA from these clones faced challenges, were ultimately unsuccessful and the chromosome paints degraded. Remarkably, until recently, the smallest microchromosomes (chromosomes 33–39) had no associated sequences in the genome assembly. The latest comprehensive chicken genome sequence produced chromosome-scale contigs

for all 38 autosomes plus the Z and W. Only 26 gaps remain on the W chromosome, primarily located within long stretches of satellite DNA or simple repeats (Huang et al., 2023). Exploring aspects of genome structure as reflected in karyotype organization is an area that requires further comparative investigation, taking into account the still-understudied roles of many DNA segments within vertebrate genomes and the smallest of the microchromosomes 29-38. Addressing these issues is now achievable by using the newest BAC, cosmid, and fosmid libraries with FISH, and other technologies to generate more complete comparative physical maps. This enables larger sequence datasets to be generated for extensive genome analyses, including conservation-focused studies of avian genomes (e.g., Romanov et al., 2009).

When attempting to define microchromosomes in multiple bird species by comparison with chicken, the method of Damas et al. (2017) took BAC libraries from genome sequencing assemblies and selected a set that effectively hybridized to all avian microchromosomes and some other reptiles. Leveraging a bioinformatic approach to identify a high proportion of conserved sequences they became suitable for hybridization across multiple species, providing a reliable anchor point for tracking chromosomal rearrangements over time. The result was clear, punctate signals, akin to those observed in chicken metaphases, obtained for all microchromosomes across most species tested including some non-avian reptiles (O'Connor et al., 2018a,b, 2019). In all tested species, regions homologous to chicken chromosomes 22, 24, 26, and 27 appeared to have remained intact as discrete, solitary microchromosomes (e.g., Fig. 7), showing no signs of chromosomal fusion (reviewed in O'Connor et al., 2024).

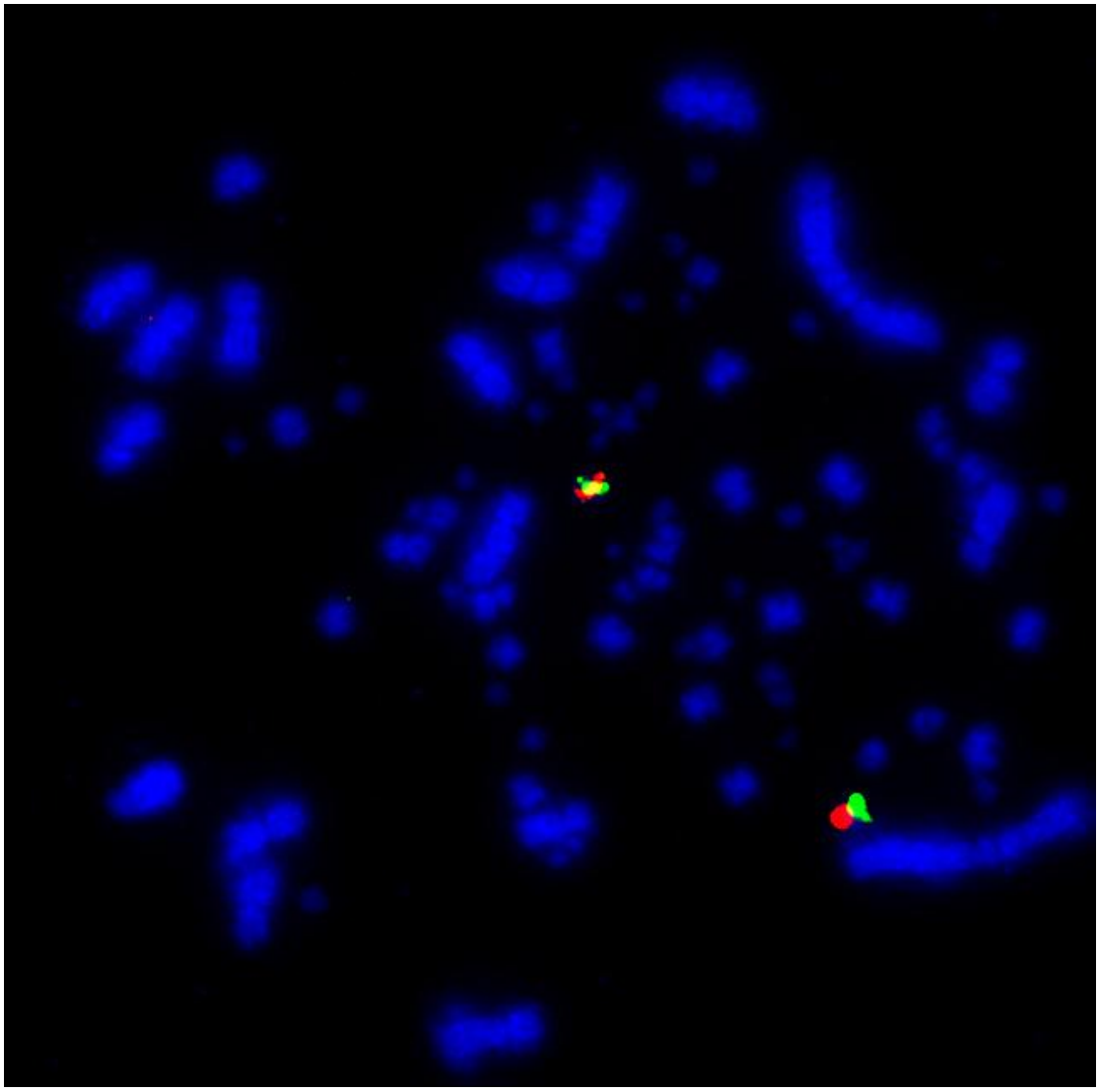

Fig. 7. Dual colour hybridization of ancestral chromosome 21 to *Jacana jacana* (wattled jacana) metaphases using selected chicken chromosome BACs

A BAC library for the zebra finch with approximately 16-fold coverage was developed at the Arizona Genome Institute, and an emu library was created with 13.5-fold coverage at the DOE Joint Genome Institute; both were also useful for comparative genomic studies (Clayton, 2004; Kellner et al., 2005). This generated further comparative maps, enhancing the application and analysis of the latest iterations of the chicken genome sequence assembly (Huang et al., 2023). Orthologous BACs across various mammals (including primates, cats, dogs, cows, and pigs) and across vertebrate orders can be identified using Universal OVERGO probes, or Uprobes, as demonstrated by Thomas et al. (2002).

This technique involves synthesizing OVERGO probes by annealing two 22- or 24-base oligonucleotides with an 8-bp overlap and then labelling them in vitro using radiolabelled nucleotides. OVERGOs are created from regions of high sequence conservation and are employed to probe new, non-sequenced genomes. Researchers can also leverage the searchable database of Uprobes to facilitate cross-species hybridization (Sullivan et al., 2008). Due to a more balanced representation of repetitive versus single-copy DNA elements compared to mammals, avian genomes represent an ideal platform for evaluating strategies of structural mapping and the mapping of insertions, deletions and duplications; this has opened new avenues for understanding ordinal and familial relationships (Edwards et al., 2005). Furthermore, the genomes of species like the anole lizard, American alligator, garter snake, tuatara, and several turtles can serve as reptilian outgroups for linking avian evolution with that of reptiles (Griffin et al., 2024).

The introduction of new technologies enabling chromosome-level genome assemblies without the need for chromosome preparations is creating new opportunities for avian genome research (Bravo et al., 2021). Recent advancements in mammalian genome assemblies, as well as more recent avian assemblies, underscore this progress. Technologies such as long-read sequencing, optical mapping, plus others, including de novo PacBio long-read as well as phased avian genome assemblies, can complement and refine reference genomes previously created using short and intermediate reads (Schwartz et al., 2014; Sutton et al., 2018; Peona et al., 2018; Huttener et al., 2021; Korlach et al., 2017). Long- intermediate reads have revealed, for instance, significant variation in the number and structure of the major histocompatibility complex loci in birds (He et al., 2021). Additionally, single-molecule long-read sequencing has illustrated the potential influence of post-transcriptional regulation on the effects of gene dosage on the Z chromosome (Wang et al., 2022); optical mapping data was beneficial for improving genome assembly, such as in the ostrich (Zhang et al., 2015). Recent avian studies have demonstrated that PacBio/SMRT long reads can reveal evolutionary divergence and adaptation by resolving complex genome architectures. For example, Weissensteiner

et al. (2020) combined short- and PacBio long-read data to catalogue approximately 220,000 structural variants in crows, uncovering an approximately 2.25 kb LTR retrotransposon insertion in the NDP that probably contributes to premating isolation. Similarly, Lundberg et al. (2023) used PacBio HiFi reads and optical mapping to assemble willow warbler genomes, discovering three large inversions (0.4–13 Mb) associated with migratory behaviour and environmental gradients, with divergence times indicating that the inversions arose in separate refugia and persisted through hybridisation. Zhang et al. (2025) produced eight chromosome-level duck genomes, demonstrating continuous female-biased gene flow during speciation, and identifying structural variants that distinguish domestic and wild ducks (affecting candidate genes like GHR and FER), as well as a flurry of LTR retrotransposons reshaping genes (e.g., MITF, IGF2BP1) in domestication (cell.com). Collectively, these studies emphasise that high-quality long-read assemblies and genotyping reveal hidden structural variation and gene flow, providing new insights into bird speciation and adaptive evolution.

In contrast to mammals, most birds possess a conserved ZW sex chromosome system (heterogametic females (ZW) homogametic males (ZZ)). That is, in all birds apart from the Palaeognathae, the sex chromosomes differ in size and morphology; the W is predominantly heterochromatic, gene-poor, and substantially smaller than the Z. In some species, however, the blocks of heterochromatin on the W make it of similar size to, or larger than, the Z (Schartl et al., 2016). In ratites, the W chromosome is similar in size to the Z and is largely homologous, apart from a small pericentric region in emus. Changes in chromatin conformation due to the accumulation of transposable elements (TEs) represent an important initial step in the differentiation of sex chromosomes (Ellegren, 2013). A putative ZW system existed before the Palaeognathae-Neognathae divergence, with the differentiation in size occurring later (Deakin and Ezaz, 2014). Although the ZW system superficially resembles the XY system in mammals, the XX/XY (mammalian) and ZZ/ZW (avian) systems do not share homology and have entirely separate origins (Bellott et al., 2010). The avian Z chromosome

shows homology with human chromosomes 5, 9, and 18, whereas the human X chromosome shares homology with a segment of the log arm of chicken chromosome 1, plus a 20 Mb section of the short arm of chicken chromosome 4 (which is a larger microchromosome in most other birds) (O'Connor et al., 2024). The sex-determining gene in birds is not SRY, as it is in mammals (the homologue of SRY is located on chicken chromosome 4). Instead, the gene DMRT1, found on the Z chromosome, is believed to play a critical role in sex determination via a dosage-dependent mechanism. Male sex determination requires two copies of this gene, as found in ZZ males, and DMRT1 is also essential for testis development. However, there remains considerable debate regarding the mechanisms of sex determination in birds, with potential candidates—including W-specific genes that may influence ovarian function. Advances in the Z chromosome assembly have been made using a BAC-based approach, along with ongoing efforts to enhance the W chromosome assembly (Bellott et al., 2010). Recent studies revealed unexpected dynamism in avian sex chromosomes, challenging previous notions of their stability. Notably, studies identified a multiple-sex chromosome system (♂Z1Z1Z2Z2/♀Z1Z2W) in the Adélie penguin (*Pygoscelis adeliae*) (Gunski et al., 2017). Additionally, genomic analyses have uncovered instances of independent fusions between autosomes and sex chromosomes in Sylvioidea species (Sigeman et al., 2020). Neo-sex chromosomes have also been detected in parrots (Huang et al., 2022), with similar discoveries reported in certain cuckoo species (Kretschmer et al., 2021).

6. The (Lack of) Diversity of the Avian Karyotype - ~30 microchromosomes in most species

Unlike the phenotypes of birds, which are remarkably diverse (Fig. 2a,b), avian karyotypes paradoxically appear to change very little between species, particularly considering the number of chromosomes involved. The diploid number approximating to 2n=80 hardly changes from species to species, and the more investigations that are performed, the more similarities we find (Griffin et al., 2007, 2024; O'Connor et al., 2024). Nonetheless, there are rare exceptions to the rule. These include species with particularly low diploid numbers like the stone curlew (*Burhinus oedicnemus*; 2n = 42),

443 the beach thick-knee (*Esacus magnirostris*; 2n = 40), the trumpeter hornbill (*Bycanistes buccinator*;
444 2n=40) and the merlin (*Falco columbarius*; 2n = 40). On the other end of the scale, species such as
445 kingfishers (*Alcedo atthis*; 2n = 132), the grey go-away bird (*Corythaixoides concolor*; 2n = 136 to 142)
446 and the hoopoe (*Upupa epops*; 2n > 120) exhibit unusually high diploid numbers. It is important to
447 emphasize here, however, that these species are not necessarily representative of the entire avian
448 orders to which they belong. For instance, in Charadriiformes (shorebirds), and Bucerotiformes
449 (hornbills, hoopoes), these are exceptions, not the norm within these groups. Conversely orders like
450 Ciconiiformes (storks), Pelecaniformes (ibis, herons, pelicans, hamerkop, and shoebill), Falconiformes
451 (falcons), and Psittaciformes (parrots) generally feature lower diploid numbers, while toucans (e.g.,
452 *Ramphastos toco*; 2n = 114) often exhibit higher diploid counts. At the risk of stating the obvious,
453 more chromosomes mean more microchromosomes: Those with the least number of
454 microchromosomes (4–12) are found in the family Accipitridae (Falconiformes) compared to those
455 with more than 100 microchromosomes are found in the Coraciiformes (kingfishers etc.). In terms of
456 the lowest proportion of microchromosomes the bald eagle (*Haliaeetus leucocephalus*; 2n = 58) has
457 4 microchromosomes, with the highest proportion (48 microchromosomes, 2n = 50 is the African
458 grass owl (*Tyto capensis*)) (all reviewed in O'Connor et al., 2024). In general terms however, around
459 two thirds of all birds studied have a karyotype of 2n=74-86 and in around 90% 2n=66-86.
460
461 A significant advance in comparative avian cytogenomics occurred when whole-genome sequences
462 were generated for 48 species covering all Neoaves orders (Zhang et al., 2014). This analysis provided
463 detailed insights into the evolutionary history, early branches in the tree of life, and the genomic
464 evolution and adaptation of modern birds. Through the Bird 10,000 Genomes (B10K) initiative, the
465 genome alignments across all sequenced bird species facilitated cross-species comparisons, yielding
466 new perspectives on avian genetic diversity and evolutionary dynamics. Utilizing these genomes and
467 their alignments, the B10K team is retracing the evolution of birds and uncovering the genomic
468 architecture underlying the diverse phenotypic traits of the avian class. Each sequenced genome,

469 importantly, aids efforts to conserve species and explore their unique characteristics. As bird
470 genomes continue to accumulate and, increasingly, incorporate long-read sequencing data, the
471 resolution of genomic features such as the W chromosome and germline-restricted chromosomes is
472 substantially enhanced, thereby facilitating the integration of genotype with karyotype (Feng et al.,
473 2020; Bravo et al., 2021). The current count of microchromosomes, however, even in long-read
474 genome assemblies supported by HiC data, could differ significantly from the karyotypic
475 chromosome count. This likely occurs because the small size of these chromosomes makes them
476 hard to distinguish from unplaced small scaffolds, such as some diverged alternative haplotypes.
477 There is a need for improved assembly of centromeres and telomeres to identify these small
478 chromosomes. Missed microchromosomes may cause issues with the reconstruction of ancestral
479 avian and deeper ancestral genomes using genome assemblies, as well as reduce our ability to use
480 these genomes for genotype-phenotype association studies.
481
482 **7. Detailed Comparative Cytogenomic Analysis of Birds**
483 The relevance of chickens to developmental biology and agriculture and of zebra finch to
484 developmental biology and neuroscience ensure that these key reference species' genomes are well
485 described in molecular and functional terms. Such references help address broader biological
486 questions concerning avian/all vertebrate genomes more effectively (Romanov et al., 2004), although
487 work on avian genomes is relatively underdeveloped compared to mammalian cytogenomics
488 (Carbone et al., 2006a). At the sequence, as well as the cytogenetic, level, bird genomes have
489 evolved at a moderate pace compared to mammals, making them suitable for effective cross-species
490 hybridization experiments (Derjusheva et al., 2004; Romanov and Dodgson, 2006; Modi et al., 2009;
491 Romanov et al., 2011; Sazanov et al., 2023; Ray-Chaudhuri, 1973; Takagi and Sasaki, 1974; Tegelström
492 and Ryttman, 1981; Tegelström et al., 1983; Griffin et al., 2007, 2024; Kretschmer et al., 2018a;
493 O'Connor et al., 2024). Different rates of change nonetheless exist amongst groups. For instance,
494 Passeriformes, being the most recently evolved bird order, exhibit a higher rate of karyotypic

evolution at the intrachromosomal (but not interchromosomal) level and, concurrently, show a heightened speciation rate (Tegelström et al., 1983; Griffin and Skinner, 2012; Kretschmer et al., 2014). The most studied Passeriform species is the zebra finch and early studies involved comparative genomics between the first three sequences avian species – this plus chicken and turkey (Volker et al., 2010; Griffin and Skinner, 2012). Chromosome painting experiments (chicken chromosomes 1-9 +Z) revealed considerable homology between the three species, with the only exception being the chicken chromosome 4 - a fusion of the ancestral chromosome 4 and a smaller chromosome. Isolation of zebra-finch BACs homologous to those in the chicken along with bioinformatic approaches (Genalyzer tool) allowed for the detailed comparative genomics of chicken and zebra finch (Volker et al., 2010), while Griffin and Skinner (2012) used bioinformatic approaches to map evolutionary cytogenomic changes between the three species and gave the first indication of the relative speed of intrachromosomal change in Passeriformes. Initial studies on these species also included Romanov and Dodgson (2005, 2006) who conducted cross-species hybridizations using OVERGO probes derived from chicken genomic data and zebra finch Expressed Sequence Tags (ESTs) to probe BAC libraries of both turkey and zebra finch. As anticipated, the hybridization success was significantly higher for chicken-turkey pairings compared to chicken-zebra finch or zebra finch-turkey combinations, particularly for OVERGOs located within coding sequences rather than within untranslated regions, introns, or flanking sequences. This facilitated a “one sequence, multiple genomes” methodology. A substantial collection of orthologous data points corresponding to BACs linked to chicken, turkey, and zebra finch genes through interspecies hybridization was made available online (Romanov et al., 2003; Romanov and Dodgson, 2006). Additionally, the success rates of comparative physical mapping among avian genomes using cross-species OVERGO-BAC hybridization aligned with their evolutionary divergence (Romanov and Dodgson, 2006; Romanov et al., 2006, 2011, 2020).

Early comparative analyses of avian karyotypes using chromosome banding techniques (see above) indicated that large microchromosomes can, rarely, fuse by Robertsonian translocation to form metacentric macrochromosomes. Concurrently, microchromosomes can translocate preferentially to telocentric macrochromosomes, resulting in a shift of centromeric positions from telocentric to submetacentric (reviewed in Griffin et al., 2024). As mentioned in the previous paragraph, chicken chromosome 4 results from a fusion between ancestral chromosome 4 and a smaller chromosome and similar fusions are seen in other species such as other Galliformes and a least one species of goose (Shetty et al., 1999; Schmid et al., 2000, 2005; Raudsepp et al., 2002; Itoh and Arnold, 2005; Griffin et al., 2007). In guinea fowl, chromosome 4 is formed through a centric fusion between chromosome 9 and the long arm of chicken chromosome 4 (Shibusawa et al., 2002) which has strong conservation to human chromosome 4 indicating its presence in the common ancestor that lived ~310 MYA (Chowdhary et al., 1998; Sazanov et al., 2004b,c). In Galliformes and Anseriformes (Galloanserae) no further interchromosomal rearrangements in chromosomes 1-9+Z are observed (Schmid et al., 2000; Shibusawa et al., 2002; Kasai et al., 2003; Galkina et al., 2006).

As mentioned in the previous section diploid chromosome numbers in certain groups of birds, such as Falconiformes, Psittaciformes, and Ciconiiformes, are lower (Nishida et al., 2008; Furo et al., 2020; Seligmann et al., 2023) and this has been studied at the individual chromosome level. Falconiformes (falcons and caracaras) possess the most atypical chromosomal organization of all birds studied, characterized by few microchromosomes (typically 1-6 pairs) (Nanda et al., 2006; Joseph et al., 2018; Kretschmer et al., 2018a; O'Connor et al., 2018b, 2024). Significant rearrangements occurred, with regions homologous to chicken microchromosomes 10, 12, 13, 14, 15, 17, 18, 19, 20, 21, 23, and 28 fused to regions of chicken macrochromosomes in peregrine, gyr and saker falcons (*Falco peregrinus, F. rustcolus and F. cherrug)* (O'Connor et al., 2018b; Joseph et al., 2018). Evidence suggest that lineage-specific rearrangements quickly became fixed within falcons with minimal interchromosomal rearrangement thereafter. In other birds of prey, the harpy eagle (*Harpia harpyja,* Accipitriformes)

546 lacks a clear distinction between micro- and macrochromosomes (de Oliveira et al., 2005) and Old-
547 World vultures, contrastingly, show extensive reshuffling of macrochromosomes (Nanda et al., 2006).
548 The California condor is a New World vulture closely related to Falconiformes (Ericson et al., 2006;
549 Jarvis et al., 2014; Chesser et al., 2016). Comparative cytogenomic analysis reveals 2n = 80
550 (potentially with an additional pair of microchromosomes) with broad similarity to the ancestral
551 avian (chicken) karyotype (Raudsepp et al., 2002). A large-insert BAC library of the California condor
552 was developed at the BACPAC Center (Romanov et al., 2006), yielding approximately 14-fold
553 coverage of the condor genome. Utilizing this library, a first-generation comparative physical map
554 between the chicken and condor was established through an OVERGO hybridization approach
555 (Romanov et al., 2006). Subsequently, a BAC-based comparative map of chicken and condor was
556 updated, containing 192 loci anchored to condor BACs (Romanov et al., 2009). Among nearly 200
557 genes identified in the condor BAC library, several functional candidate genes involved in bone and
558 cartilage development were found, including aggrecan 1 (AGC1), which has been shown to
559 contribute to skeletal dysplasia in various model species, including chicken, turkey, Japanese quail,
560 mouse, and human (Li et al., 1993; Gleghorn et al., 2005). A FISH study involving ~70 condor/chicken
561 BAC clones (Modi et al., 2009) confirmed considerable conserved synteny between Condor and
562 chicken. Interestingly, tandemly repeated *Hae*III satellite DNA sequences, previously identified only in
563 other New World vultures (Keyser et al., 1996), were also detected in the California condor. Utilizing
564 the established polymorphic microsatellite loci, parentage analysis in condors revealed two instances
565 of parthenogenetic reproduction (Ryder et al., 2021), and a first-generation genetic linkage map for
566 condors has been created (Romanov et al., 2022). These efforts have established Californian Condor
567 as one of the few genomes with a high-quality, chromosome-length assembly (Ryder et al., 2014,
568 2016; Robinson et al., 2021). Comparative analyses were also performed on the genomes of two
569 closely related species—the turkey vulture (*Cathartes aura*) and the Andean condor (*Vultur gryphus*).
570 Studies of historical population decline in all three species revealed a history of purifying selection
571 against linked deleterious alleles but pointing to promising prospects for future conservation efforts

572 (Robinson et al., 2021). The iconic North American bird of prey the bald eagle *(Haliaeetus*
573 *leucocephalus* Accipitridae, Accipitriformes*)* has a karyotype consisting of 66 chromosomes, including
574 only four pairs of microchromosomes, with small satellites present on the fourth pair (De Boer and
575 Sinoo, 1984). The genomic data for the bald eagle were generated as part of the Avian Phylogenomic
576 Project (Jarvis et al., 2015) and the analysis identified 16,526 protein-coding genes with an average
577 length of 19 Kb (Warren et al., 2014). Judkins et al. (2020) generated data through RAD-tag and low-
578 coverage whole-genome resequencing, which were mapped to the bald eagle reference genome
579 (Warren et al., 2014).
580
581 Other, comparatively rare, examples that have shown a reduction in chromosome number include
582 some Psittaciformes, Ciconiiformes and Pelecaniformes. Within the Psittaciformes (parrots and
583 relatives), four species have been studied: the kakariki (*Cyanoramphus novaezelandia*), the cockatiel
584 (*Nymphus hollandicus*), the budgerigar (*Melopsittacus undulatus*) and the monk parakeet (*Myiopsitta*
585 *monachus*). No rearrangements were found in common between these species. Fusion events were
586 nonetheless identified for the homologs of chicken chromosomes 10, 11, and 14 in the kakariki,
587 cockatiel, and budgerigar, with an additional fusion of the chicken chromosome 13 homolog noted in
588 the budgerigar. The monk parakeet (*Myiopsitta monachus)* exhibited several fusions between
589 microchromosomes, as well as fusions between macro- and micro-chromosomes, resulting in a
590 karyotype containing a modest diploid number of 48 (O'Connor et al., 2018, 2019; Furo et al., 2020).
591 In storks (Ciconiiformes) an intriguing variation in diploid chromosome numbers has been noted,
592 ranging from 2n = 52 to 78 possibly involving fusions of microchromosomes. In Pelecaniformes,
593 recent investigations using chromosome painting methods with chicken and/or stone curlew (*B.*
594 *oedicnemus*) paints have shown that the karyotype is substantially reorganized. Chromosome
595 painting on *Ardea cinerea, Egretta garzetta,* and *Nipponia nippon* revealed that different lineages
596 within Pelecaniformes exhibit distinct chromosomal rearrangements, the primary changes observed
597 being fusion events involving both macro- and microchromosomes.

598

599 Other species studied include Indian rollers (*Coracias benghalensis,* Coraciidae, Coraciiformes),
600 featuring a diploid number of ~88, with only two pairs of large macrochromosomes, a medium-sized
601 Z chromosome, and a small W chromosome; all other chromosomes are small or microchromosomes
602 (Belterman and De Boer, 1984). The species was included in a study aimed at examining the
603 phylogenetic relationships among 16 Coraciidae birds through sequences generated from fifteen
604 nuclear genes and their complete mitochondrial genomes (Johansson et al., 2018). The subspecies *C.*
605 *benghalensis affinis* from Southeast Asia clusters with the purple-winged roller (*C. temminickii*) from
606 Sulawesi, forming a sister group with *C. benghalensis benghalensis* from western Asia and India.
607 Recently, the genome sequencing of the Indian roller was announced, but further public data is yet
608 to be released (NCBI BioProject, 2024). Among other species studied, other Passeriformes, plus
609 Caprimulgiformes and Suliformes species display the typical avian karyotype, with only one species
610 (*Tolmomyias sulphurescens*, 2n = 60, Rhynchocyclidae) out of seven studied exhibiting
611 microchromosomal fusions. Within the Cuculiformes, *Crotophaga ani* (smooth billed ani) is currently
612 the only species thus far studied using BAC FISH, revealing extensive chromosome reorganization
613 involving both macro- and microchromosomes (reviewed in O'Connor et al., 2024).

614

615 Among the Palaeognathae, the emu *(Dromaius novaehollandiae,* Casuariiformes*)* has a karyotype
616 consisting of 80 chromosomes thought to resemble the avian ancestral avian karyotype (Shetty et al..
617 1999; Nishida-Umehara et al., 2007). This species also has a high-quality draft genome bolstered by
618 extensive long-read sequencing data (Liu et al., 2021) and has one of the most fully assembled
619 genomes (Sackton et al., 2019). The centromeres of the small gene-rich emu microchromosomes are
620 clustered in the nuclear centre, away from the macrochromosomes located in the periphery, and
621 show numerous interchromosomal connections associated with housekeeping genes. In contrast to
622 non-ratite birds, regions of the emu W chromosome have diverged between the sexes and lost
623 homologous recombination in less than one-third of its regions. The W chromosome is divided into

two sections: WS0, highly heterochromatic, and WS1, a more recently evolved region with marginal sequence divergence from the Z chromosome. The spread of heterochromatin from WS0 appears to have diminished interactions with neighbouring regions, increased chromatin interactions within WS1 itself, and expanded its inactive chromatin compartment. These observations suggest that changes in chromatin conformation play a vital role early in the evolution of sex chromosomes (Liu et al., 2021).

Fig. 8 summarises the findings in terms of the issue of chromosome 4, the sex chromosomes, the overall pattern seen in a range of species and exceptions to the rule in terms of wholesale fission and fusion.

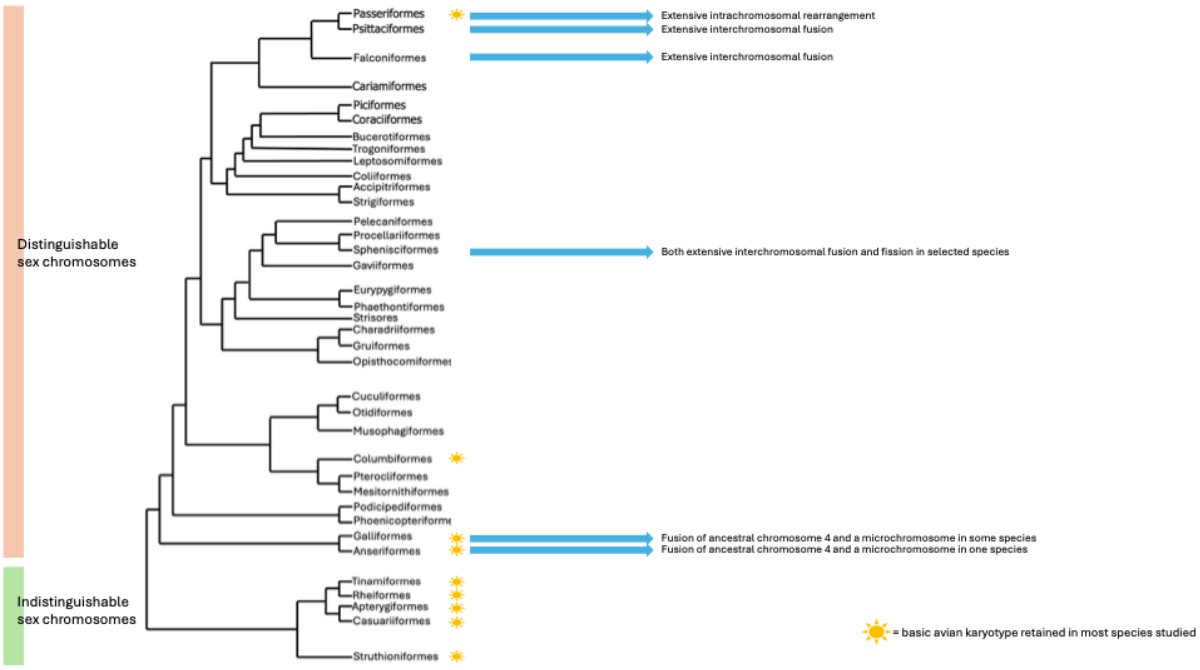

Fig. 8. Superimposed on the cladogram, overall chromosomal patterns of genome structure in avian orders. Paleognathe have indistinguishable sex chromosomes, whereas, in Neognathe, a ZW system is obvious cytogenetically. Most orders retain the signature avian karyotype while wholesale fission or fusion is characteristic of certain orders (or individual species within them).

In general terms, this figure (Fig. 8) is a culmination of work that began with basic karyotyping in the 1960s, 70s and 80s that, from the late 90s began to be supplemented with data from chromosome painting. These approaches continue to the present day supplemented by cross species BAC mapping from about 2014 which also is ongoing. The publication for the first avian genome (chicken) in 2004 followed up with the second and third (zebra finch and turkey) in 2010 then the first multi-species analysis in 2014 complemented the classical and molecular cytogenetic approaches leading to the current state of the art summarised in Fig. 8 and throughout this article.

8. Why Some Genomes do not Follow the Rules

The reason why certain species, as discussed in the previous section, rarely display a high degree of inter-chromosomal rearrangement, particularly in terms of microchromosomal fusion, remains a topic of discussion and ongoing research. As previously noted, other groups, such as kingfishers, exhibit unusually high chromosome number (2n = 130+) through extensive fission. Both higher *and* lower than typical deviations from the standard karyotype (approximately 2n = 80) can occur within the same group; for example, the Adélie penguin (*Pygoscelis adeliae*) has a diploid number of 96 (in males) and 95 (in females), while the Magellanic penguin (*Spheniscus magellanicus*) has a diploid number of 68. This variation suggests that similar mechanisms might lead to both rapid decreases and increases in chromosome numbers. The short time frame in which these changes have occurred in the penguins, along with the rearranged karyotypes observed in the Falconiformes (but not in their sister group, the Strigiformes (owls)) and in Psittaciformes (but not in their sister group, the Passeriformes (songbirds)) implies that these modifications can arise rapidly in evolutionary terms (reviewed in O'Connor et al., 2024).

Vertebrates that have large, repeat-rich genomes, such as amphibians and mammals, commonly exhibit rapid intra- and inter- chromosomal rearrangements. The findings reviewed here, however, suggest that birds can also experience similar changes in selected groups, although there is little

evidence indicating that these grossly rearranged avian genomes are significantly larger or more repeat-rich than those of other birds. Comparative studies of zebra finches and budgerigars show a similarly high rate of chromosomal changes in both species, but these characteristics appear to stem from fixed interchromosomal rearrangements that developed in response to the utilization of specific evolutionary niches. In contrast, most other avian species seem to prevent such fixation, allowing for the preservation of the typical avian karyotype (reviewed in O'Connor et al., 2024).

9. Mechanistic Aspects of Karyotype Evolution in Birds:

Eukaryotic genomes undergo evolution through both micro- and macro-rearrangements (Seoighe et al., 2000; Fischer et al., 2001; Britten et al., 2003). Micro-rearrangements encompass small-scale changes such as the inversion of a few genes, as well as single-gene insertions and deletions. In contrast, macro-rearrangements involve large chromosomal alterations that significantly affect genome structure and adaptability. Biémont and Vieira (2006) posited that transposable elements and endogenous retroviruses are crucial sources of genetic innovation with regulatory roles across various species, based on analyses of numerous eukaryotes. Comparative sequence studies in mammals reveal that macro-rearrangements tend to cluster around telomeres and centromeres (Eichler and Sankoff, 2003).

Research into mammalian genome evolution highlights a phenomenon where certain genomic regions are reused as independent evolutionary breakpoints across different lineages (Murphy et al., 2002). There also appear to be hotspots that are more susceptible to rearrangements (Carbone et al., 2006b). The reasons some rearrangements become fixed while others do not remain unclear; however, gene ontology (GO) terms found within homologous synteny blocks (HSBs) and evolutionary breakpoint regions (EBRs) might offer insights. Claeys et al. (2023) demonstrated that the specificity of GO terms in HSBs suggests that microchromosomes may have been preserved through evolution. Some EBRs were specific to bird lineages, while others were identified in the

693 genome of the anole lizard, indicating commonality across all sauropod descendants. The notion that
694 microchromosomes possess double the gene density of macrochromosomes is supported by
695 estimates of gene richness in HSBs (Claeys et al., 2023). In order to establish whether there are gene
696 pathways associated with bird and/or reptile multi-species HSBs (msHSBs) Farré et al. (2016) assayed
697 gene ontology (GO) enrichment terms in these regions. By examining 10,830 genes with one
698 orthologue in both chicken and human they established functional enrichment in all five sets of
699 msHSBs. The “development of primary sexual characteristics” GO term-related genes were
700 significantly enriched in avian, archosaurian and archosaurian/testudines msHSB sets. A bird-specific
701 CNE found 100 bp upstream from *BMPR1B* containing two transcription factor binding sites (TFBSs)
702 for *NF-E4* and for *AP-1*. The transcription factor superfamily of the latter plays a role in the regulation
703 of apoptosis during chick limb development and may contribute to the expression differences
704 pertaining to *BMPR1B* in birds comparison to other vertebrates Farré et al. (2016). Indeed, GO terms
705 for 19 “appendage and limb development” genes (present in 12 avian msHSBs from 8 chicken
706 chromosomes) were also significantly enriched in the avian msHSB set only.
707
708 The question of why some rearrangements became fixed while others did not is still relatively
709 unexplored, although insights may emerge from examining gene ontology terms found in
710 evolutionary breakpoint regions (EBRs). Farré et al. (2016) identified a correlation between EBRs and
711 specific adaptive traits in certain avian species. That is, to establish possible associations between
712 functional groups of genes and lineage-specific EBRs, Farré et al. (2016) also performed GO
713 enrichment analysis in these regions, analysing a total of 20 avian genomes. Methodologically,
714 alignment of 20 avian genome assemblies and that of five outgroup species were performed in
715 relation the chicken genome assembly using the SatsumaSynteny tool (Grabherr et al. 2010). This
716 thence facilitated defining syntenic fragments using to SyntenyTracker tool (Donthu et al. 2009) to
717 detect chromosomal rearrangements. EBRs and multispecies (ms) HSBs were subsequently identified
718 and verified; then functional analysis of genes contained within them was performed using the

719 Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (Huang et al. 2008).
720 The authors identified gene networks that were a) preferentially reshuffled during avian
721 chromosome evolution, or 2) conserved in msHSBs for several MYs of evolution. EBRs unique to the
722 budgerigar and hence following the Passeriformes/Psittaciformes divergence appeared to reshuffle
723 genes involved in *forebrain development*. These EBRs contained genes related to the *NOTCH1-NUMB*
724 pathway and *DRAXIN* all of which are involved in neuron differentiation. Forebrain development in
725 budgerigar, is unique in that it distinguishes this species not just as a vocal learner but also in terms
726 of its unique neuronal connections relative to other vocal learners Further GO terms enriched in
727 specific species are given in Table 1 (adapted from Farré et al. (2016))
728
729 Table 1. Gene Ontology terms that appear to be enriched in lineage-specific EBRs according to Farré
730 et al. (2016)

Species	GO term	Number of genes	Number of EBRs	Fold-enrichment	False discovery rate (%)
Budgerigar	Forebrain development	12	11	2.74	5.47
	Neuron differentiation	15	13	2.33	6.83
	Neuron development	12	11	2.62	8.19
	Response to wounding	11	11	2.77	8.35
Common cuckoo	Mitotic cell cycle	11	11	3.57	1.14
	Condensed chromosome	7	5	4.88	2.67
	M phase	10	9	3.25	4.50
Little egret	Passive transmembrane transport	10	5	4.15	0.59
	Cation channel activity	7	4	4.32	5.61
Anna's hummingbird	Hexose metabolic process	10	8	2.90	9.70
Peregrine falcon	RNA degradation	6	6	6.13	2.29
	Soluble fraction	5	4	6.23	8.35
Downy woodpecker	Histidine metabolism	6	5	10.30	0.16

731
732 Analyses led the first exposition of chromosome evolution in birds and other reptiles using sequence
733 alignment comparison. It showed that comparative genomics, in this way, can detect ancestral-

734 specific genome rearrangements, lineage- specific genome rearrangements and evolutionary stable
735 chromosomal intervals. The study further demonstrated how chromosome evolution could have
736 acted upon the development of various phenotypes in birds. As more genomes are sequenced and
737 assembled with greater accuracy, these analyses may reveal adaptive phenotypic features specific to
738 various avian orders and families.
739
740 Centromere repositioning is a widespread phenomenon in eukaryotic organisms (Carbone et al.,
741 2006a). This process involves inactivating an existing centromere and establishing a new one on the
742 same chromosome. After a centromere repositioning event, the major constriction and centromeric
743 function shift to new positions, although the physical structure of the chromosome remains
744 unchanged. Such events profoundly influence chromosomal architecture, as evidenced through
745 studies using locus-specific BAC/PAC clones in various groups, including primates (Ventura et al.,
746 2004; Misceo et al., 2005; Cardone et al., 2006; Carbone et al., 2006b), equids (Carbone et al.,
747 2006a), birds (Kasai et al., 2003), reptiles (including snakes, lizards, geckos, and crocodiles)
748 (Srikulnath et al., 2009, 2011, 2013; Singchat et al., 2018; Romanenko et al., 2022), as well as other
749 species. Results suggest that centromere repositioning could play a crucial role in the karyotype
750 formation of certain species, potentially impacting speciation and population dynamics.
751
752 While centromere organization and centromere repositioning in mammals have been extensively
753 studied, significantly less data is available regarding these processes in birds. The DNA sequences of
754 centromeric regions are largely unknown and currently represented by gaps in avian chromosome
755 sequences (Damas et al., 2015). Some well-defined centromeric repeat units identified in birds
756 include the chicken nuclear-membrane-associated (CNM) 41/42-bp tandem repeat, primarily located
757 within the microchromosomes that include the W chromosome (Matzke et al., 1990), and a partially
758 inverted repeat specific to chicken chromosome 8 (Wang et al., 2002). In the draft sequence of the
759 chicken genome, CNM repeats with ≥95% identity were found solely on chromosomes 23 and 28,

760 with their centromeres appropriately assigned. Additionally, 53 CNM repeats were identified on non-
761 aligned contigs (Romanov et al., 2005). Generally, these sequences are not conserved among species
762 within the same order or even family, limiting their utility for cross-species hybridization and
763 centromere localization and indicating a dynamic role for repeat families. For instance, an analysis of
764 the satellitome—a collection of satellite DNAs in a genome—in two Charadriiformes species revealed
765 no shared satellite families between them. Centromeric sequences in *Vanellus chilensis* (Southern
766 Lapwing) showed pronounced localization of the satellite DNA VchSat01 at the centromeres of all
767 chromosomes, including both autosomes and sex chromosomes (Kretschmer et al., 2024). In
768 contrast, *Jacana jacana* (Wattled Jacana) exhibited no satellite hybridization signals at the
769 centromeric regions of any chromosomes (de Oliveira et al., 2024). In monitor lizards, VSAREP
770 satellite DNAs are conserved in Asian and Australian species but are absent in those from Africa. Four
771 VSAREP subfamilies were identified, showing greater similarity within individual subfamilies than
772 across different ones. In Australian lizards, VSAREP sequences are localized near centromeric regions
773 but display different chromosomal arrangements across species (Prakhongcheep et al., 2017).
774 Thongchum et al. (2019) found that the diversity of PBI-DdeI satellite DNA in snakes has a correlation
775 with rapid evolution and varied functions. The presence of PBI-DdeI in distantly related species
776 suggests differences in chromosomal locations and repeat numbers. Satellite DNA families in *Daboia*
777 *russelii* (Viperidae) and *Pantherophis guttatus* (Corn Snake, Colubridae) exhibit high conservation of
778 nucleotide sequences and chromosomal locations, challenging the notion that these elements evolve
779 quickly (Lisachov et al., 2023).
780
781 FISH mapping of BAC clones from chicken chromosome 4 to the metaphases of the Red-legged
782 Partridge (*Alectoris rufa*) demonstrated that the ordering of loci was preserved in both species,
783 indicating the occurrence of a neocentromere during their divergence (Kasai et al., 2003). Similarly,
784 BAC FISH mapping on lampbrush chromosomes revealed neocentromere formation on chromosome
785 4 of the Japanese quail (Galkina et al., 2006). As mentioned earlier, the centromeres of chromosome

4 in both chickens and quails likely arose independently following the centric fusion of ancestral chromosome 4 with a microchromosome. Structures enriched with cohesin, resembling centromere protein bodies (PB), are characteristic of lampbrush chromosomes Galliformes, which Krasikova et al. (2006) demonstrated using labelled antibodies against cohesin subunits. Their centromeric placements were verified using various DNA probes, including BACs. However, a different location for the centromere was proposed, as regions expected to be centromeric according to the current chicken chromosome 3 sequence assembly actually correspond to a non-centromeric cluster of CNM repeats on the long arm of chicken chromosome 3. Thus, in at least Galliformes, the centromeres on chicken chromosomes 3 and 4seem to have formed de novo during the evolution of avian karyotypes.

There is growing evidence to suggest that repeat elements, such as retroposons, significantly contribute to dynamic chromosomal evolution, encompassing both micro- and macro-rearrangement events. Crombach and Hogeweg (2007) explored a model of evolution involving retroposons and a breaking/repair mechanism sensitive to environmental changes. Their findings indicated that rearrangements driven by retroposons might serve as beneficial mutational processes for short-term adaptations to novel environments. However, this observation does not imply that genomes capable of chromosomal rearrangement are inherently superior to those that primarily undergo single-gene insertions and deletions. Instead, genomic restructuring occurs because genes that require amplification or elimination in response to novel environments tend to cluster together, facilitating rapid adaptation through genomic rearrangement. As demonstrated by Crombach and Hogeweg (2007), genomes enriched in retroposons will ultimately organize themselves from a random gene order, enabling rapid rearrangement-based responses to environmental shifts.

The proliferation and reduction of retroelements over evolutionary timescales are the primary mechanisms influencing genome size in eukaryotes. The repetitive landscape profile within major

amniotic clades can offer insights into the molecular processes explaining the nearly 380-fold variation in genome sizes observed among extant vertebrates (Kazazian, 2004). Implementing an efficient BAC-end-sequencing approach to identify major repetitive families across phylogenetically diverse avian taxa facilitates the utilization of newly identified repeat motifs to characterize repeat content and organization in paracentromeric regions. It also assesses whether centromeric regions exhibit dynamic turnover of non-coding DNA in relation to the conservation of synteny.

Analyses of mobile elements in the genomes of extant sauropsids indicate that the chicken repeat 1 (CR1), long interspersed nuclear elements (LINEs) and the related mammalian-wide interspersed repeat (MIR)-like short interspersed nuclear elements (SINEs) are the most common repetitive elements observed. These elements were likely active in the common ancestor of archosaurs approximately 250 million years ago (Shedlock et al., 2007). Additionally, CR1 retrotransposons appear to be the sole source of LINE elements in avian genomes (Shedlock, 2006), even though intact, full-length CR1 elements are rare in chicken genomes (Hillier et al., 2004), implying that LINE elements in this species may be relatively diminished. The chicken genome contains nearly 100,000 CR1 repeats, divided into at least six subfamilies, each approximately 300 bp long and exhibiting significant sequence similarity. The presence of CR1-like elements in the genomes of various invertebrates and mammals underscores their importance for genome structure and function, as well as their potential regulatory roles in gene expression (Coullin et al., 2005).

Organ et al. (2007), utilizing RepeatMasker across 24 extant vertebrate species, estimated that a substantial portion of the genomes consists of repetitive DNA, particularly interspersed mobile elements. Their analysis included over 119 Mb of BAC end and scaffold DNA, sourced from online databases supplemented by data from de-novo whole-genome sequencing projects. The findings suggest that these archaic retroelements likely experienced rates of lineage-specific expansion that varied between ornithischian and saurischian dinosaurs, resulting in a 50% difference in these

genomic components and leading to the repetitive landscape observed in modern birds. Estimates of the repetitive fraction inferred for extinct dinosaur genomes indicate that the decline in CR1 activity likely began around 230-250 MYA, close to the origin of saurischian or carnivorous therapod dinosaurs. In a megabase-scale phylogenomic analysis of reptiles, Shedlock et al. (2007) revealed diverse retroelement landscapes and simple sequence repeats (SSRs) reminiscent of those seen in mammals, which are not present in chickens.

Extensive investigations into major classes of retroelements and repeats have been conducted in Galliformes, griffon vultures and other avian species (Coullin et al., 2005; Kaiser et al., 2007; Watanabe et al., 2006). Coullin et al. (2005) found that CR1 repeats are extensively distributed across nearly all chicken chromosomes, demonstrating greater density on macrochromosomes, particularly clustered in subtelomeric hotspots on chromosomes 1, 2, 3q, 4q, and 5q. Regardless of the karyotypes or reorganizations in the studied Galliformes, the CR1 distribution pattern appears consistent across their chromosomes. CR1 primers also produced similar signals on the chromosomes of more distantly related bird orders, including Anseriformes, Passeriformes, and Falconiformes, supporting the significance of these sequences in the broader context of avian evolution and chromosomal structure.

Furthermore, the Reptile Genome Consortium (Modi and Crews, 2005) conducted related studies in reptiles and birds, including turtles, alligators, anole lizards, tuataras, emus, and zebra finches (Wang et al., 2006; Shedlock et al., 2007). Examining the chromosomal distribution of repetitive elements like CR1 in vertebrates is crucial for understanding the evolution of genome structure and function (Coullin et al., 2005). The introduction of "MicrosatNavigator," a new tool for detailed analysis of microsatellites in DNA sequences, has been applied across 186 vertebrate genomes. This tool has identified trends such as the prevalence of (AC)n motifs and variations in microsatellite characteristics among different lineages. Notably, longer microsatellites are typically found on sex

chromosomes in birds and mammals but not on autosomes. GC content also varies among clades, with high-GC microsatellites observed in fish and low-GC microsatellites in non-fish vertebrates (Rasoarahona et al., 2023).

10. Microchromosomes, Whether Discrete or Fused

At the molecular level, microchromosomes are notably unique in that they are extremely GC-rich and gene-dense, comprising less than a quarter of the genome while harbouring nearly half of the genes (Hiller et al., 2004). Microchromosomes to remain as discrete units with similar GC content, repeat density and recombination rate, whether they are fused to a larger chromosome or not. Additionally, in birds and snakes, microchromosomes tend to have low content of transposable elements and high recombination rates (Hiller et al., 2004). Microchromosomal rearrangements have traditionally been regarded as infrequent compared to other chromosomal changes in birds. These gene-rich chromosomes are believed to have undergone minimal alteration throughout the last 100 million years of avian evolution, with a significant degree of conservation potentially tracing back to the common vertebrate ancestor 400 million years ago. Indeed, Burt (2002) proposed that microchromosomes seen in avian species were present in the ancestral vertebrate karyotype around 400 million years ago. This hypothesis is supported by research from Nakatani and colleagues (2007), which indicated that many avian microchromosomes correspond directly to protochromosomes from the gnathostome ancestor. The adoption of BAC-based methodologies allowed for the analysis of representatives from 17 different avian orders, all sharing a common ancestor over 100 million years ago (Damas et al., 2018; O'Connor et al., 2018). The findings from these studies reveal an exceptional level of microchromosomal conservation, with 9 of the 17 orders showing no visible changes from the microchromosomal pattern found in chickens. These results support the hypothesis that the avian ancestor's karyotype most closely resembled the chicken, with rare exceptions like the aforementioned chromosome 4 (Romanov et al., 2014). Conversely, the highly rearranged order Psittaciformes shows evidence of inconsistent microchromosomal fusions across species, indicating

ongoing karyotypic evolution since their common ancestor as well as the presence of species-specific rearrangements (reviewed in O'Connor et al., 2024). Despite lineage-specific rearrangements, four microchromosomes (chicken chromosomes 22, 24, 26, and 27) have been conserved in their entirety across all tested bird species, showing no signs of fusion.

Other distinctive characteristics of microchromosomes include specific spatial arrangements within the interphase nucleus, with macrochromosomes positioned peripherally. Studies on both avian and primate species indicate that fusions between gene-dense chromosomes and gene-poor ones do not alter their nuclear positioning. Notably, microchromosomes consistently show high levels of interchromosomal interaction—especially with other microchromosomes—co-localizing in a central nuclear domain. This pattern is observed in all the microchromosomes of reptiles and birds, suggesting it may represent an ancestral trait (Haberman et al., 2001). Interestingly, this characteristic persists even after integration into a macrochromosome, though it may diminish over time. However, newly formed microchromosomes quickly establish high interaction rates with other microchromosomes, possibly due to having a greater proportion of open chromatin compared to the macrochromosomes.

The microchromosomes of turtles, lizards, snakes, and tuatara are, on average, larger and less numerous than in birds. Snakes display a relatively constrained range of karyotypic variation, typically possessing a diploid number of 2n = 36, comprising eight pairs of macrochromosomes and ten pairs of microchromosomes (reviewed in Matsuda et al., 2005; Oguiura et al., 2010; Olmo and Signorino, 2022; Singchat et al., 2018, 2020a). Lizards likewise exhibit limited karyotypic variation, with chromosome counts ranging from 32 to 44 (e.g., Lamborot, 1998; Dos Santos et al., 2003; Srikulnath et al., 2009, 2011, 2013; Singchat et al., 2020a), ranging from extremes of 16 (Schmid et al., 1994) to 62 (Pellegrino et al., 1999). An example of an intermediate diploid number (2n = 48) is seen in the lizard *Anolis monticola*, (foothill anole lizard) which features 24 macrochromosomes alongside 24

916 microchromosomes. Fission of chromosomal elements has been shown to play a role in species with
917 lower diploid numbers (Webster et al., 1972). Notably, chromosome numbers vary significantly
918 within the Gekkonidae family (Trifonov et al., 2011; Srikulnath et al., 2015). Sphenodon, the tuatara
919 genus native to New Zealand, has a diploid count of 2n = 36 consisting of 14 pairs of
920 macrochromosomes and four pairs of microchromosomes. The similarities in the karyotypes of
921 Sphenodon and Testudines suggest a derived ancestral karyotype characterized by 14 pairs of
922 macrochromosomes and the capability to produce varying numbers of microchromosome pairs
923 (Norris et al., 2004).
924
925 In crocodilians, chromosome numbers typically range from 30—found in species like *Crocodylus*
926 *palustris* and *C. siamensis, C. rhombifer* (American/Cuban), and *Mecistops cataphractus* (African)—to
927 42, which is common among all Neotropical Caimaninae species of the Alligatoridae family (Cohen
928 and Gans, 1970; King et al., 1986; Amavet et al., 2003; Shedlock et al., 2007; Kawagoshi et al., 2008;
929 Srikulnath et al., 2015; Olmo and Signorino, 2022; Oliveira et al., 2021; Sales-Oliveira et al., 2023).
930 Notably, unlike other reptiles and birds, crocodilians are characterized by the absence of
931 microchromosomes.
932
933 Waters et al. (2021) demonstrated that avian in particular, but also diapsid, microchromosomes
934 generally have synteny with single chromosomes of amphioxus (invertebrate chordate), suggesting
935 that they appeared as a result of the two gnathostome genome duplications, followed by gene loss.
936 Secondly, they showed that microchromosomes can 'disappear' via a mechanism of micro-micro or
937 micro-macro chromosomal fusion, or else by acquiring transposable elements that expand the
938 chromosome (such as is seen in therian mammals). Third, they demonstrated that
939 microchromosomes can arise *de novo* as a result of macrochromosome fragmentation, rapidly
940 adopting microchromosomal properties including locating to a central nuclear compartment during
941 interphase as well as cell division (mitosis and meiosis). Finally, they provide evidence that

macrochromosomes are more prone to intrachromosomal rearrangements than the microchromosomes, which are more gene-rich and more prone interchromosomal rearrangements. Moreover, there appears to be a quicker rate of protein evolution in microchromosomal genes (which are GC-rich owing to GC-biased gene conversion during meiosis; Huttener, 2021), thereby clarifying the organisation of the chicken genome, including all of the microchromosomes (Huang, 2023).

11. Comparative Genomics of Birds with Other Reptiles

In contrast to studies on mammalian chromosomes, hybridization over greater evolutionary distances (beyond the phylogenetic class) is feasible with chicken chromosome paints. For instance, homology has been identified between chickens, turtles, and crocodiles, all of which last shared a common ancestor more than 250 million years ago. Matsuda et al. (2005) generated comparative cytogenetic maps for the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and the Japanese four-striped rat snake (*Elaphe quadrivirgata*) using FISH and cDNA clones of functional reptile genes. Their research revealed high conservation of homology between turtle and chicken chromosomes, with the six largest chromosomes exhibiting near-identical structures. In contrast, the degree of homology between snake and chicken chromosomes was lower than that between turtles and chickens.
The genomic region corresponding to the chicken Z chromosome is highly conserved across 30 reptile species, including squamates, crocodilians, and turtles (Pokorná et al., 2011). Notably, macrochromosomes from chickens and red-eared sliders demonstrate substantial conservation despite diverging over 200 million years ago (Kasai et al., 2012). Additionally, macrochromosomes in Nile crocodiles exhibit evolutionary changes through fission and fusion processes derived from ancestral chromosomes (Kasai et al., 2012). Chicken macrochromosome probes have also identified homology in various squamate species, indicating that chromosomal fusion events likely occurred prior to the divergence of these species from a common Squamata ancestor (Pokorná et al., 2012).

968 The chicken Z chromosome aligns in synteny with turtle chromosome 6q and snake chromosome 2p
969 (Singchat et al., 2018, 2020b). These findings suggest that conserved sequence blocks exist within
970 turtle and avian genomes, maintained throughout the evolutionary paths of Testudines and
971 Archosauria. The more frequent interchromosomal rearrangements between macrochromosomes
972 and between macro- and microchromosomes may have contributed to the evolution of a karyotype
973 characterized by larger macrochromosomes and fewer microchromosomes within the snake lineage
974 (Matsuda et al., 2005). The greater conserved synteny observed in comparisons between chicken
975 and turtle chromosomes, as opposed to those between chickens and snakes, further supports the
976 most recent molecular phylogenetic connections among the three groups, indicating that Testudines
977 and birds share a closer relationship (Zardoya and Meyer, 1998; Cao et al., 2000).
978
979 Among the early large-insert genomic BAC libraries for reptiles were those from five species
980 representing all five major lineages of contemporary reptiles: the American Alligator (*Alligator*
981 *mississippiensis*), the Garter Snake (*Thamnophis sirtalis*), the tuatara (*Sphenodon punctatus*), the
982 Chinese Painted Turtle (*Chrysemys picta*), and the Gila Monster (*Heloderma suspectum*) (Wang et al.,
983 2006; Shedlock et al., 2007). The Green Anole Lizard (*Anolis carolinensis*) became the first reptile for
984 which a complete genome sequence was produced (Alföldi et al., 2011), followed by the painted
985 turtle (Shaffer et al., 2013), the American alligator and the garter snake (Modi and Crews, 2005;
986 Green et al., 2014; Rice et al., 2017; Perry et al., 2018). The American Alligator (*Alligator*
987 *mississippiensis*) has made a remarkable recovery thanks to various conservation efforts,
988 management practices and captive breeding programs that, in part, involve genomics. This species
989 has a small karyotype (2n = 32) and notably lacks both microchromosomes and sex chromosomes.
990 The draft genome of the American alligator was produced in conjunction with two other crocodilians:
991 the Indian Gharial (*Gavialis gangeticus*) and the Saltwater Crocodile (*Crocodylus porosus*) (Green et
992 al., 2014). Researchers found that the rate of genomic evolution in crocodilians is unusually slow
993 across various metrics, including nucleotide substitutions, insertions and deletions, the content and

mobility of transposable elements, gene family evolution, and chromosomal synteny. The Garter Snake *(Thamnophis sirtalis)* is found throughout the United States and southern Canada. Its karyotype consists of 36 chromosomes, including macrochromosomes, microchromosomes, and sex chromosomes (Z and W). To investigate the various adaptations that snakes have developed for hunting and capturing prey, the genome of the garter snake was sequenced and analysed (Perry et al., 2018), uncovering features of the snake's genomic structure that illuminate the evolution of amniote genomes in general. Identification of scaffolds specific the Z and W chromosomes emphasized the diverse origins of the snake sex chromosome systems and highlighted the genome's importance for studying sex chromosome evolution. Investigations into gene duplication and loss within visual and olfactory gene families indicated that olfactory receptor repertoires expanded early in snake evolution, suggesting an ancestral adaptation to low-light conditions. A major breakthrough in avian-reptilian cross species BAC mapping came, however, with the approach of Damas et al. (2017) and O'Connor et al. (2018), hybridizing chicken BAC probes with highly conserved sequences to the metaphases not only of other birds but other reptiles also. This, and all the above-mentioned approaches and analyses, have allowed us a glimpse into what the genome organization of extinct dinosaurs might look like and, more fundamentally, why this mode of genome organization evolved and persisted.

12. Conclusions: Did Extinct Dinosaurs Have Microchromosomes and Why?

O'Connor et al. (2018a) successfully hybridized chicken BAC clones to metaphases of Anole Lizard (*Anolis carolinensis*), Red-eared Slider Turtle (*Trachemys scripta*) and Spiny Soft-shelled Turtle (*Apalone spinifera*) indicating close homology for most chromosomes in the latter. Herein, we gained a unique insight into the karyotypes of extinct dinosaurs. That is, for most chromosomes, there were very few chromosomal rearrangements between *A. spinifera* (2n=66) and modern chickens. While individual similarities might be put down to convergence (homoplasy) similarities of most of the karyotype can only mean identity by descent. In other words, in all likelihood, the common ancestor

of birds and turtles (testudines) probably had a similar number of chromosomes to *A. spinifera* – a number that had risen from about 2n=40 in around 15 million years. A continuing rate of increase in the overall diploid number would mean that a similar chromosome count would have reached 2n=80 around the time the pterosaurs and dinosaurs emerged ~240 million years ago, with very little chromosome differences (at least interchromosomally) from what we see in modern birds such as chickens (Fig. 9).

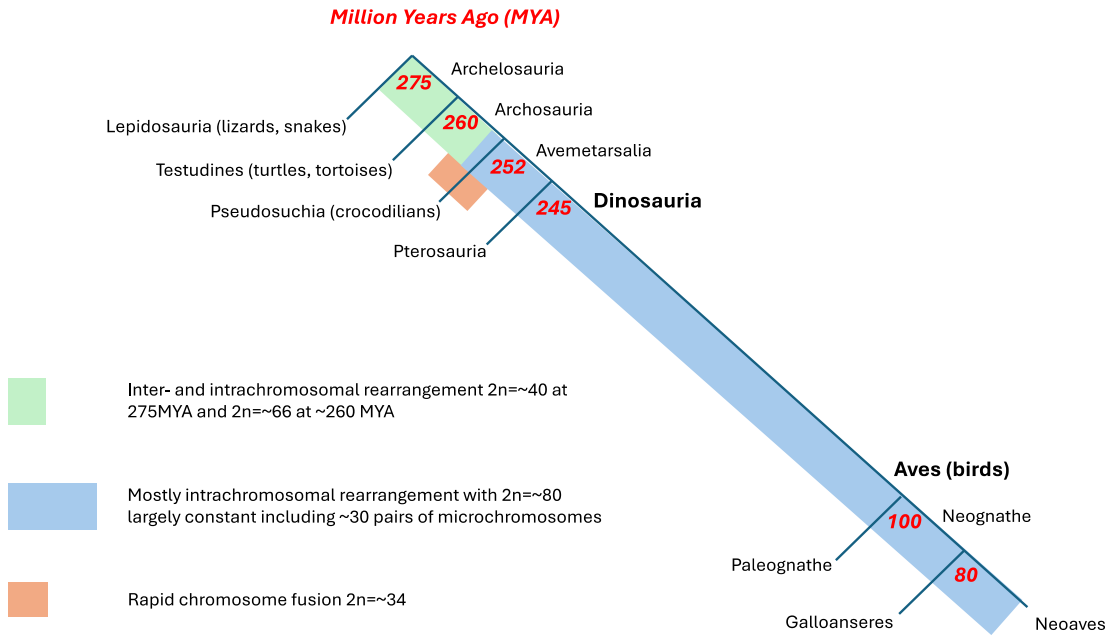

Fig. 9. Cladogram indicating chromosome copy number changes in avian ancestors and their descendants over evolutionary time (millions of years). The Saurian karyotype most likely had ~2*n*=40 (half of which were microchromosomes). There were predominantly fissions until the turtle (testudines) divergence ~260 MYA with a chromosome number of ~2*n*=64-68. At a similar rate of fission, the avian pattern (2*n*=80) was established before the time the dinosaurs emerged. The Crocodilians diverged earlier but re-fused their chromosomes (~2n=34); this constituted ~16 fusions (with no remaining microchromosomes). Extensive comparative FISH and bioinformatic evidence

1035 although suggests that the pattern of 2n=~80 (with ~30 pairs of microchromosomes) became largely
1036 fixed (i.e., changed very little) before the emergence of the dinosaurs (Fig. 9 adapted from O'Connor
1037 et al., 2018a). In other words, it is likely that dinosaur (and pterosaur) karyotypes closely resembled
1038 those of most modern birds.
1039
1040 The work tallied with insights into lizard microchromosomes, particularly from the genome
1041 sequencing of the Dragon Lizard (*Pogona vitticeps*), and to a lesser extent anole lizards (*Anolis*
1042 species), which revealed that most microchromosomes exhibit homology with chicken
1043 microchromosomes, with exceptions attributed to interchromosomal rearrangements (Deakin et al.,
1044 2016; Kichigin et al., 2016).
1045
1046 *12.1. When did the signature avian karyotype first appear?*
1047 Burt (2002) suggested that some microchromosomes were present in the common dinosaur
1048 ancestor of birds, which, he stated, likely had a diploid number of approximately 2n = 60. He
1049 suggested that a number of fissions in the avian lineage resulted in a stable karyotypic pattern of
1050 2n=80 (approximately 30 pairs of microchromosomes) that became fixed before the divergence of
1051 Palaeognathae and Neognathae 100 MYA. O'Connor et al. (2018a), however, combined genome
1052 assembly information from the Anole lizard (*Anolis carolinensis*) used as an outgroup with FISH data,
1053 establishing that the bird-like karyotype seen in two soft shell turtle species (*Apalone spinifera* and
1054 *Pelodiscus sinensis*) was, incredibly, similar at a molecular cytogenetic (cytogenomic) level and thus
1055 Burt's estimation required revisiting. That is, most microchromosomes in these turtle species proved
1056 to be direct homologues of chicken, indicating identity by descent. This paper (O'Connor et al.,
1057 2018a) thus provided compelling evidence that that fixation of the avian karyotype, with all its
1058 microchromosomes, likely occurred much earlier than proposed by Burt, i.e., around 255 MYA. Of
1059 course, we can only speculate how many of the well-known species such as *Brontosaurus,*
1060 *Ankylosaurus* and *Stegosaurus* had avian-like karyotypes. Like the Falconiformes, there were, most

likely, exceptions to the rule. Species such as *Tyrannosaurus rex* and *Velociraptor*, however, like birds, also being Therapods, are most likely to have avian-like karyotypes (O'Connor et al., 2018a).

12.2. Why did the signature avian (dinosaurian) karyotype appear and persist?

Conceptually, if a pattern in nature changes very little over 250 or so million years in evolutionary time, then it is a reasonable assumption that there is a scientific explanation why this is the case. The absence of interchromosomal rearrangements between birds (and to some degree between birds and other reptiles) could indicate an evolutionary advantage associated with retaining this signature configuration (karyotype) characteristic of avian, dinosaur and pterosaur genomes (as described above). On the other hand, it could reflect a lack of opportunities for change (O'Connor et al., 2018a). Given that considerable intrachromosomal (but not interchromosomal variation) has been documented in Columbiformes (pigeons) and Passeriformes (songbirds) species, the evidence is that intrachromosomal changes can occur, and may accelerate in line with rapid speciation events, without changing the overall structure of the karyotype. Indeed, the near absence of interchromosomal rearrangements does not hinder diversity, as a direct correlation has been noted between speciation rates and intrachromosomal rearrangements (O'Connor et al., 2018a). Below we consider the evidence for lack of opportunity for change, and for the possibility that maintaining a karyotypic structure comprising numerous compact, gene-rich microchromosomes may confer an evolutionary advantage:

12.3. Lack of opportunity for change

It is reasonable to assume that the stable overall avian karyotypic structure offers a reduced likelihood of interchromosomal rearrangement, as evidenced by the low number of recombination hotspots, fewer repeat sequences such as transposable elements, and a scarcity of endogenous retroviruses. All these genomic characteristics have previously been shown to provide substrates for interchromosomal rearrangements and are less common in avian genomes compared to those of

other organisms (Ellegren, 2013). Previous studies suggested that the evolution of the signature avian karyotype may have been a response to reduced genome size as a result of the metabolic demands of flight (see earlier sections). However, the findings reviewed here indicate that the foundational karyotypic structure was in place long before the reduction in avian genome size (see above). For instance, the average genome size in non-dinosaurian and non-avian saurians (such as lepidosaurs, turtles, and crocodilians) is approximately 3 Gb, which is significantly smaller in saurischian dinosaurs (1.78 pg) compared to ornithischian dinosaurs (2.49 pg) (Organ et al., 2007). Although the evolution of flight may contribute to genome size reduction—evidenced by reports of smaller genomes in pterosaurs compared to other avemetatarsalians—and in bats compared to other mammals—additional factors clearly influence genome size (Organ and Shedlock, 2009). The evolution of flight in theropods occurred only around 150 MYA, the dinosaurs and the pterosaurs emerged about 240 MYA and the basic karyotypic structure was most likely in place 255 MYA. Thus, the development of the karyotype may have been a driving force behind genome size reduction rather than vice versa. The reduced opportunity for interchromosomal change does not preclude intrachromosomal rearrangement (e.g., inversions) and we see this in many species.

12.4. Evolutionary advantages of the avian (dinosaurian) karyotype

Approximately seven years ago (O'Connor et al., 2018a) we proposed the hypothesis that there possible advantages to retaining multiple chromosomes in a karyotype, First, the potential for a greater genetic combination of gametes increases with the number of chromosomes. That is, leaving genetic recombination aside for a moment, a species with one chromosome pair can segregate only two genetically different gametes. With two chromosome pairs the number is 2^2=4, with three, in increases to 2^3=8 and so on, each time increasing exponentially. Now factoring in genetic recombination, each chromosome pair needs to pair, synapse and segregate, requiring at least one cross-over event. Two fused microchromosomes are likely to have only one chiasma because interference would not allow more, if the microchromosomes were discrete (i.e., not fused),

however, then there would be two cross-overs at least. The hypothesis that we proposed therefore is that the mere fact of having more chromosomes generates more genetic diversity. Genetic diversity in turn generates phenotypic biodiversity, and phenotypic biodiversity is the driver of natural selection (O'Connor et al., 2018a). In other words, if our hypothesis is correct, a group of species with more chromosomes (e.g., microchromosomes) might be more likely to be phenotypically diverse and more likely to recover from catastrophic extinction events such as K–Pg.

While we accept that there is an element of speculation to this hypothesis and that it would be difficult to provide further evidence for it, Burt (2002) proposed that a greater recombination rate contributes to the genomic characteristics that are unique to microchromosomes. These include elevated GC content, low levels of DNA repeats, as well as a greater gene density. Herein, the signature typical avian karyotype may be preserved evolutionarily.

12.5. Dinosaurs survive extinction

It is a thus common misconception that the dinosaurs were a largely unsuccessful group of animals that, by and large, did not survive because they did not adapt to change. On the contrary, Dinosaurs did not go extinct; birds are dinosaurs, and they are highly speciose and successful (Griffin et al., 2018). Dinosaurs survived Carnian–Norian extinction event 228 MYA; they survived End-Triassic mass extinction event, 201 MYA and, despite the ravages of the Cretaceous-Paleogene (K–Pg) extinction event 66 MYA (caused by the Chicxulub impactor meteor strike), they emerged again as birds (Clarke et al., 2005). The avian ancestor was probably a terrestrial bipedal, feathered Jurassic dinosaur, not entirely unlike a chicken phenotypically; (see Fig. 4), karyotypically it was probably similar too.

12.6. Future prospects

If our hypothesis is correct, then the overall avian karyotypic structure provides the substrate for the vast phenotypic diversity and adaptation to change that we see now in birds and that has been

previously present in other dinosaurs. Most commentators agree that we are now living the 6th and latest extinction event (Holocene, also known as Anthropocene) this time caused by our own actions. Of course, we will not be around to observe whether the birds will adapt better than other terrestrial vertebrates to this event or specifically better than mammals such as ourselves. The next tens of millions of years (or perhaps sooner) will ultimately test the hypothesis that increased number of chromosomes in the karyotype, predominantly microchromosomes, is an effective evolutionary strategy.

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