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Insights into avian molecular cytogenetics—with reptilian comparisons

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Abstract

In last 100 years or so, much information has been accumulated on avian karyology, genetics, physiology, biochemistry and evolution. The chicken genome project generated genomic resources used in comparative studies, elucidating fundamental evolutionary processes, much of it funded by the economic importance of domestic fowl (which are also excellent model species in many areas). Studying karyotypes and whole genome sequences revealed population processes, evolutionary biology, and genome function, uncovering the role of repetitive sequences, transposable elements and gene family expansion. Knowledge of the function of many genes and non-expressed or identified regulatory components is however still lacking. Birds (Aves) are diverse, have striking adaptations for flight, migration and survival and inhabit all continents most islands. They also have a unique karyotype with ~10 macrochromosomes and ~30 microchromosomes that are smaller than other reptiles. Classified into Palaeognathae and Neognathae they are evolutionarily close, and a subset of reptiles. Here we overview avian molecular cytogenetics with reptilian comparisons, shedding light on their karyotypes and genome structure features. We consider avian evolution, then avian (followed by reptilian) karyotypes and genomic features. We consider synteny disruptions, centromere repositioning, and repetitive elements before turning to comparative avian and reptilian genomics. In this context, we review comparative cytogenetics and genome mapping in birds as well as Z- and W-chromosomes and sex determination. Finally, we give examples of pivotal research areas in avian and reptilian cytogenomics, particularly physical mapping and map integration of sex chromosomal genes, comparative genomics of chicken, turkey and zebra finch, California condor cytogenomics as well as some peculiar cytogenetic and evolutionary examples. We *conclude* that comparative molecular studies and improving resources continually contribute to new approaches in population biology, developmental biology, physiology, disease ecology, systematics, evolution and phylogenetic systematics orientation. This also produces genetic mapping information for chromosomes active in rearrangements during the course of evolution. Further insights into mutation, selection and adaptation of vertebrate genomes will benefit from these studies including physical and online resources for the further elaboration of comparative genomics approaches for many fundamental biological questions.

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Introduction

A vast amount of knowledge has been gathered over the past century about the karyology, genetics, physiology, biochemistry, and evolution of bird species. The chicken (*Gallus gallus*; GGA) genome project generated the genomic resources [1–3] that can be used in comparative aspects and for elucidating fundamental evolutionary processes. Domestic fowl and other birds are also significant species involved in the study of disease ecology and zoonotic disease transmission, many, part from chicken, are of huge economic importance.

Advances in understanding the architecture of karyotypes and complete nucleotide sequence of genomes are contributing significantly to the knowledge of population processes, evolutionary biology, and genome function [4, 5]. Utilizing genomics databases to advance molecular cytogenetics makes further computational insights into chromosomal rearrangements and abnormalities possible [6–8]. Comparative cytogenetics and genome sequencing in mammals, insects, and plants has provided new insight regarding the role of repetitive sequences, transposable elements, and expansion of gene families as an evolutionary process facilitating radiation and adaptation. Notwithstanding these advances, knowledge of the function of many genes and the non-expressed or identified regulatory components of the genome sequence is still lacking. Avian genomes, derived from therapod dinosaurs and most closely related to reptilian genomes, can be studied utilizing comparative genomics approaches involving the use of the chicken, zebra finch and other avian pivotal genomes [4, 9–12]. This will further facilitate comprehending vertebrate genome evolution and the use of this information in understanding population processes.

Extant birds belong to the class Aves, which is a large, diverse vertebrate group, consisting of around 11,000 species in approximately 2,390 genera, 254 families and 44 orders [13]. Aves manifest striking adaptations for flight, migration and survival in diverse environments on land and water. They inhabit all continents and distant oceanic islands, including the harsh climatic zones of Arctic and Antarctica, high mountain altitudes and hot deserts. Avian species tend to be divided into two large groups: the Palaeognathae (these are the ratite plus the single palaeognathous carinates, i.e., tinamous) and the Neognathae (i.e., all other carinates). Taxonomy is based on morphology of the palatal form and this has since been confirmed molecularly using DNA to DNA hybridization as well as genomic sequencing and comparative molecular cytogenetics (reviewed in [4, 14–17]).

Because birds (Aves) and reptiles (Reptilia) are considered evolutionarily close classes of vertebrates, essential knowledge and understanding about the evolution and adaptation of their genomes can be derived when examining them in comparative studies. In this respect, we aimed here to overview avian molecular cytogenetics with reptilian comparisons, shedding light on their karyotypes and genome structure features that will facilitate further comprehension of how these groups of vertebrates developed and evolved, shaping the unique appearance of the living nature of our planet.

Avian evolution

Evolutionarily, birds are a monophyletic group; they are homoeothermic animals sharing a common ancestor with humans and other mammals. The divergence between synapsids (mammals and their extinct ancestors) and anapsids (turtles) plus diapsids (other reptiles and birds) occurred around 310–350 million years ago (MYA) (Fig. 1). Birds are thought to have evolved from theropod dinosaurs some MYA (e.g [18–20]). , . Among the earliest described birds is *Archaeopteryx* from the late Jurassic (~ 150 MYA). Prehistoric Cenozoic times (65–0 MYA) see the first fossilization of the majority of existing bird orders. Mitochondrial DNA comparisons with living reptiles suggest that birds are most closely related to crocodilians, diverging around at 210–250 MYA (reviewed in [21]).

Right hand side: Evolutionary relationships and divergence periods of extinct and extant birds. According to molecular clocks, the shaded region in Neoaves denotes the time when the majority of ordinal and superordinal lineages split. Paleontological evidence suggests that the lineages of Mesozoic birds and *Archaeopteryx* ended arbitrarily at the Cretaceous/Tertiary boundary; however, some lineages may have vanished earlier. (The shown timescale and branches are adapted from [23]; bird silhouettes are sourced from Wikimedia Commons and conform to public domain or CC licenses).

Despite decades of research by morphologists and molecular phylogeneticists, the phylogeny of modern birds remains incompletely understood. Discrepancies in findings have been attributed to the diversity of species sampled, the choice of phylogenetic methods, and the selection of genomic regions [4, 16, 17, 24]. While a complete detailed tree of birds with clear resolution remains a future task, some of the higher-level clades are now firmly established [4, 16, 17, 24]. For example, there is wide agreement that modern birds (Neornithes) form three major clades: Palaeognathae (tinamous and

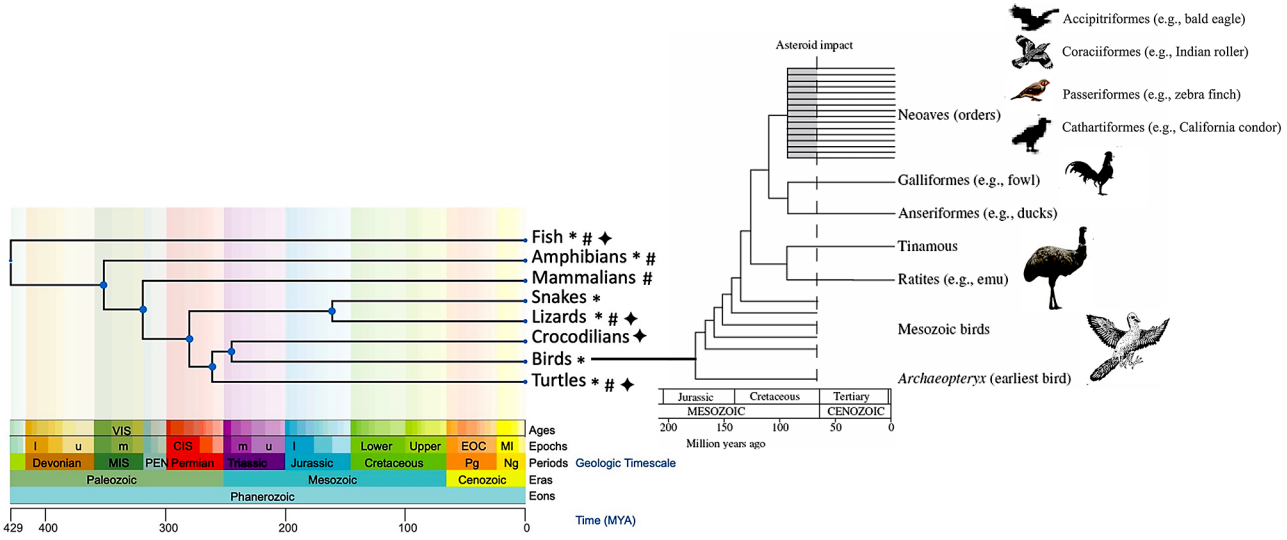

Fig. 1 Vertebrate phylogeny and sex determination modes in different taxa. The phylogenetic tree was sourced from TimeTree databases [22] using the following species representing the major clades, *Danio rerio* (fish), *Gallus gallus* (birds), *Homo sapiens* (mammalians), *Xenopus tropicalis* (amphibians), *Caretta caretta* (turtles), *Pantherophis guttatus* (snakes), *Anolis carolinensis* (lizards), and *Crocodylus palustris* (Crocodilians). * = Female heterogamety, # Male heterogamety and ✦ = Temperature-dependent sex determination (TSD)

ratites), Galloanserae (e.g., fowl, ducks), and Neoaves (all other birds) (Fig. 1). In addition, DNA sequence analyses indicate that Galloanserae and Neoaves are sister taxa (reviewed in [19]). Neoaves is the third major clade of living birds and accounts for 95% of the species. Within Neoaves, four major clades have been recently identified [17]: Mirandornithes (these are flamingos and grebes), Columbaves (the Columbimorphae, that is, pigeons/doves, mesites and sandgrouse, and Otidimorphae, the cuckoos, turacos and bustards), Telluraves (so-called higher landbirds, including Afroaves and Australaves), as well as the newly recognized phenotypically diverse clade of Elementaves (Aequornithes, that is, penguins, pelicans, tubenoses, and loons; Phaethontimorphae (tropicbirds, kagu, and sunbittern); Strisores (hummingbirds, swifts, and nightbirds); Opisthocomiformes (hoatzin); and Cursorimorphae (cranes and shorebirds). The clade Elementaves was supported by coalescent-based analyses of intergenic regions and ultraconserved elements (UCEs), but not by exons, introns, or when using concatenated analyses of intergenic regions [17].

Avian karyotype and genome features

Birds have not only undergone a remarkable evolutionary radiation and diversification, but also possess peculiar karyotypic and genome organization features, including a compact genome architecture, small genome size, decreased number of repeats and gene duplications, and presence of multiple microchromosomes (reviewed in [25–27]).

Among vertebrate, the class Aves demonstrates the greatest conserved genome size, with an average haploid genome size of 1.45 pg of DNA (1 pg=978 Mb) [28]. Genome sizes, typically reported as gametic nuclear DNA contents ('C-values'), range from the lowest value of 0.91 pg – that of the black-chinned hummingbird (*Archilochus alexandri*) up to the largest of 2.16 pg in the common ostrich (*Struthio camelus*) [28]. Overall, larger genomes are seen in flightless species such as the common ostrich [28]. Hence, compared to other vertebrate classes, birds have a smaller and more homogeneous genome. This could be explained by proposing a hypothesis that avian genomes has evolved from a small ancestral genome that had been reduced before emergence of the protoavian or by the "necessity of flight" (physiological constraints of flight), i.e., as a response to selection for high metabolism/flight [28–30]. Organ et al. [31] provided evidence that bone-cell size correlates well with genome size in living vertebrates as well as extinct dinosaurs and birds.

Small genome sizes in birds may be associated with the relatively low abundance of repetitive sequences in their genomes. Most avian genomes contain fewer repeat elements (~4 to 10%) [9] compared to other tetrapod vertebrates, such as mammals, where repeat content ranges from 34 to 52% [32]. However, a few exceptions exist, including the downy woodpecker (*Picoides pubescens*) and the snowy owl (*Bubo scandiacus*). In the downy woodpecker, transposable elements (TEs) constitute approximately 22% of the genome, primarily due to species-specific expansion of LINE (long interspersed element) type CR1 (chicken repeat 1) transposons [9]. In the snowy owl, repeat DNA comprises 28.34% of the genome, predominantly consisting of centromeric satellite DNA,

which is believed to have originated from an endogenous retrovirus (ERV1) [33].

The main distinctive feature of the avian karyotype is a larger than average diploid count as well as heterogeneity of chromosome size, i.e., macrochromosomes (3–8 microns) and microchromosomes (0.3–3 microns) [34]. The latter are difficult to count and identify accurately. Females are the heterogametic sex (ZW) in birds. One of the major challenges in research of this kind is linking genome sequence to karyotype – soo called "cytogenomics" or "chromosomics".

Karyotype comparative studies in birds were first attempted in the 1960s and 1970s using banding techniques. It was demonstrated that birds, being a monophyletic group, show a striking similarity in genome organization in general, but nonetheless have example of considerable variation (reviewed in [7, 26, 28, 35]) including the species that have:

a) the least [$2n = 40$, trumpeter hornbill (Coraciiformes: *Bycanistes bucinator*) and merlin (Falconiformes: *Falco columbarius*)] and the largest [$2n = 136$ to 142, grey go-away bird (Musophagiformes: *Corythaixoides concolor*)] number of chromosomes;
b) the least (2–12, Accipitriformes: Accipitridae) and the largest (100+, Coraciiformes) number of microchromosomes;
c) the least [4 microchromosomes, e.g., $2n = 58$, bald eagle (Accipitriformes: Accipitridae: *Haliaeetus leucocephalus*)] and the largest [48 microchromosomes + sex chromosomes, $2n = 50$, African grass owl (Strigiformes: *Tyto capensis*)] proportion of microchromosomes.

In cytogenetic research, fluorescence in situ hybridization (FISH) methods like chromosomal painting provided a more potent instrument. Most of comparative FISH studies made use of painting libraries (whole-chromosome or partial chromosome paints) as a basic tool for karyotype comparison [26, 27, 36–38]. This approach offers the advantage of producing rapid results but often lacks resolution, with marker order frequently remaining undetermined. The introduction of locus-specific clones, BAC/PAC probes in particular, has strongly contributed to a detailed analysis of chromosomal evolution (reviewed in [27]).

In recent decades, the use of BAC probes derived from chicken and zebra finch has significantly enhanced the resolution of avian cytogenetic analyses, particularly for investigating microchromosomes and intrachromosomal rearrangements. This approach offers higher resolution compared to traditional chromosome banding and chromosome painting techniques. In these studies, two or more BACs were chosen from each chicken chromosome that had been sequenced (from GGA1 to GGA28, excluding GGA16) to investigate interchromosomal rearrangements, or multiple BACs employed for intrachromosomal analyses [27, 39–41]. In the species examined, the microchromosomes homologous to chicken microchromosomes 22, 24, 26, and 27 consistently remain intact as whole segments, showing no evidence of chromosomal fusion. Additionally, microchromosomes appear resistant to breakage, even when fused to other chromosomes [39]. The only known exception is the white-spotted Woodpecker (*Veniliornis spilogaster*), where a break in a microchromosome, resulting from an inversion was identified. This involved ancestral chromosome 12 (homologous to chicken microchromosome 12) after it had fused with an unidentified macrochromosome [42].

The most studied avian genome, both in molecualar and cytogenetic terms, the chicken, is at 2.8-fold smaller than the mean mammalian genome [28, 43]. It is organized on 38 autosomes, including several macrochromosomes and numerous microchromosomes, plus Z and W sex chromosomes. The chicken karyotype is considered to be similar to an ancestral type of avian karyotypes [19, 44–46]. Chicken microchromosomes represent about 23% of the genome and are relatively gene-rich, containing not less than 50% genes [47]. Primitive amphibians and most reptiles have microchromosomes as well, indicating that some or most of the microchromosomes in birds represent archaic vertebrate syntenies [48, 49]. From the comparative genomic perspective, the level of conserved synteny between human and chicken is greater than that between human the mouse [50]. In particular, FISH evidence has been instrumental in demonstrating more shared ancestry between the chicken and human genomes, e.g., orthology between chicken microchromosomes 12, 14 and 15 and human chromosome 3 [51, 52].

The published chicken genome sequence [1] and the identification of genetic polymorphisms [3] have provided an ideal model for developmental and evolutionary studies, as well as comparative research across approximately 11,000 extant avian species [13, 53]. The most recent and comprehensive chicken genome sequence resulted chromosome-scale contigs for all 38 autosomes and Z and W chromosomes, with only 26 gaps remaining on the W, primarily located within long arrays of satellite DNA or simple repeats [43]. Aspects of genome structure reflected in organization of karyotypes is an area requiring further comparative investigation taking into consideration still understudied role of much of the DNA content of vertebrate genomes. Elucidating these problems is now feasible by employing BAC, cosmid and fosmid libraries, FISH and other technologies to generate comparative physical maps and larger sequence datasets in order to do large-scale genome analyses and

investigate evolution of avian genomes including those that are subject to conservation.

A further breakthrough in comparative avian genomics was achieved when whole genome sequences were produced for 48 species encompassing all Neoaves orders [4, 9]. Their analysis provided detailed information with respect to the history, early branches in the tree of life, genome evolution and adaptation of modern birds. Within the subsequent Bird 10,000 Genomes (B10K) initiative [54, 55], the alignments of the genomes for all bird species allow for cross-species comparisons that provide fresh insights into avian genetic diversity and evolutionary processes. Using these genomes and alignments, the B10K group is retracing the development of birds and identifying the genomic architecture underlying the variety of avian phenotypic traits. With each genome separately, the attempts to conserve the sequenced species and their relatives can be aided and species-specific features can be examined [54]. As bird genomes accumulate and increasingly include long-read sequence data, the resolution of genomic characteristics like the W chromosome and germline-restricted chromosomes is substantially improved, and the comparative integration of genotype with phenotype is made easier [55].

Reptilian karyotype features

The traditional phylogeny considers the order Testudines (turtles, tortoises, and terrapins) as the sole descendant of a primitive anapsid reptile group and places them separate from the diapsid reptiles, namely the Lepidosauria (lizards, snakes, and tuatara) and Archosauria (birds, crocodilians, and extinct dinosaurs), the former having diverged approximately 250 MYA [20] (Fig. 1).

As in birds, the karyotypes of turtles, lizards, snakes and tuatara are principally composed of two major components, that is, macro- and microchromosomes. The spread of karyotypic variation in snakes is somewhat narrow, the most typical diploid number being $2n=36$, including eight pairs of macrochromosomes plus 10 pairs of microchromosomes (reviewed in [21, 56–59]). Lizards also have a lesser karyotypic variation, i.e., 32–44 chromosomes (e.g [59–64]. , and the extremes being 16 [65] and 62 [66]. With 24 macrochromosomes and 24 microchromosomes, the lizard *Anolis monticola* (La Hotte bush anole or foothill anole) represents an example of an intermediate diploid number ($2n=48$) amongst reptiles. Fusion of elements has been demonstrated to be involved in congeners with lower diploid numbers [67]. Notably, in the family Gekkonidae, chromosome numbers vary significantly [68, 69].

The endemic New Zealand reptile genus *Sphenodon* (tuatara) has a karyotype unchanged for at least 1 MY. Its diploid chromosome count is $2n=36$, comprising 14 pairs of macrochromosomes plus four pairs of microchromosomes. Similarity between *Sphenodon* and Testudines (turtles) karyotypes allows us to derive an ancestral karyotype with a macrochromosomal complement of 14 pairs plus the ability to generate variable numbers of microchromosome pairs [70].

The chromosome number in crocodilians generally ranges from 30, as observed in species such as the Asian *Crocodylus palustris* and *C. siamensis*, the American/Cuban *C. rhombifer*, and the African *Mecistops cataphractus*, to 42 in all Neotropical Caimaninae species of the family Alligatoridae [57, 71–78]. Interestingly, unlike other reptiles and birds, crocodilians are characterized by the absence of microchromosomes.

A proposed molecular phylogeny, which was established from the nucleotide sequences of complete mitochondrial genomes plus nuclear genes, indicated that turtles should be placed in the Archosauria alongside birds and crocodilians, while squamates (scaled reptiles including lizards and snakes) can be classified into a different clade of the Lepidosauria (e.g [21, 79–82]). , .

Matsuda et al. [21] produced comparative cytogenetic maps of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) as well as the Japanese four-striped rat snake (*Elaphe quadrivirgata*) using FISH and cDNA clones of functional reptile genes. The chicken and turtle chromosomes were found to have highly conserved homology, with the six biggest chromosomes nearly identical to one another. Conversely, the snake's homology to chicken chromosomes is lower than that of turtle's. The chicken Z chromosome is preserved in synteny with the turtle chromosome 6q and the snake chromosome 2p [59, 83]. These results suggest that conserved sequence blocks occur in the turtle and avian genomes that have been maintained during the evolution of the Testudines and Archosauria. A higher frequency of interchromosomal rearrangements that occurred between macrochromosomes plus between macro- and microchromosomes, led to the evolution of a karyotype with a number of large-sized macro- but fewer microchromosomes in the snake lineage [21]. A greater conserved synteny in the chicken-turtle comparison compared to the chicken–snake comparison supports the latest published molecular phylogenetic relationships among the three genera, with testudines and birds more closely related [79, 81].

Among the first reptilian genomic large-insert BAC libraries, these became available for five species, i.e., the American alligator (*Alligator mississippiensis*), the garter snake (*Thamnophis sirtalis*), the tuatara (*Sphenodon punctatus*), the painted turtle (*Chrysemys picta*) and the gila monster (*Heloderma suspectum*), that represent all five major lineages of extant reptiles [74, 84]. A completed genome sequence for the green anole lizard (*Anolis carolinensis*) was the first reptilian target species [85], with the painted turtle [86], American alligator and/

or garter snake following [87–90]. One may now examine the evolutionary relationships and genome history of higher vertebrates (birds, mammals, and reptiles) in a more comprehensive manner thanks to these developments and the advancements in avian genomics. Further comparative mapping of birds and reptiles might yield more precise details regarding the evolution of amniotes [21, 58, 91, 92].

Genome evolution: synteny disruptions, centromere repositioning, and repetitive elements

Eukaryotes and their genomes appear to evolve by micro- and macrorearrangements [93–95]. Microrearrangements include inversions of a couple of genes, single-gene insertions and deletions, and macrorearrangements are large chromosomal rearrangements that are very important for the evolution of genome structuring and adaptability. Biémont and Vieira [96] concluded that transposable elements and endogenous retroviruses are sources of genetic innovation and have regulatory roles in many species, based on the sequencing of various eukaryotes. Comparative sequence analysis in mammals shows that macrorearrangements are localized at the telomeres and centromeres (e.g [97]). , . Chromosomal rearrangements have also been reported in monitor lizards [98].

Studies dedicated to the dynamics of mammalian genome evolution suggest a "reuse" of chromosomal regions as independent evolutionary breakpoints in different lineages [99] as well as the existence of hotspots more prone to rearrangements (reviewed in [100]). It is not well understood why some rearrangements become fixed and others do not; however, gene ontology (GO) terms found in homologous synteny blocks (HSBs) and evolutionary breakpoint regions (EBRs) may provide some insight. Claeys et al. [101] established that, because of the specificity of GO terms inside HSBs, microchromosomes may have been conserved throughout evolution. Some of the identified EBRs were specific to bird lineages, whereas others were discovered in the genome of the anole lizard, indicating that they were shared by all sauropod descendants. The idea that microchromosomes have twice the density of genes as macrochromosomes was corroborated by estimates of gene richness in HSBs [101].

Centromere repositioning (CR) is a biological characteristic that eukaryotes may experience widely (reviewed in [102]). It comprises the inactivation of the previous centromere and the appearance of a new one along a chromosome. Following a CR, the major constriction and the centromeric function adopt new locations, but the arrangement of physical markers on the chromosome endures. These events profoundly affect chromosomal architecture as shown using locus-specific BAC/PAC clones in primates (e.g [10, 103–105]). , , equids [102], birds [106], reptiles—such as snakes, lizards, geckos, and crocodiles [58, 62, 64, 107], and other organisms. According to these results, the CR phenomenon may have been crucial in some species' karyotype formation, which could have an impact on speciation and population dynamics.

Although an important phenomenon in mammalian chromosomal evolution, comparatively little information on centromere organization and CR in birds has yet been produced. The DNA sequences in centromeric regions are mostly not known and are thus represented by gaps in the current avian chromosome sequence assemblies [108]. The centromeric repeats of chicken macrochromosomes, whether metacentric or submetacentric, are well characterized [109]. In contrast, acrocentric centromeres in chickens are almost universally associated with tandem arrays of a 41/42-bp sequence known as the chicken nuclear-membrane-associated (CNM) repeat [43, 110]. These CNM repeat are also present in two acrocentric macrochromosomes, chromosome 6 and chromosome 9 [111]. The CNM monomer frequently forms higher-order repeats (HORs) in acrocentric chromosomes in spite of their large intra- and inter-chromosomal divergence [43] and is conserved in all galliform species [112]. However, usually the centromeric repeats are not conserved between species within the same order or even family and cannot be used for cross-species hybridization and centromere localization suggesting a dynamic role for repeat families. For example, the analysis of the satellitome (the collection of satellite DNAs in a genome) in two Charadriiformes species revealed no shared satellite families between them. In terms of centromeric sequences, *Vanellus chilensis* exhibited conspicuous localization of the satellite DNA VchSat01 at the centromeres of all chromosomes, including both autosomes and sex chromosomes [113]. In contrast, *Jacana jacana* showed no satellite hybridization signal in the centromeric regions of any chromosomes [114]. In monitor lizards, VSAREP satellite DNAs are conserved in Asian and Australian species but absent in African ones. Four VSAREP subfamilies were identified, with higher similarity within each subfamily than between subfamilies. In Australian lizards, VSAREP sequences are co-localized near centromeric regions but show different chromosomal arrangements across species [115]. Thongchum et al. [116] found that PBI-DdeI satellite DNA diversity in snakes correlates with rapid evolution and varied functions. PBI-DdeI is present in distantly related species, indicating differences in chromosomal location and repeat number. Satellite DNA families in *Daboia russelii* (Viperidae) and *Pantherophis guttatus* (Colubridae) show high conservation of nucleotide sequences and chromosomal locations, challenging the view that these elements evolve rapidly [117].

FISH mapping of BAC clones from GGA4 to metaphases of the red-legged partridge revealed that the order of loci was the same in both species, though indicating the occurrence of a neocentromere during divergence [106]. A similar neocentromere formation on Japanese quail chromosome 4 was found by BAC FISH mapping on lampbrush chromosomes of the chicken and quail [118]. The centromeres of chromosomes 4 in chicken and quail appear to have formed independently after centric fusion of ancestral chromosome 4 and a microchromosome. Cohesin-enriched structures resembling the so-called centromere protein bodies (PB) are a feature of galliform lampbrush chromosomes, as demonstrated by Krasikova et al. [111] using labelled antibodies against cohesin subunits. Using FISH, their centromeric location was verified with certain DNA probes including BACs. A different location for the centromere was suggested, as the gap that the current GGA3 sequence assembly expected to be centromeric actually corresponds to the noncentromeric cluster of CNM repeat on the q-arm of GGA3. So, at least in the Galliformes, the centromeres on GGA3 and GGA4 appear to form *de novo* during the evolution of avian karyotypes.

FISH hybridization of BAC probes allows for the identification of organizational and structural changes within avian genomes that can point the way for further whole genome sequencing studies to follow. While preceding comparable approaches in other vertebrate classes and further genome sequencing studies in birds and reptiles, the FISH and BAC-based investigative approaches and targeted aims offer to advance broadly the knowledge of comparative aspects of avian genome organization and implicate genomic changes in the evolutionary diversification and adaptive radiation of birds [27, 41, 119–121]. Focusing, for example, on homologs to GGA3 and GGA4 provide particular insights into these processes.

Evidence supports a significant role for repeat elements, e.g., retroposons, in dynamic aspects of chromosomal evolution, including both micro- and macrorearrangement events. A model of evolution with retroposons and a breaking/repair mechanism sensitive to environmental changes was investigated by Crombach and Hogeweg [122]. It was shown that retroposon-mediated rearrangements may be a beneficial mutational operator for short-term adaptations to a novel environment. However, this does not mean that a genome with the capacity to rearrange its chromosomes is superior to one with merely single-gene insertions and deletions. Rather, a restructuring of the genome is required because genes that must be amplified (or eliminated) in a novel environment frequently group together, facilitating quick environmental adjustments by rearrangement. As demonstrated by Crombach and Hogeweg [122], genomes containing retroposons will eventually become ordered from a random gene order, allowing for (quick) rearrangement-based environmental responses. Put simply, this model presents a "proof of principle" showing that genomes can organize themselves to maximize the advantageous effects of chromosome rearrangements.

Proliferating and attenuating copies of retroelements across evolutionary time are the primary mechanisms mediating genome size in eukaryotes. The repetitive landscape profile in the major amniotic clades' genomes can shed light on the molecular mechanisms governing the almost 380-fold variation in genome sizes seen in extant vertebrates [123]. Incorporating an efficient BAC end sequencing approach [124] to identify major repetitive families in phylogenetically diverse taxa of birds enables to utilize the newly identified repeat motifs to characterize repeat content and organization in paracentromeric regions and evaluate whether centromeric regions are dynamic in turnover of non-coding DNA relative to conservation of synteny. Furthermore, significantly expanding the understanding of CR in birds facilitates further studies through establishment of cell cultures and identification of informative hybridization probe sequences.

Comparative avian and reptilian genomics

Comparative cytogenetics and genome mapping in birds

The chicken genome sequence is typically used as a reference for comparative mapping and sometimes compensates for the lack of knowledge in genetics and genomics of most other birds. Having both a well defined karyotype as well as a deep-sequenced genome assembly means that global questions in biology of avian and vertebrate genomes can be more easily addressed through comparative means (reviewed in [125]). While progress has been made in understanding the evolutionary processes driving chromosomal organization in birds, this has been relatively sparce compared to the advances in mammalian cytogenetics and genomics [102].

According to earlier avian karyotype analyses using chromosome banding [126], large microchromosomes are fused by Robertsonian translocations to create small metacentric macrochromosomes. At the same time, there is a parallel process where microchromosomes are translocated preferentially to telocentric macrochromosomes, shifting the centromeric position from telocentric to subtelocentric or submetacentric. As a consequence of these processes, the diploid number of chromosomes in some groups of birds is lower, such as in Falconiformes, Psittaciformes and Ciconiiformes species [127–129].

It appears that bird karyotypes have relatively reduced rates of evolution [26, 27, 38, 126, 130–132] compared to mammals for which more drastic rearrangements have been described. However, certain avian groups, such as Passerines, display high rate of intra-chromosomal

rearrangements (e.g [133]). , . Additional evidence in favor of a slow karyotypic evolution in birds is that their ability to hybridize interspecifically has gradually diminished. Compared to only 11% in placental mammals and exceedingly rare intergeneric hybridization in frogs, 44% of documented incidences of hybridization in birds occur between genera (reviewed in [132]).

Using comparative cytogenetics including Zoo-FISH (BAC clones and chromosome painting) plus G-banding, it is established that GGA4 is a fusion between chromosome 4 and a smaller chromosome in many other birds (e.g [19, 34, 38, 134–136]). , . In the guinea fowl, chromosome 4 represents a centric fusion of GGA9 with the q arm of GGA4 [137]. The fusion involving ancestral avian chromosome 4 is particularly noteworthy since the ancestral chromosome 4 (q arm of GGA4) is well conserved in humans, indicating that it must have been in the common ancestor, implying 310 million years of genome conservation [138–140]. On the other hand, chicken chromosome-specific paints derived from chromosomes 1–9+Z hybridized to metaphases of the other Galliformes revealed no inter-chromosomal rearrangements [34, 106, 117, 137]. Comparative FISH mapping of specially-selected chicken BAC clones hybridizing to chromosomes 1–8+Z provided evidence of strong conservation between the genomic sequences of the chicken, quail, turkey and duck [19] that represent two early evolutionary avian lineages diverging nearly 90 MYA (Fig. 1). Small numbers of intrachromosomal rearrangements, fusions or fissions were detected in four species, with an unusually common feature being the fusion/fission event on GGA4.

In striking contrast from most birds with basic avian karyotype, the karyotypes of species from some orders, such as Falconiformes, Accipitriformes, Psittaciformes and Ciconiiformes are very different [127–129]. Most peculiar and intensively investigated are the members of the order Falconiformes (falcons and caracaras). Based on detailed cytogenetic analyses of diverse Falconiformes, it was suggested that they have the most 'atypical organization' among birds, because of extremely low numbers of microchromosomes (1–6 pairs) in most of the cases and a relatively low diploid number. This suggests that, unlike many birds, evolutionary karyotypic rearrangements in Falconiformes favor the formation of macrochromosomes (reviewed in [26, 27, 141]).

To expand knowledge about the considerable changes occurring in the genomic reorganization of diurnal birds of prey and, in comparing them to other birds, de Oliveira et al. [142] studied the world's largest eagle, the harpy eagle (*Harpia harpyja*, Accipitriformes) using chromosome painting. The findings demonstrated that the harpy eagle has lost its organization into micro- and macrochromosomes, apparently without preference or restriction. Nanda et al. [141] hybridized chicken macrochromosome paints to metaphase preparations of three Old-World vultures that came from two different evolutionary clades within the family Accipitridae to evaluate the degree of chromosomal conservation between each of these species. The analysis of the karyotypes of Old-World vultures provided a detailed description of an extensive re-shuffling of macrochromosomes, the pattern of which, however, completely differs from that in eagles.

Use of large-insert BAC libraries for comparative mapping can provide a critical part of genomic research in avian species. For instance, a zebra finch BAC library [143] with ~16-fold coverage was generated at the Arizona Genome Institute, and that of the emu (13.5·) at the DOE Joint Genome Institute [144]. Large-insert physical maps of more bird genome assemblies, aligned to the chicken sequence, would be further valuable resources. Moreover, these comparative maps would aid in the analysis and application of the chicken latest iterations of the genome sequence assembly.

Orthologous BACs in a range of mammals (primates, cats, dogs, cows, and pigs) and between vertebrate orders [144] can be identified using so-called Universal OVERGO probes, or Uprobes, as Thomas et al. [145] showed. In this approach, the OVERGO probes were synthesized by annealing two 22- or 24-base oligonucleotides that had an 8-bp overlap, followed by labeling in vitro with radiolabeled nucleotides. OVERGOs are designed from regions of high sequence conservation and then used to probe new, un-sequenced genomes. Mapping BAC contig maps of other birds alongside the chicken genome sequence and creating interspecies comparison maps are two applications for cross-species OVERGO hybridization. To do cross-species OVERGO hybridization, one can also take advantage of using the searchable database of Uprobes [145, 146].

As has been noted in DNA sequence based phylogenetic analyses in other vertebrate classes, e.g., mammals, genomic information, including structural (mapping; insertions/deletions; duplications) and DNA sequence data, have contributed to new hypotheses about ordinal and familial relationships and provided fundamental insights and testable hypotheses. A variety of comparative genomics strategies, including contiguous DNA sequences analyses using large-insert genomic libraries and the identification of retroposon insertions and other unusual genomic changes all give hope for an integrated insight into genome evolution. Owing to a more equal representation of repetitive and single-copy DNA sections than in mammals, the avian genome provides an ideal platform for evaluating such strategies [147]. In addition to the selected avian genomes, the genomes of anole lizard, American alligator, garter snake, tuatara and

turtle can be used as reptilian outgroups to link avian evolution with reptiles.

Comparative genomic and chromosome evolution in reptiles

Molecular cytogenetic techniques are pivotal in understanding the evolutionary history of reptile chromosomes and linking genome assemblies with karyotypes. Traditional cytogenetics, such as G-banding, show that crocodilians and turtles have the most conserved karyotypes, while squamates (snakes and lizards) exhibit greater variability in chromosome number and morphology [57, 71, 148, 149]. Molecular approaches, like chromosome painting and gene mapping, have provided deeper insights, though they have been applied to only a few reptile species.

Chromosome painting has revealed significant levels of homology among reptilian species, especially in macrochromosomes. Studies have shown that the genomic region corresponding to the chicken Z chromosome is highly conserved across 30 reptile species, including squamates, crocodiles, and turtles [150]. Notably, chicken and red-eared slider macrochromosomes are remarkably conserved despite diverging over 200 million years ago [106]. Furthermore, in the Nile crocodile, macrochromosomes have evolved through fission and fusion processes from ancestral chromosomes [106]. Probes for chicken macrochromosomes have identified homology in various squamate species, suggesting that chromosomal fusion events occurred before these species diverged from a common ancestor within Squamata [151].

Reconstructing the evolutionary history of reptile microchromosomes is challenging due to their small size and variability, which contributes significantly to karyotypic diversity among reptiles. Understanding their gene content is crucial for comparative genomics. The chicken genome is a common reference for reptile studies [152], and its complete genome has been recently published [43]. Cytogenetic maps have been used to link genes or genome sequences to reptile microchromosomes, but these assignments often lack specificity regarding which microchromosome the markers belong to. For instance, the soft-shelled turtle (*Pelodiscus sinesis*) shares gene locations on microchromosomes with the chicken, indicating high karyotype conservation [153]. However, in the painted turtle (*Chrysemys picta*), some regions corresponding to chicken microchromosomes are found on macrochromosomes, highlighting the complexity of chromosome evolution in turtles [154].

Emerging data on lizard microchromosomes, particularly from the genome sequencing of the dragon lizard (*Pogona vitticeps*), show that most microchromosomes share homology with chicken microchromosomes, with some exceptions due to interchromosomal rearrangements [155]. Similar patterns were observed in anole lizards (*Anolis species*), where most microchromosomes corresponded to chicken microchromosomes, with some regions diverging, further indicating the need for more detailed studies across squamates [156].

Z- and W-chromosomes and sex determination in birds and reptiles

Evidence suggests that the origin of sex chromosomes in birds, mammals and reptiles is different and independent. The ancestral state in amniotes is most likely to be temperature-dependent sex determination (TSD), and this is still found in many living reptiles, e.g. crocodilians as well as some turtles and lizards. Genetic sex determination evolved later in birds, ultimately utilizing the ZZ/ZW chromosome system, and also independently in mammals, using the XX/XY system. The ZZ/ZW system is also found in most snakes thus far studied. Matsubara et al. [157] provided evidence that the ZZ/ZW system evolved independently in snakes and birds.

There are considerable differences between the avian ZZ/ZW and the mammalian XX/XY sex chromosome systems, although the inheritance of sex-specific chromosomes during fertilization determines sex in both groups. Despite the availability of the chicken whole genome sequence, the structure and function of the avian W sex chromosomes is still incomplete. One of the major obstacles is an incomplete assembly of the chicken W sex chromosome sequences. Repetitive sequences comprise 87% of the chicken W chromosome, including 4.9 Mb satellite DNA, however, some satellite DNA is still absent from the current assembly [43].

Cytogenetic studies suggest that a common ancestor exists for the Z and W chromosomes. Typically making up 7–10% of the total genome, the Z chromosome is a medium-sized macrochromosome. Significant structural alterations to the Z chromosome during avian evolution are suggested by the chromosome's extremely varied appearance across bird karyotypes. For example, within the dove genus *Columbina* of the family Columbidae, the morphology of the Z chromosome can vary: it is telocentric in *Columbina picui*, while in *Columbina passerina* and *Columbina talpacoti*, it is metacentric [158]. As such, one could anticipate that the Z chromosomes of different bird species will have rather different gene orders. The average W chromosome is considerably smaller than its partner, the Z, and sometimes only marginally larger than the microchromosomes. It is predominantly heterochromatic and lacking in genes (reviewed in [19, 159]). However, the number of cases where the W chromosome is the same size [128, 160] or even larger than the Z chromosome has recently increased [161]. Previous research indicates that in all Neognathae taxa studied, the ZW pair exhibits highly restricted recombination, confined to a small pseudoautosomal region (PAR) [162].

Conversely, palaeognathous birds possess a significant PAR, unlike other birds [163–165]. For instance, despite being over 100 million years old, the W chromosome of the ostrich (*Struthio camelus*) still retains 65% the size of the Z chromosome [164]. There are several published W-linked genes in the PAR that have counterparts on the Z, reflecting their shared ancestry from homologous chromosomal pairs: *CHD1*, *HINT1*, *SPIN1*, *UBAP2*, *ATP5F1AZ*, *KCMF1*, *HNRNPK*, *UBQLN1*, etc [19, 166]. (Fig. 2). A number of repeating DNA sequences unique to females have been cloned for chicken and a few other neognathous and palaeognathous birds. As quickly evolving molecules, several of them have greatly diverged

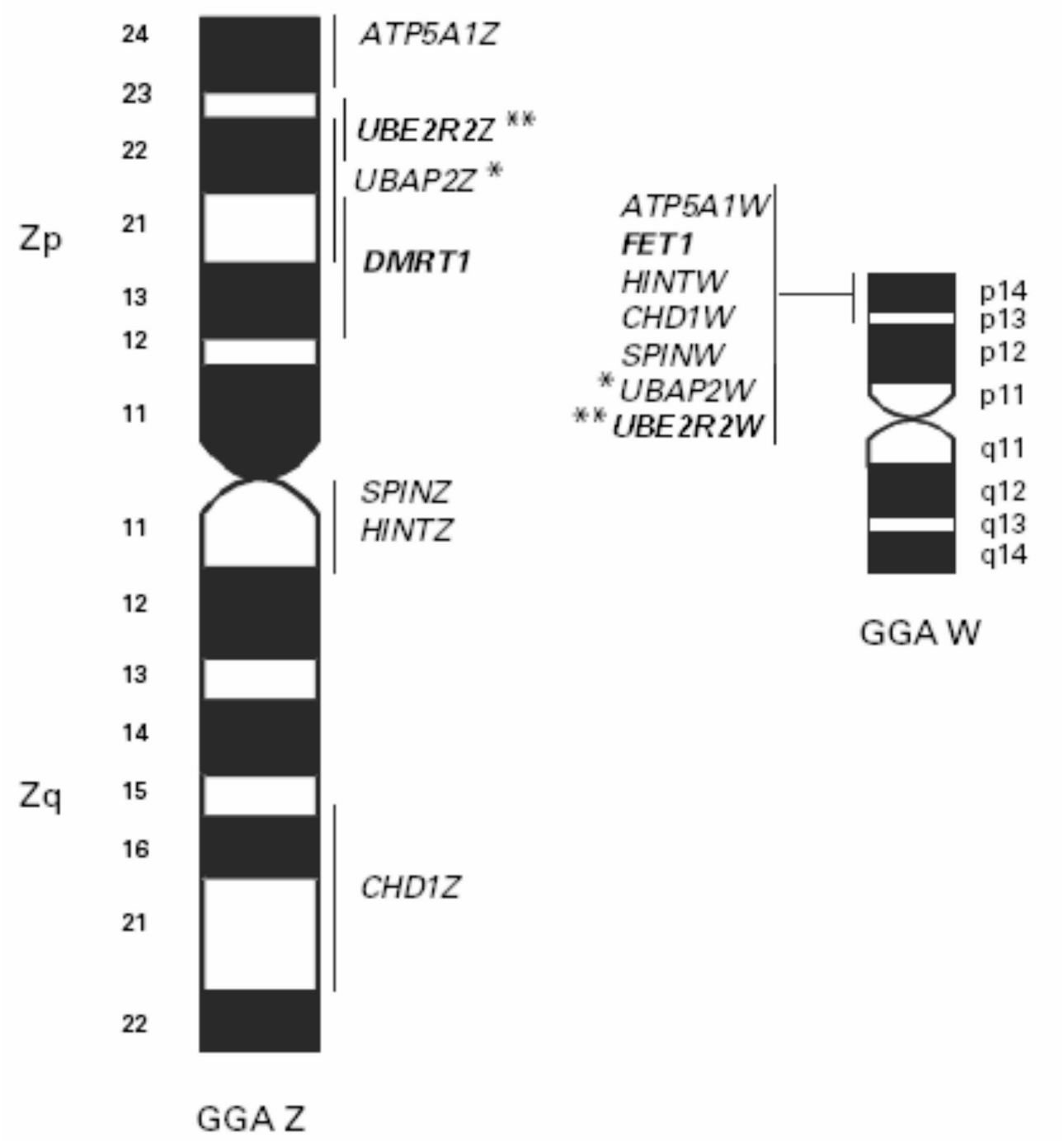

Fig. 2 Chicken sex chromosome G-banded ideogram displaying the shared genes between the Z and PAR on the W chromosome as well as the cytological position of sex determining candidate genes (bold). (adapted from [19]; *Sazanov et al. [169]; **Sazanov et al. [170])

between species and are important parts of the W-heterochromatin (reviewed in [14]).

Comparison of genome assemblies between amniotes has revealed significant linkage homology and chromosomal rearrangements over millions of years. The snake W chromosome is a key model for understanding the genetic divergence of sex chromosomes in amniotes. Studies suggest that sex chromosomes across various amniote lineages share genomic blocks, indicating the possible divergence of an ancestral super-sex chromosome as a result of chromosomal rearrangements. Major findings on sex chromosomal profiles in amniotes highlight repeat-mediated sex chromosome conformation and the genomic landscape of snake Z and W chromosomes, including the role of transposable elements. Advances in complete telomere-to-telomere assembly offer new insights into the evolutionary origins of reptilian and avian sex chromosomes [58, 83, 167].

There are considerable differences in the constitution of sex chromosomes between the two avian groups, Palaeognathae and Neognathae [165]. According to Takagi and Sasaki [131] and Tsuda et al. [14], neognathous birds have highly distinct W chromosomes that are late replicating, highly heterochromatized, and somewhat smaller than Z counterparts. Alternatively, the earliest types of bird sex chromosomes—which are essentially homomorphic between the Z and W chromosomes—remain in the palaeognathous ratites, such as ostrich, emu, cassowary, and rhea (reviewed in [14, 165, 168]). The significant molecular homology between the Z and W chromosomes is likewise conserved in emus as evident through comparative chromosomal painting using the chicken Z chromosome-specific DNA [134].

A primitive stage of W chromosome differentiation from the proto-sex chromosomes was demonstrated in ostriches [14]. Unlike chickens, this species shows limited differentiation of the W chromosome, with deletions occurring in a region from near the centromere to a site proximal to the *RPS6–NTRK2–PKCI* (*HINTW*) genes. The W chromosome differentiation in the tinamou lineage (Fig. 2) is at a transitional stage between that in the ostrich, which has a partially deleted W chromosome, and neognathous birds, which have much more degenerated and heterochromatic W chromosomes [14]. Hence, the Palaeognathae sex chromosomes diverged from each other at a lower rate after the recombination was suppressed [171]. An analysis of W-linked genes across several bird species, representing the three major avian clades— Palaeognathae, Galloanserae, and Neoaves—revealed that W chromosomes display highly conserved gene content, despite the independent evolution of recombination suppression in these lineages [166]. The retained W-linked genes tend to be more dosage-sensitive and exhibit higher expression levels compared to those that have been lost, suggesting that purifying selection plays a key role in shaping the gene content of W chromosomes [166].

Avian sex chromosome function during sex determination and sex differentiation is likely to differ from that of the mammalian sex chromosomes. Moreover, the Z and W chromosomes carry different sex-determining genes than the X and Y [19]. In mammals, expression of the *SRY* gene from the Y chromosome triggers sexual development in heterogametic (XY) individuals. Since birds lack a counterpart for *SRY*, it has been speculated that some W- and Z-linked genes function as dominant gonad-determining factors in female and male birds. In particular, most intriguing among potential ovary-determining genes was the *FET1* (female expressed transcript 1) gene. Reports suggested that this was expressed in female chicken gonads, and located in the W short arm's euchromatic region and not having a Z homolog. If FET1 plays a role in gonadal sex differentiation, it would have represented a very interesting case of viral co-option by the embryo for a developmental process. It should be noted however that this has not been studied recently and the paper was retrated by the authors [172].

The *DMRT1* (Doublesex and Mab3-related transcription factor) gene was found to be more expressed in the testes in ZZ male chicken embryos and down-regulated on the single Z chromosome of female embryos. It has no counterpart on the W, shows a conserved testis-specific expression pattern across several vertebrate groups, including birds (Fig. 2), and is conserved on the Z chromosomes of both neognathous and palaeognathous birds, including chicken, zebra finch and emu (reviewed in [19]). This all support its role in avian sex determination. In this model, a gene dosage effect governs avian sex: in ZZ males, two *DMRT1* dosages are necessary for testis production, while in ZW females, a single dosage results in ovary differentiation. The *DMRT1* dosage effect hypothesis is being further elucidated. Itoh et al. [173] used microarray analysis to compare the male: female ratio of expression of sets of Z-linked, though excluding *DMRT1*, and autosomal genes in two bird species, zebra finch and chicken, and in two mammalian species, mouse and human. In various tissues from finches and chickens, Z genes expressed at much higher male: female ratios than autosomal genes. In contrast, when studying human and mouse, the male: female ratio of expression of X-linked genes is somewhat similar to that of autosomal genes, indicating effective mechanisms of dosage compensation. Seemingly in birds, genes on one sex chromosome are expressed on average at constitutively higher levels in one sex than the other. Sex-chromosome dosage compensation is this unusually ineffective in birds, suggesting that some genomes can cope perfectly well

without effective mechanisms of sex-specific sex-chromosome dosage compensation.

Reptiles seem to have different ways of determining sex. Only snakes have the ZZ/ZW mechanism, but lizards and turtles have both the XX/XY and ZZ/ZW mechanisms. The sex Z chromosomes of birds and snakes were produced from distinct autosomes in a shared ancestor, suggesting that birds and snakes may have different sex-determining genes. In addition, TSD is widely spread in reptiles, including all crocodilians, the tuatara, most turtles and many lizards (reviewed in [21, 87]). Although sex chromosomes are not morphologically distinguishable from other chromosomes of the Chinese soft-shelled turtle, its chromosome 6 has extensive conserved linkage homology to that of human chromosomes 5 and 9, known to be homologous to the chicken Z chromosome [34, 48, 139, 174]; moreover, the three chicken Z-linked genes were localized to the turtle chromosome 6 [21]. These results suggest that the ancestral chromosomes of avian sex Z chromosomes have been conserved as an autosome in testudine genomes for approximately 230–240 MY [21, 79].

The evolution of sex determination provides a significant model system for researching how genes regulate development. The availability of contemporary genomics technologies such as chromosome sorting, FISH, high-throughput sequencing, subtractive hybridization and cDNA arrays are poised to bring about rapid increases in investigation of, and knowledge about, evolution of sex differentiation and sex determination [87].

Evolution of repetitive elements in avian and reptilian genomes

Mobile element analyses of living sauropsid genomes provide evidence that 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 predominant repetitive elements and were probably active in the common archosaur ancestor living some 250 MYA [74]. Furthermore, it appears that CR1 retrotransposon elements are the only source of LINE elements in avian genomes [175], despite the fact that full-length, intact CR1 elements are scarce in chicken genomes [1], suggesting that LINE elements in this host species are relatively extinct. Divided into at least six separate subfamilies, each with a length of 300 bp and significant sequence similarity, almost 100,000 repeats are scattered across the chicken genome. CR1-like elements were discovered in the genomes of invertebrates and mammals, suggesting their importance for genome structure and function as well as their implication in regulation of gene expression [176].

Organ et al. [31], using RepeatMasker in 24 extant vertebrate species, estimated that the dominant fraction was repetitive DNA (interspersed mobile elements). These studies comprised > 119 Mb of BAC end and scaffold DNA sampled from online databases, supplemented with data derived from *de novo* whole-genome sequencing projects. Results provided evidence that these ancient retroelements most likely underwent different rates of lineage specific expansion within ornithischians and saurischian dinosaurs, leading to a 50% difference in these genomic components, and resulting in the repetitive landscape currently observed in extant birds. Estimates of the repetitive fraction inferred for extinct dinosaur genomes based on the correlation between repetitive element composition and genome size of extant species, suggest that the reduction in CR1 activity began close to the base of carnivorous therapod or saurischian dinosaurs around 230–250 MYA.

Based on a megabase-scale phylogenomic analysis of the Reptilia, Shedlock et al. [74] revealed diverse, mammal-like landscapes of retroelements and simple sequence repeats (SSRs) not found in the chicken. The results suggest a diverse array of interspersed and SSRs in the common ancestor of amniotes and a genomic conservatism and gradual loss of retroelements in reptiles that culminated in the minimalist chicken genome.

Major classes of retroelements and repeats have already been investigated in Galliformes, griffon vulture and other birds (e.g [176–178]). , . Based on CR1 distribution pattern, Coullin et al. [176] found that CR1 repeats are distributed over nearly all chicken chromosomes with a greater density on the macrochromosomes and in particular with hot spots on sub-telomeric regions of chromosome 1, 2, 3q, 4q, and 5q. Regardless of the karyotypes or reorganizations of the Galliformes under study, CR1 distribution pattern seems to be retained on their chromosomes. On the chromosomes of phylogenetically more distantly related birds (Anseriformes, Passeriformes, and Falconiformes), CR1 primers likewise display comparable signals. This evidence supports the significance of these sequences at the macro scale of bird evolution and in chromosomal structure.

In addition, the Reptile Genome Consortium [87] initiated and performed a number of similar studies in reptiles and birds including turtle, alligator, anole lizard, tuatara, emu and zebra finch (e.g [31, 84]). , . Evaluating the chromosomal distribution of the repetitive elements such as CR1 in vertebrates is of great interest in elucidating the evolution of genome structure and function [175]. The new tool, MicrosatNavigator [179], allows for the detailed examination of microsatellites in DNA sequences. Applied to 186 vertebrate genomes, it identified trends such as the prevalence of (AC)n motifs and varied microsatellite characteristics across lineages.

Notably, longer microsatellites are found on sex chromosomes in birds and mammals but not autosomes. GC content varies between clades, with high-GC microsatellites in fishes and low-GC ones in non-fish vertebrates. These insights aid understanding of microsatellite roles in sex chromosome differentiation [180].

Examples of pivotal research in avian and reptilian cytogenetics and genomics

Here, we briefly describe a few examples of previous studies in genetics, cytogenetics, and genomics of the chicken, the most commonly cited model avian species, and other avian species. In particular, we review a few seminal studies aimed at physical and comparative genome mapping in the chicken, turkey, zebra finch, and California condor. The first three birds were the first avian species whose whole genomes were sequenced. The California condor exemplifies a successful conservation research landmark among birds. All studies alos highlight the impoirtance of linking genome assembly with karyotype.

Physical mapping and map integration in chicken

A BAC-based whole-genomic physical map of the chicken genome has been integrated with the genetic (linkage) map by hybridizing probes containing molecular markers onto filter-spotted arrays [2, 180–182]. For integrating genetic and physical maps, a high throughput screening technique was employed that involved BAC filter hybridization using highly specific OVERGO probes [182]. This aided in alignment of the first- [181] and second-generation BAC-contig physical map [2], developed alongside the whole genome sequence, to the linkage map and resulted in the assignment of BAC contigs in specific chicken chromosomes. The integrated map incorporated approximately 91% of the chicken genome and has since been used for identification of chicken clones aligned comparatively to positions in other sequenced genomes.

In addition, the chicken physical map has been integrated with the chromosomal (cytogenetic) genome map. Many BACs corresponding to genes and markers have been hybridized by FISH to numerous chicken chromosomes (e.g [139, 183, 184]). , , and a detailed analysis of microchromosome GGA17 using FISH has been conducted [185]. These studies demonstrated that the GGA17 map orientation is reversed from that currently proposed for the linkage map and draft sequence. Experimental confirmation of GGA17's reversed orientation and centromere placement was achieved by dual-color FISH, employing terminal BACs and the centromere-specific CNM oligonucleotide as probes. An advantage of this cytogenomic approach is the improved alignment of the sequence and linkage maps with chromosomal features such as the chromosome arms, staining patterns indicating AT vs. GC content, centromeres and telomere. Incorporating these approaches helps efficiently evaluate genomic changes in an evolutionary context.

Z- and W-linked genes

Using BAC-based FISH, it was possible to investigate in detail the genes harbored by the chicken Z and W chromosomes. One of them, *UBAP2Z*, was identified with its exact cytogenetic location on the Z chromosome. Its W-linked orthologue, *UBAP2W*, was also mapped [169]. Also, it was possible to map cytogenetically the sixth, previously undiscovered pair of Z- and W-linked homologs, *UBE2R2Z* and *UBE2R2W* [170] (Fig. 2), and the fine FISH mapping of two more homologs, *ATPA5A1Z* and *ATPA5A1W*, was also performed. These studies can be extended by FISH mapping the Z- and W-linked genes to the chromosomes of other birds and reptiles. Investigating homologous genes found on both the Z and W chromosomes, or avian gametologous genes, yields important insights into the fundamental mechanisms governing the evolution of both chromosomes [120, 169, 170].

Most caenophidian snakes exhibit genotypic sex determination (ZZ/ZW) with Z sex chromosomes homologous to chicken chromosomes 2 and 27, which are among the largest metacentric chromosomes in most snake species [106, 186–189]. Recent sequence analysis of the *CTNNB1* and *WAC* genes has provided insights into the evolutionary process of sex chromosome differentiation in snakes [106, 190, 191].

Comparative avian cytogenomics: turkey and zebra finch

Romanov and Dodgson [192, 193] performed cross-species hybridizations using OVERGO probes designed from chicken genomic and zebra finch EST sequences to turkey and zebra finch BAC libraries. Cross-hybridization between chicken and turkey, as opposed to chicken–zebra finch or zebra finch–turkey, was, as predicted, more successful with OVERGOs contained within coding sequences than in untranslated region, intron, or flanking sequences; this enabled a "one sequence, multiple genomes" approach. A large collection of ortholog data points for BACs assigned to chicken, turkey and zebra finch genes using interspecies hybridization were made available online [180, 193]. Using cross-species overgo–BAC hybridization, success rates of comparative physical mapping within avian genomes were also concordant with the degree of their evolutionary divergence [121, 192–194]. Overall, bird genomes are thought to have evolved just moderately over the course of evolution, making them suited for effective cross-species hybridization using chromosome paints, BAC-based FISH as well as overgo-based BAC library screens [170, 193, 195–197].

California condor cytogenetics and genome assembly

The California condor is an endangered avian species that was previously assigned to the order Ciconiiformes, the family of the New World vultures or Cathartidae. The taxonomy of cathartids has been an arguable issue as earlier classifications related this group to the Old-World vultures within the order Falconiformes (see for review [197, 198]). A preliminary study, based on 5000-bp sequences from five nuclear genes and used novel phylogenetic methods, raised New World vultures to the rank of an independent order that is more associated with a clade that also includes Falconiformes than with a clade of storks and related birds [199]. Currently, Cathartidae are recognized as the sole family within the separate order Cathartiformes [4, 200]. California condors are one of the largest North American flying birds, with 9–10 ft wingspan. They used to be an important element of the ecological systems across the wide range in North America including the western and southern U.S. and Mexico. Like other scavengers, they are part of the nature's cleanup crew. These birds can travel 150 miles a day in search of carrion, reach speeds of up to 55 mph, climb to altitudes of 15,000 ft and go without food for several days. The karyotype ($2n$) consists of 80 chromosomes and seems to maintain basic avian karyotype features.

To support genomic analysis of the endangered California condor and take advantage of progress in chicken genomics, an extensive cytogenetic analysis in the condor identified a chromosome number of 80 (with a likelihood of an extra pair of microchromosomes), and provided information pertaining to the telomeres, centromeres and nucleolar organizing regions [135]. A comparative map of condor and chicken macrochromosomes was generated by using individual chicken chromosome-specific paints for GGA1–9 and Z and W on condor metaphase spreads. Apart from chromosomes 4 and Z, each chicken macrochromosome painted a single condor macrochromosome. The GGA4 paint detected homology with two condor chromosomes, 4 and 9, providing additional evidence (as has been established with many other birds) that the latter are ancestral chromosomes among avian species. The GGAZ paint hybridized to both sex chromosomes (Z and W) in the condor, suggesting that the condor sex chromosomes have not completely differentiated, unlike in other non-ratites [135].

A genomic large-insert BAC library of the California condor was generated at the BACPAC Center [194]. It represented ~ 14–fold coverage of the condor genome. Using this library, a first-generation comparative chicken-condor physical map was developed using OVERGO hybridization approach [194]. The two avian genomes have a high degree of conserved synteny, as indicated by a comparison of specific condor BAC sequences with orthologous chicken sequences. Later, the BAC-based chicken-condor comparative map was updated and contained 192 loci anchored to condor BACs using the probes derived from sequences of several avian species: chicken, California condor, other New-World vultures, and zebra finch [201]. This work also aids in identification and characterization of candidate loci for a chondrodystrophy mutation in condors and advance genetic management of this disease. Among the almost 200 genes identified in the condor BAC library, there are several functional candidate genes that are involved in bone and cartilage formation. One of them, aggrecan 1 (*AGC1*), was found to be affected and cause skeletal dysplasia in model species including chicken, turkey, Japanese quail, mouse and human (e.g [202, 203]). , .

To establish the applicability of cross-species hybridization of the condor BAC library to other species and build the condor cytogenetic map, a FISH study was performed using around 70 condor and chicken BAC clones [196]. Most BACs mapped in the condor were found to be homologous to the appropriate chicken genes and chromosomes, suggesting a very high degree of conserved synteny between two genomes. On chromosome 4, one intrachromosomal rearrangement was detected. Additional intrachromosomal rearrangements were also identified on the Z chromosome. In a few cases, a condor clone for the Z-linked gene was mapped to an autosome [197]. More FISH tests need to be carried out in the California condor to confirm these intra- and interchromosomal rearrangements.

By sequencing clones from a condor microsatellite-enriched library, 951 short genomic sequences were obtained. 30% of them were discovered to be homologous to avian sequences in some cases and nearly all chicken chromosomes throughout the in silico mapping process. Numerous of these sequences include retroviral LTRs, (micro)satellites, CR1, other LINEs, and other repetitive components that are found in chickens [201]. Interestingly, tandemly repeated *Hae*III satellite DNA sequences previously detected only in the lineage of other New World vultures [204] were also found in the California condor. Using the established polymorphic microsatellite loci, parentage analysis in condors showed two cases of parthenogenetic development [205] and a first-generation condor genetic linkage map was developed [206]. In addition, a total of 13 BACs orthologous to human chromosome 7 (HSA7) and six chicken chromosomes were sequenced in collaboration with the NISC at NIH. A condor–human comparative physical map for a region corresponding to human chromosome 7 was designed to make it available online through the NISC web site [201]. In the collaboration with the Washington University Genome Sequencing Center, nearly 440,000 cDNA sequences were generated from a condor fibroblast cell line using a novel 454 technology and deposited in the NCBI Trace

Archive. These data provided first insight into the condor transcriptome and be used in the future condor genomics and avian comparative genomics research [201]. Eventually, a high-quality chromosome-length condor genome assembly was created and its genome-wide diversity was examined [207–209]. Genomes of two close relatives, the turkey vulture (*Cathartes aura*) and the Andean condor (*Vultur gryphus*), were analyzed for comparison. All three species' genomes contain evidence of past population decreases. Interestingly, the great degree of variety that the California condor genome preserves is a relic of its historically high abundance. A history of purifying selection against connected harmful alleles was further shown by correlations between genome-wide diversity and recombination rate, which bodes well for future restoration [209].

A few other peculiar cytogenetic and evolutionary examples of birds and reptiles

Here, a few avian and reptilian species that are interesting in terms of comparative cytogenetic mapping, being representatives of significant clades for understanding evolution of birds and reptiles, are mentioned. These have, however, been less studied so far by means of modern genomic tools.

Emu (Dromaius novaehollandiae)

The emu is a palaeognathous ratite bird and the sole extant species of the tribe Dromaiini, which, along with cassowaries, belong to the order Casuariiformes. Emus and cassowaries had a common ancestor in the Pliocene period (5–10 MYA). Emus live in open woodland and semi-desert regions of Australia and Tasmania; they are easy to keep and rear in captivity and have been bred (mainly for meat) on farms in Western Australia since 1970. Farms are now being established in Tasmania, New South Wales and Queensland raising the Australian national flock in 1994 to >30,000 birds. Emus are gaining in popularity in Australia and some other countries because of the market for their meat, feathers, oil and hide [210]. The karyotype (2*n*) includes 80 chromosomes and is likely to be similar to the avian protokaryotype [168].

The emu's nuclear genome is a high-quality draft genome that was enhanced by considerable long-read data [211]. Previously, it was one of the most fully assembled genomes of any paleognath [212]. The emu genome sheds light on the evolution of the avian chromosomes' nuclear architecture and genome arrangement. Centromeres of the small, gene-rich emu microchromosomes are grouped in the nuclear center, distant from the macrochromosomes in the nuclear periphery, and exhibit many inter-chromosomal connections linked to housekeeping genes. In contrast to nonratite birds, the emu W chromosome regions have diverged between the sexes and lost homologous recombination in fewer than one-third of them. The emu W is separated into two regions: WS0, which is strongly heterochromatic, and WS1, which is a more recently evolved area with only mild sequence divergence from the Z chromosome. Perhaps as a result of heterochromatin from WS0 spreading, WS1 has decreased interactions with neighboring regions, increased chromatin contacts inside the region, and widened its inactive chromatin compartment. These patterns imply that chromatin conformation modification is a crucial early stage in the evolution of the sex chromosome [211].

Bald eagle (Haliaeetus leucocephalus)

The species is a bird of prey found only in North America, and a member of the family Accipitridae, order Accipitriformes, most recognizable as the national bird of the United States. As for conservation status, it is classified as threatened in southern Canada and most of the United States by the U.S Fish and Wildlife Service; still abundant in its northern range, especially in Alaska. The karyotype is 66 chromosomes, with only four pairs of microchromosomes. The members of the pair 4 bear small satellites [213]. The bald eagle's genomic data were generated within the Avian Phylogenomic Project [214] and included 1.26 Gb of high-quality sequencing scaffolds built, with contig and scaffold N50 values of 10 Kb and 670 Kb, respectively. Also, a total of 16,526 protein-coding genes with a mean length of 19 Kb were found [215]. Judkins et al. [216] generated data from RAD-tag and low-coverage whole genome resequencing approaches. These pooled datasets were mapped to the bald eagle reference genome [215] in order to produce a 50 K SNP array and reveal genetic structure for bald eagles [216].

Indian roller (Coracias benghalensis)

A bird that breeds throughout tropical southern Asia, from Iraq to Thailand, the Indian roller belongs to the family Coraciidae of the order Coraciiformes. It travels occasionally during the seasons but is not migratory. The karyotype is characterized by certain unusual features. It has a diploid number of approximately 88 and shows only two pairs of large macrochromosomes, the medium-sized Z chromosome, and the small W chromosome, all remaining autosomes being small or microchromosomes [217]. This species was included in a study to assess phylogenetic relationships between 16 Coraciidae birds based on generation of sequences for fifteen nuclear genes and their entire mitochondrial genomes [218]. The subspecies *C. benghalensis affinis* from Southeast Asia forms a group with the purple-winged roller (*C. temminickii*) from the Sulawesi and then a sister group with *C.*

benghalensis benghalensis from western Asia and India. Just recently, genome sequencing of the Indian roller was announced with no further public data linked to this project [219].

American alligator (Alligator mississippiensis)

The species is a member of the family Alligatoridae, order *Crocodylia*. It was once considered endangered, but through various conservation plans, management, and captive propagation it has made a staggering comeback. The karyotype is small ($2n=32$) and, remarkably, has neither microchromosomes nor sex chromosomes. The draft American alligator genome was originally produced along with two other crocodilians: the Indian gharial (*Gavialis gangeticus*) and the saltwater crocodile (*Crocodylus porosus*) [88]. It was found that the rate of genome evolution in crocodilians is abnormally slow at all levels: nucleotide substitutions, indels, transposable element content and mobility, gene family evolution, and chromosomal synteny. In the comparative context, birds have the relatively rapid evolution, while the common ancestor of all these taxa (i.e., crocodilians, birds and turtles) also displayed slow genome evolution. Additionally, the data allowed for the analysis of crocodilians' heterozygosity, which suggests that all three taxa's populations likely shrank throughout the Pleistocene. Improved American alligator genome assembly demonstrated conserved estrogen signaling architecture as a primary cause of female-biased gene expression in gonads during the post-temperature sensitive stage in the process of temperature-dependent sex determination [89].

Garter snake (Thamnophis sirtalis)

This is a species found across the United States and into southern Canada that belongs to the family Colubridae, suborder Serpentes, order Squamata. The karyotype consists of 36 chromosomes including macro-, micro- and sex (Z and W) chromosomes. To demonstrate how snakes have developed a variety of adaptations for detecting and seizing prey, the genome of the garter snake was generated and analyzed [90]. Characteristics of snake genome structure that shed light on the evolution of amniotic genomes were also discovered. Studies of the genomes of the garter snake and other squamate reptiles showed changes in the expansion and abundance of repeat elements among snakes, revealed genes that are subject to positive selection, and updated estimates of the neutral substitution rate for squamates. Discovery of scaffolds specific to the Z and W sex chromosomes supported the idea that the snake sex chromosome systems have various origins and highlighted the usefulness of this genome in the study of sex chromosome evolution. Olfactory receptor repertoires expanded early in snake evolution, according to investigation of gene duplication and loss in visual and olfactory gene families, suggesting a dim-light ancestral situation in snakes. Also, new genomic evidence was obtained for the coevolutionary arms race between highly toxic newt prey and garter snakes, which resulted in toxin resistance in garter snakes, as well as origins of, and connections between, genes encoding secreted venom proteins [90].

Conclusions

On a conceptual basis, comparative molecular cytogenetic and genomic studies are contributing strongly to new approaches in population biology, developmental biology, physiology, disease ecology, systematics and evolution. Realistically, a genome is only complete when the sequences are all assigned and aligned on the chromosomes. The close relationship between sequence assembly and chromosome (both structure and function) brings into focus the need to integrate both the genomic and the cytogenetic information to gain a greater insight into the part that genome architecture plays in genome function and evolution. Deakin et al. [220] suggested the terminology 'chromosomics' to unite the discplines of whol genome sequencing/assembly, (molecular) cytogenetics and cell biology. More recently, the term "karyotype coding" [221] has been introduced to mean the unique order of genes on and within chromosomes. The purpose of karyotype coding is to establish the structural basis of the emergent genetic network, thereby searching for new genomic inheritance patterns. Karyotype coding (including posting an hypothesis, creating a model, and making predictions) is intended to facilitate a deeper unserstanding of bio-systems wirth specific reference to how their inheritance is preserved. It provides a new conceptual framework for appreciating that information on genome organization-is essential for evolutionary and genomic studies in the future.

Strategically, developing enhanced cytogenetic and genomic resources, including but not limited to, whole genome sequencing studies, structured by a phylogenetic systematics orientation, represents essential steps in contributing to a broader understanding of genome evolution and the nature of the mutational processes upon which natural selection acts. This also benefits from producing genetic mapping information for chromosomes that have been active in rearrangements during the course of avian evolution. Furthermore, a special research focus on the sex chromosomes has shown that its dynamic evolution has followed different trajectories in different vertebrate clades. Further insights into mutation, selection and adaptation of vertebrate genomes will benefit from the studies that are targeted to facilitate the generation of cytogenomic data and reagents (probes) that can become community resources for the further elaboration of comparative genomics approaches to a

variety of significant questions in vertebrate biology and evolution.

Fascination with the diversification of life forms is a human propensity that has broad impact on human activities and society. Biotic evolution is a fundamental underlying principle for considering the diversification of living organisms and their extinct ancestors. Studies of molecular evolution, that now include whole genome sequencing studies, are providing fundamental new insights regarding the process of evolution over time when large segments of the public express confusion about or distrust of scientific explanations of the process of evolution. Development of the field of comparative genomics as a tool in understanding population processes and evolutionary biology requires incorporation of additional taxa representing major clades of vertebrates. This makes comparative genomics approaches especially important for generating more insights into the relatively unexplored classes of birds and reptiles. This also facilitates further studies to verify major events in genome organization in diverse avian and reptilian lineages, create the opportunity for generating new and intriguing examples of evolutionary processes that will be of interest to the general public.

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Author contributions
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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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