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Comparative painting reveals strong chromosome homology over 80 million years of bird evolution

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Abstract

Chickens and the great flightless emu belong to two distantly related orders of birds in the carinate and ratite subclasses that diverged at least 80 million years ago. In the first ZOO-FISH study between bird species, we hybridized single chromosome paints from the chicken (*Gallus domesticus*) onto the emu chromosomes. We found that the nine macrochromosomes show remarkable homology between the two species, indicating strong conservation of karyotype through evolution. One chicken macrochromosome (4) was represented by a macro- and a microchromosome in the emu, suggesting that microchromosomes and macrochromosomes are interconvertible. The chicken Z chromosome paint hybridized to the emu Z and most of the W, confirming that ratite sex chromosomes are largely homologous; the centromeric region of the W which hybridized weakly may represent the location of the sex determining gene(s).

Introduction

Fluorescent *in-situ* hybridization (FISH) has become a very powerful implement in mammalian gene mapping strategies. ZOO-FISH (comparative chromosome painting) has been adapted to compare genomes of distantly related species by detecting DNA sequence homology between whole chromosomes. Such comparative mapping studies have been performed by hybridizing DNA from single human chromosomes to the chromosomes of great apes, cat, seal, horse, cattle, muntjac, pig, whale, and mouse (Solinas-Toldo *et al*. 1995, Scherthan *et al*. 1994, Rettenberger *et al*. 1995, Schmid *et al*. 1995, Raudsepp *et al*. 1996, Frönicke *et al*. 1997, Yang *et al*. 1997, Ferguson-Smith *et al*. 1998). Recent chromosome painting in kangaroos has led to the confirmation of their ancestral karyotype (Glas *et al*. 1999). These chromosome painting studies have confirmed and refined homologies established by gene mapping studies and conventional G-banding results.

Although this technique has been successfully used to compare different mammalian genomes, no comparative painting has been attempted with birds. Here we describe comparative chromosome painting between two distantly related extant bird species, the emu and the chicken. These species represent Paleognathous birds ('ancient jaw') and Neognathous birds ('new jaw'), the divergence of which represented the first stage in the evolution of modern birds. Extant Paleognathous birds include the ratites and tinamous, and the Neognathous birds include all the remaining

higher taxa of carinate birds. The modern Galliformes (including the chicken) and Anseriformes diverged early from other Neognathae, which radiated to more than 9000 species of higher land and water birds (Figure 1).

Ratites and carinates therefore represent the earliest divergence among living birds. Based on DNA–DNA hybridization comparisons between different species, Sibley & Ahlquist (1990) proposed that the ratites and tinamous diverged from the main avian lineage 80–90 million years ago (MYrBP). However, recent comparisons of the mitochondrial DNA of different species of birds including ratites (Härlid 1998) suggest a more recent split. A tree constructed from evidence of α-crystallin A sequences supports the hypothesis that the ratites are monophyletic, and depicts them as the sister group of all other birds (Stapel 1984).

The living ratites include the ostrich (*Struthio camelus*) of Africa and Arabia, the greater and the lesser rheas of South America (*Rhea americana* and *Rhea pennata*), the Australia emu (*Dromaius novaehollandiae*), three species of cassowaries (genus *Casuarius*) of northeastern Australia and New Guinea, and three species of New Zealand kiwis (genus *Apteryx*).

The bird genome is highly conserved in size at about one third that of the human genome (Stevens 1997), and has a high proportion of unique to repetitive (non-coding) DNA. Birds have a very high chromosome number, and a curious karyotype which includes normal-sized 'macrochromosomes', as well as a number of tiny 'microchromosomes'. Most birds have chromosome numbers of 2n = 76–84 (Rodoinov 1997). This chromosome number is especially conserved in all Paleognathae and the earliest diverged carinates (Galliformes and Anseriformes) but is more variable in higher carinates.

In most species, the largest 9 pairs of chromosomes (including the Z and W) are considered macro-

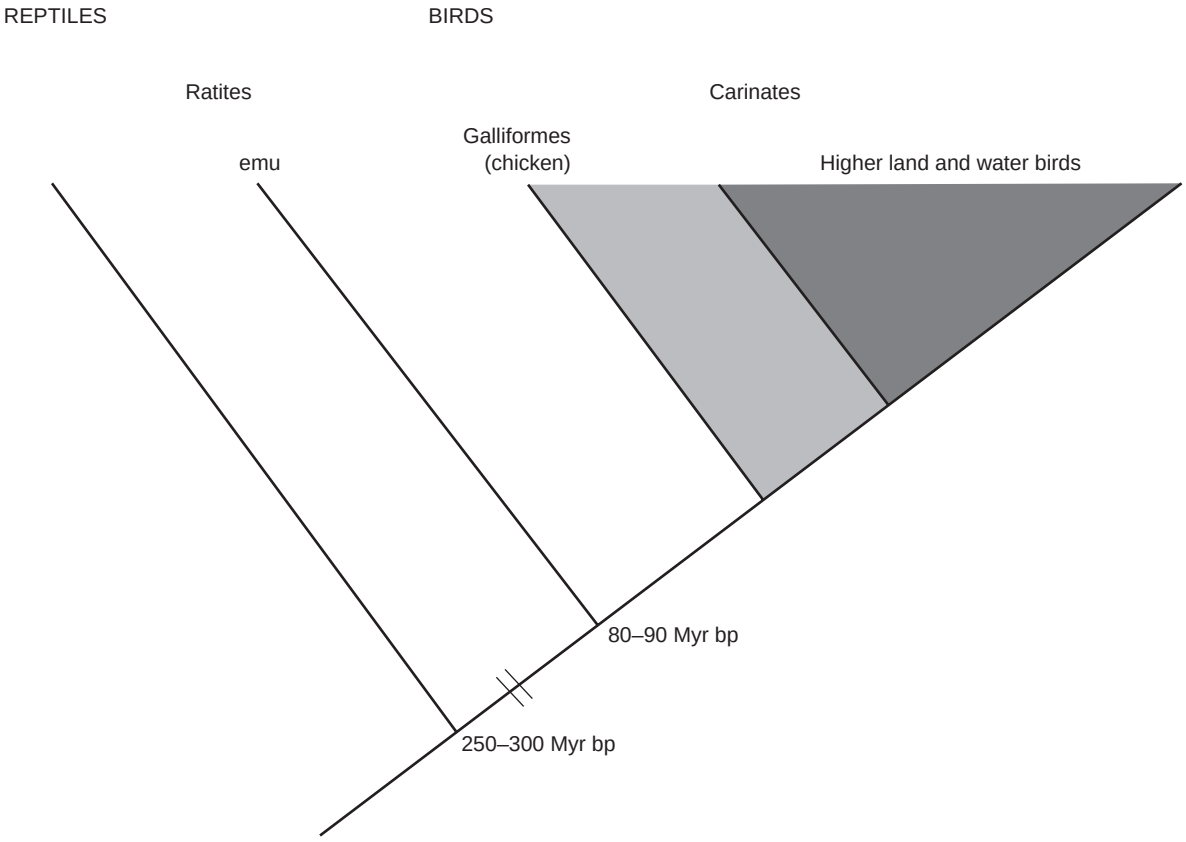

Figure 1. Relationships of bird taxa

chromosomes (Ladjadi *et al*. 1993). The average number of microchromosomes is 60 but this varies, being much lower in some orders (Accipitridae have only 6–12 and Ciconiiformes 20) and higher in others (Coraciiformes have more than 100). Initially considered to be inert and variable accessory elements, perhaps circular and lacking centromeres and telomeres, microchromosomes are now known to have a normal chromosome structure and contain many CpG islands (Fillion 1998). More than 50% of the genes mapped in chicken are on the microchromosomes, which may therefore represent ancient conserved elements of great genetic significance. Whether the microchromosomes are the result of subdivision of large chromosomes, or whether ancestral microchromosomes fused to form macrochromosomes is an important question which still needs to be answered.

Taking into consideration the millions of years that separate the ratites from other orders of birds, it is surprising that the chromosome number and morphology seems to have undergone very little change. The ratites all have 9 pairs of macrochromosomes and 62 or 64 microchromosomes. Several (the ostrich, emu, cassowary and Darwin's rhea) have $2n = 80$, and the greater American rhea has $2n = 82$. This $2n = 80$ karyotype is considered to be ancient, and perhaps conserved for about 100 million years (De Boer 1980) because it is shared by many neognathes. For instance, the chicken has $2n = 78$, with 9 pairs of macrochromosomes and 60 microchromosomes. Macrochromosomes morphology has also been conserved between some orders of Neognathae (Columbiformes and many orders of higher water birds) and Paleognathae (Rodionov 1997).

In all species of birds, males are homogametic, with a ZZ pair of sex chromosomes, and females are heterogametic, with a ZW pair. In most birds, the ZW pair is the fifth or the sixth largest of the karyotype. The Z shows morphological and G-banding homology between all species, but the W is strikingly different in ratites and carinates. The carinate Z and W are strongly differentiated with the W comparatively smaller and conspicuously rich in heterochromatin. However, the ratite W is usually indistinguishable from the Z, and completely lacks constitutive heterochromatin (Ansari *et al*. 1988). It pairs at meiosis with the Z chromosome except over the short arm and pericentromeric regions of the W which lack recombination nodules in *Rhea americana* (Pigozzi & Solari 1997). This suggests that the ratite sex chromosomes remain in a primitive stage of differentiation. How the chromosomes determine sex is unclear, with some evidence from aneuploids pointing to a gene on the W (Solari 1993) and evidence from triploids to a Z dosage effect (Thorne & Sheldon 1993).

The present study involves comparative chromosome painting. We used flow-sorted chromosomes from the chicken (*Gallus domesticus*) to hybridize to the chromosomes of a ratite bird, the emu (*Dromais novaehollandiae*).

Materials and methods

Emu samples and embryonic fibroblast culture

Five emu eggs were obtained from a commercial breeder in Marysville, Victoria, after 15 days of incubation at 37°C. Eggs were held under La Trobe University Animal Ethics Committee permit number AEC98/20(BG) and Department of Natural Resources and Environment permit number 10000081.

The embryo was decapitated and internal organs and limbs removed. The remaining tissue was cut into small pieces and disaggregated in 0.25% trypsin for 15 min. The suspension was then passed through a syringe and diluted 15 times in Dulbecco's modified Eagle's medium with 10% fetal calf serum, benzyl penicillin (60 μg/ml), dihydro streptomycin (50 μg/ml) and L-glutamine (100 μg/ml). Cultures were observed daily. They reached confluence in 2 days, when they were trypsinised and seeded at a 1:3 ratio into new culture flasks with the same medium. They could be subcultured once every 4–5 days for at least 9 passages before they showed signs of aging.

To sex the 5 cell lines, DNA was extracted from pelleted cells and used as a template in a PCR reaction with primers which gave sex specific bands in the female (PE AgGen).

Chromosome preparations

Cells were harvested with trypsin after 2 h incubation with 0.1 μg/ml of Colcemid. The cell suspension was incubated for 30 min in 0.75 mmol/L KCl, fixed in 3:1 methanol : acetic acid, then dropped onto acetone-cleaned slides and air dried. Slides were

passed through an alcohol series of 70%, 90% and 100% for 3 min each and stored at −70°C.

Preparation of chicken single chromosome paints

In order to prepare chromosome paints, chromosomes were isolated by dual laser flow-cytometry. Degenerate oligo-primed PCR (DOP-PCR) was carried out to amplify by approximately 400 1–9 and Z chromosomes (Telenius *et al*. 1992). Briefly, chromosomes were denatured for 9 min at 37°C, followed by 9 cycles of 94°C (1 min), 30°C (1.5 min) and 72°C (3 min with 0.23°C/s ramping) preceded by 30 cycles of 94°, 62°C and 72°C for 1 min each. The PCR amplification mix contained standard purified DOP primer, unlabelled nucleotides, W1 detergent (Sigma) and HC supertaq (HT Biotech, Cambridge) (Griffin *et al*. 1999).

The flow-sorted chromosomes were used in a DOP-PCR reaction using 200 μmol/L of 6MW primer (5′CCG ACT CGA GNN NNN NAT GTG G 3′) where N = any base (Telenius *et al*. 1992), 10 μl of 10 × expand buffer with $MgCl_2$, 20 mmol/L dNTPs and 1 μl of Expand enzyme (Boehringer Mannheim) in a 100-μl reaction. The reaction mixture was cycled at 94°C for 2 min, 35 cycles of (94°C for 30 s, 62°C for 1 min and 72°C for 5 min) and extended at 72°C for 10 min.

The chromosomes were labelled with biotin in a DOP-PCR reaction using 5 μl of the above PCR product in a 50 μl reaction containing 10 mmol/L of dNTPs (dGTP, dCTP, dATP), 2 mmol/L of biotin 16 dUTP (Boehringer Mannheim), 4 mmol/L of dTTP, and 2.5 μl of Expand enzyme under the conditions above.

Chromosome painting

Chicken single chromosome paints were hybridized to emu chromosomes under suppression conditions as previously described for marsupial chromosome-species painting (Toder *et al*. 1997). Chicken Cot 1 DNA (50 μg) and 40 μg of salmon sperm DNA was used for suppression. Approximately 1 μg of the labelled product was hybridized onto emu metaphase spreads for 48 h. Slides were then put through a series of washes, and blocked with 5% BSA/4 × SSC 0.2% Tween 20 for 45 min at 37°C. Avidin–FITC (fluorescein isothiocynate; Vector Laboratories) was used to detect the labelled probes. The slides were then once again put through a series of washes and chromosomes were counter-stained with DAPI (4,6-diamidino-2-phenylindole).

Hybridization signals were detected using a Zeiss Axioplan2 microscope and a Photometrics liquid-cooled CCD camera.

Results

Paints from each chicken chromosome except chromosome 4 hybridized to a single emu chromosome pair with the corresponding size and morphology. Chicken 1–3 and 5–9 hybridized solely and completely to emu 1–3 and 5–9 with the same morphology. All paints showed very strong and clean hybridization signals all along the length of the respective chromosomes in the emu spreads, including centromeres (Figure 2a).

The chicken chromosome 4 paint completely painted emu chromosome 4. However, this paint hybridized not only to the two homologues of emu chromosome 4, but also to a pair of emu microchromosomes (Figure 2b).

The chicken Z chromosome paint hybridized to both the Z and the W chromosomes of the emu (Figure 2c). Absence of signal was consistently observed around the centromeric region of the W, but not the Z chromosome.

Discussion

Hybridization of paints prepared from single chicken chromosomes onto emu chromosomes revealed strong homology between the macrochromosomes 1–9 of the two species. Thus the 9 macrochromosomes of the emu and the chicken are homologous at the DNA level. This confirms older observations that the chromosome number and morphology is conserved between ratites and carinates. In addition, G-banding studies (Ansari *et al*. 1988) on three species of ratite birds, (emu, American rhea, Darwin's rhea) and the duck (*Anas platyrhynchos*), which revealed a high degree of homology between the six largest pairs of macrochromosomes of the four species, with slight variations only in the centromeric positions of chromosomes 2 and 3 in ratites and carinates.

Compared with the level of chromosome rearrangement in mammals, this conservation is extraordin-

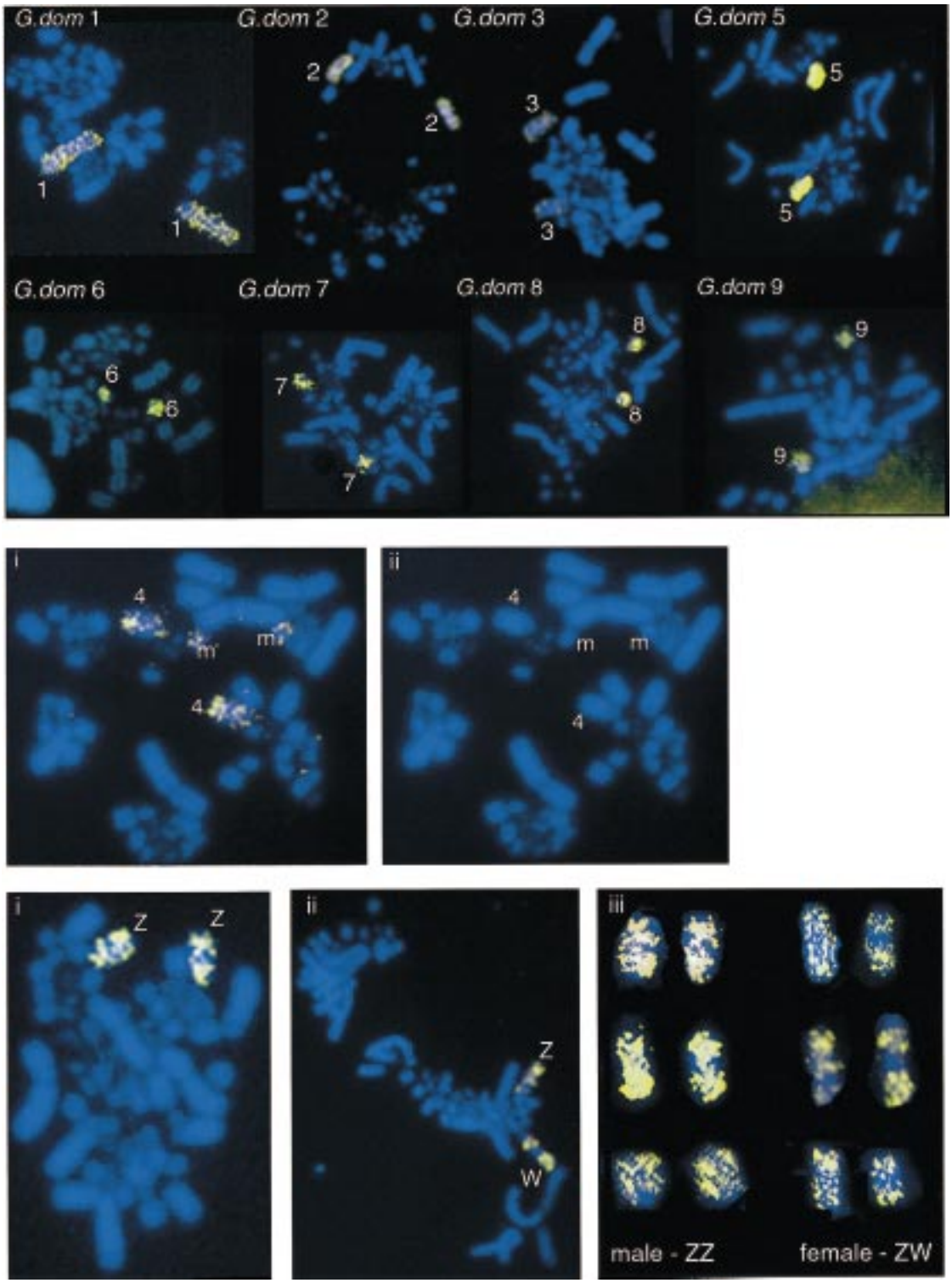

Figure 2. **a** Hybridization of chicken (*Gallus domesticus*) autosomal paints *G.dom* 1–3, 5–9 to emu chromosomes. Hybridizing chromosomes are labelled. **b** Hybridization of chicken 4 paint to emu chromosome 4 and to a pair of microchromosomes (m in i). Chromosomes were identified from DAPI-stained cell (ii). **c** Chicken chromosome Z paint hybridized to an emu male (i) and female (ii) metaphase spread. (iii) Comparison of painting patterns of ckicken Z paint on male and female emu sex chromosomes from different cells.

ary. Comparative chromosome painting reveals 10–20 interchromosomal rearrangements between humans, carnivores and artiodactyls, which diverged 60–80 MYrBP, and up to 100 between primates and rodents whose divergence is most closely comparable to ratite and carinate birds.

There are two particularly interesting features of bird karyotypes which are addressed by these results. One is the origin of the many tiny microchromosomes in all the bird karyotypes, and the other is the evolution of the ZW sex chromosomes and their function in sex determination.

The diploid numbers of the emu (2n = 80) and the chicken (2n = 78) differ by one microchromosome pair. This difference in the number of microchromosomes is explained by our present observation that the chicken chromosome 4 painted a pair of microchromosomes as well as emu chromosome 4. Thus bird macro- and microchromosomes are interchangeable. Did a larger ancestral chromosome 4 break up to in the ratite lineage, or did a smaller ancestral 4 fuse to a microchromosome in the chicken to give a larger chromosome 4 in galliformes? The observation that ratites and carinates both carry around 60 microchromosomes, as do many reptiles, whereas some of the modern carinates (e.g. eagles in family Accipitridae) have a reduced number, suggests that microchromosomes are an ancient feature of the reptile–avian lineage, but they can be fused to macrochromosomes.

The Z and W chromosomes are particularly interesting because of their evolution from an ancestral pair, and their as-yet-unknown role in sex determination. The ratite Z and W, which are barely distinguishable, may provide a good system for identifying genetic differences significant in sex determination. Our chromosome painting results show that the chicken and the emu Z chromosome are completely homologous, confirming G-banding studies that indicate homology right across birds. The mammalian X is, likewise, extremely conserved, although there have been at least two major additions in placental mammals of material that remains autosomal in marsupials and monotremes (Graves 1995). This conservation is accredited to a protection of chromosome-wide X inactivation system of dosage compensation (Ohno 1967). However, no evidence of dosage compensation of Z-linked genes has been reported in birds, and the Z chromosomes replicate synchronously. It seems more likely that the conservation of the bird Z chromosome, like the conservation of the other macrochromosomes, simply reflects the extraordinary stability of the bird karyotype.

In our chromosome painting experiments, the chicken Z chromosome paint gave a strong signal on both the emu Z and W, demonstrating that the emu W is largely homologous to the emu Z, and retains the ancient conserved bird Z sequences. This lends further support to the theory, first put forward by Ohno (1967) for snakes, that the sex chromosomes of birds were initially homologous autosomes, but the W underwent differentiation to different extents in different lineages. In ratites, the W has diverged little from the Z. Recent mapping studies with chicken Z-linked marker genes, IREBP, ZOV3 and W-linked marker sequence EE0.6 showed that these were linked to both the emu Z and W (Ogawa *et al*. 1998). In contrast, the W now shows little homology to the Z in carinate birds, although there are two DNA sequences shared between them (Fridolfsson *et al*. 1998).

Degeneration of the W chromosome is thought to have begun at the sex-determining locus, as recombination was suppressed between this locus and others conferring a female advantage or a male disadvantage. The limited regions of the emu W chromosome which show differentiation are likely to represent the region in which differentiation was initiated before the divergence of ratite and carinate birds. The absence of signal that we observed around the centromeric region of the W after painting with the Z paint may therefore point to this sex-determining locus, although centromeric regions are often enriched for species-specific heterochromatin. We are now microdissecting and characterizing sequences in this region, in an attempt to clone the bird sex-determining locus.

Our present ZOO-FISH studies therefore take us a step further in understanding the organization, function and evolution of bird genomes.

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