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Avian genomes: different karyotypes but a similar distribution of the GC-richest chromosome regions at interphase

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Abstract

The chicken karyotype, like that of the vast majority of avian species, shows a large number of dot-shaped microchromosomes that are characterized, like most telomeric regions of the macrochromosomes, by the highest GC levels and the highest gene densities. In interphase nuclei, these gene-dense regions are centrally located, and are characterized by an open chromatin structure (a similar situation also exists in mammals). Avian species belonging to the Accipitridae family (diurnal raptors) show a karyotype with no very large chromosomes, and with only a very small number of microchromosomes. To identify the GC-rich (and gene-rich) regions of the chromosomes and nuclei from Accipitridae, we performed heterologous *in-situ* hybridizations using chicken GC-richest isochores as probes. Our results clearly show that the gene-rich regions are prevalently located in the few microchromosome pairs and in the telomeric regions of the middle-sized chromosomes, as well as in the interior of the interphase nuclei. This result is consistent with a common organization of the genome in the nuclei of warm-blooded vertebrates. Indeed, in spite of the different size and morphology of the chromosomes, the gene-dense regions are always located in the interior of the nuclei.

Introduction

The avian karyotype is generally composed of 78–82 chromosomes, and typically comprises a few large macrochromosomes and many dot-shaped microchromosomes (review in Christidis 1990). This organization of the karyotype is shared by many distantly related bird species (from Ratites to Passerines) and is usually considered ancestral for living birds. In contrast, the Accipitridae family (diurnal raptors: buzzards, hawks, eagles, vultures etc.), belonging to the order Falconiformes (De Boer 1976), 'shows the most atypical karyotype known in the class Aves' (De Boer & Sinoo 1984). They have a more symmetrical karyotype with a moderate diploid number (often 66 chromosomes), without any very large chromosome and only very few dot-shaped microchromosomes.

Although the classical avian karyotype is very different from the mammalian ones, the isochore organization of the avian genome is similar to those of most mammals. In fact, the genomes from the vast majority of birds are characterized by the presence of about 15% of very GC-rich isochores belonging to

the H2, H3 and H4 families (Cortadas *et al*. 1979, Olofsson & Bernardi 1983, Kadi *et al*. 1993, Sabeur *et al*. 1993, Cacciò *et al*. 1994). The 'compositional mapping' of chicken chromosomes, namely the assessment of the GC level of chromosomal bands obtained by *in-situ* hybridization of isochore families, showed that (i) the chicken GC-richest isochores are localized on a large number of microchromosomes, as well as on almost all telomeric bands of macrochromosomes, and (ii) the GC-poorest L1 isochores are generally localized on the internal regions of macrochromosomes and are almost absent in microchromosomes (Andreozzi *et al*. 2001). An important feature of the isochores from warm-blooded vertebrates concern gene density. In fact, gene concentrations parallel GC levels, being very low and very high in the GC-poorest and the GC-richest isochore families, respectively (Mouchiroud *et al*. 1991, Zoubak *et al*. 1996).

Another important finding concerns the compositional organization of the cell nucleus. In fact, it was demonstrated that chromosomal regions located in the interior of the nucleus are very GC-rich and very gene-rich, whereas those located at the periphery are very GC-poor and very gene-poor. Moreover, these two nuclear compartments show different chromatin organization, the internal one being more open, the external one more compact, respectively (Saccone *et al*. 2002). This compositional organization of the cell nucleus is consistent with a functional compartmentalization of the chromatin inside the nucleus, as also demonstrated by other findings, such as the different replication timing of DNA located in the periphery and in the interior of the mammalian and avian cell nucleus (Strouboulis & Wolffe 1996, Ferreira *et al*. 1997, Sadoni *et al*. 1999).

In raptors, only a few species have been studied using chromosome banding: Griffon vulture (*Gyps fulvus*: Schmid *et al*. 1989), Bateleur (*Terathopius ecaudatus*: Bed'Hom *et al*. 1998), Black-winged kite (*Elanus caeruleus*: Bed'Hom *et al*. 2003) and Adalbert's eagle (*Aquila adalberti*: Padilla *et al*. 1999) for Accipitridae; Osprey (*Pandion haliaetus*: Kohler *et al*. 1989) for Pandionidae, the sister-group of Accipitridae. To advance our knowledge at the molecular level of these genomes, we studied three species from the Accipitridae family: the Black-winged kite (*Elanus caeruleus*), the Griffon vulture (*Gyps fulvus)* and the White-headed vulture (*Aegypius occipitalis*; this genus was formerly named *Trigonoceps*). *E. caeruleus* is a small (200–300 g) whitish raptor, mainly distributed in Africa, southern Europe and Asia. *G. fulvus* and *A. occipitalis* are both large vultures: up to 10 kg for *G. fulvus* and 5 kg for *A. occipitalis*. The former is distributed in northern Africa, southern Europe and eastern Asia, whereas the latter is present only in Africa. The rationale to study Accipitridae concerns the special features of their karyotypes that generally are characterized, in contrast to the majority of birds, by a very small number of microchromosomes.

In the present work, we wished to verify if the gene-dense chromosomal regions (largely corresponding to the microchromosomes of the avian karyotype) of the Accipitridae correspond to telomeric bands of the middle-sized chromosomes, as expected if taking into consideration previous data on other warm-blooded vertebrates. To verify this hypothesis, we performed *in-situ* hybridization of the well-characterized gene-poor and gene-rich isochores from chicken (see above) on chromosomes and nuclei from the Accipitridae. The choice of the heterologous *in-situ* hybridization was made because the use of homologous hybridizations would have involved (i) the preparative fractionation of the three genomic DNAs under study in order to obtain compositional fractions corresponding to the GC-poor, gene-poor isochores and to the GC-rich, gene-rich isochores; and (ii) the preparation of C_0t 1 DNA from each genome, to be added to the hybridization mixtures, in order to avoid any interference from repeated sequences. Obviously, this approach would be more laborious than the one we used.

Materials and methods

Chromosomes and nuclei preparation

Metaphase chromosomes and nuclei from chicken (*Gallus domesticus*) were obtained from primary fibroblast cell lines isolated from leg muscle biopsies of young domestic chicken. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mmol/L L-glutamine, 100 UI penicillin, 100 μg/ml streptomycin, and then incubated at 39°C in 5% CO_2. Monolayer cultures were treated with colcemid (final concentration 0.05 μg/ml) for 30 min, detached with trypsin for 10 min at 37°C, and then resuspended in hypotonic solution (75 mmol/L KCl)

for 20 min at 37°C. Conventional methanol/acetic acid fixation and slide preparation were then carried out.

Metaphase chromosomes and nuclei from Accipitridae were obtained from fibroblast cells established from skin biopsies as previously described (Bed'Hom *et al*. 2003). Conventional methanol/acetic acid fixation and slide preparation were then carried out for the preparations of nuclei and chromosomes (Saccone *et al*. 2002, Federico *et al*. 2004).

DNA probes

Compositional fractions of chicken genomic DNA were obtained by preparative ultracentrifugation in Cs_2SO_4 density gradients in the presence of the sequence-specific DNA ligand 3,6-bis (acetato-mercuri-methyl) dioxane, BAMD. Briefly, this was done at 20°C and 40 000 rpm for 65 h, using a BAMD/nucleotide molar ratio, rf = 0.14 (Cortadas *et al*. 1979). The GC-poorest and the GC-richest fractions were then separated using a fraction collector, and characterized in their CsCl profiles and modal buoyant densities, ρ°. More precisely, we used: (i) a DNA fraction containing the GC-poorest DNA (ρ° was 1.6978), and (ii) a DNA fraction containing the GC-richest DNA (ρ° was 1.7135). These chicken DNA fractions consist of isochores from the L1 and from the H3/H4 families, respectively. Then, the two DNA fractions were labelled with biotin or digoxigenin using a nick translation kit (Roche, Germany).

In-situ *hybridization and detection*

Dual colour *in-situ* hybridizations were performed with 200 ng of each of the two probes mentioned above. The labelled DNA fractions were then hybridized on chicken chromosomes and nuclei according to a standard protocol (Andreozzi *et al*. 2001). In the case of the heterologous hybridization (chicken DNA on chromosomes and nuclei from Accipitridae), this was done with some modifications. The hybridization mixture (containing 200 ng of each labelled probe) was 50% formamide/2 × SSC/10% dextran sulfate/50 mmol/L phosphate buffer, pH 7.0. Hybridizations were performed for 72 h at 37°C and the hybridized chromosomes and nuclei were washed three times in 1 × SSC at 42°C, before signal detection.

Signal detection was performed as previously described (Andreozzi *et al*. 2001, Saccone *et al*.

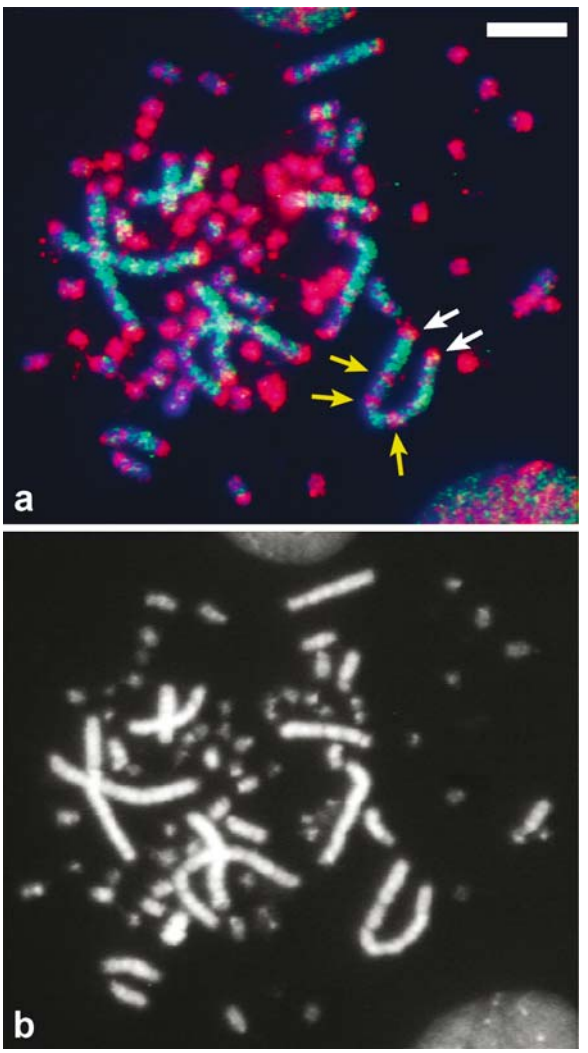


Figure 1. Hybridization of DNA probes on chicken chromosomes. Chicken DNA fractions characterized by the lowest and the highest GC levels were hybridized on chicken chromosomes. (**a**) The DNA fraction with the highest GC level (red signals) was localized on the microchromosomes and in the telomeric bands of the macrochromosomes (see white arrows as examples). Some internal bands of the macrochromosomes were also hybridized (see yellow arrows as examples). In contrast, the DNA fraction with the lowest GC-level (green signals) was localized in the internal bands of the macrochromosomes. The present results are in agreement with the previous compositional mapping of chicken chromosomes (Andreozzi *et al*. 2001). (**b**) The same metaphase shown in panel **a** is DAPI-stained in order to better show microchromosomes. The bar in the upper-right is 5 μm.

2002), using avidin conjugated with tetramethylrhodamine isothiocyanate (TRITC–avidin) and anti-digoxigenin antibody conjugated with fluorescein isothiocyanate (FITC–anti-digoxigenin antibody) to detect biotin and digoxigenin labelled probes, respectively. Epifluorescence microscopy (Olympus AX70), with the appropriate filters, and a CCD camera (COHU 4910 series), were used to capture hybridization signals, and fluorescent images were recorded using the MacProbe 4.2.3 and Photoshop 5.0 softwares.

The localization, in 2-Dimensional (2-D) cell nucleus analysis, of the GC-rich and the GC-poor isochores by *in-situ* hybridization were performed taking into consideration the following criteria: (i) each hybridized nucleus was divided into five concentric circles; (ii) when a large part of the signals was located in the four internal circles, namely when the signals were largely absent from the more peripheral circle, hybridization was considered as internal in the nucleus; (iii) when the hybridization signals covered the totality of the nucleus we considered that the localization was at the periphery of the nucleus. Because the above localization type is obtained after a 2-D analysis, we can assume that (i) the localization of the signals in the inner nucleus could be considered as definitive (the external circle being devoid of signals), whereas (ii) the localization of the signals at the periphery of the nucleus could be only considered as a strong indication because also the inner part of the nucleus contained hybridization signals; this finding is, however, not surprising in view of the fact that GC-poor regions are predominant in the avian genome and are interspersed with GC-rich regions.

Results

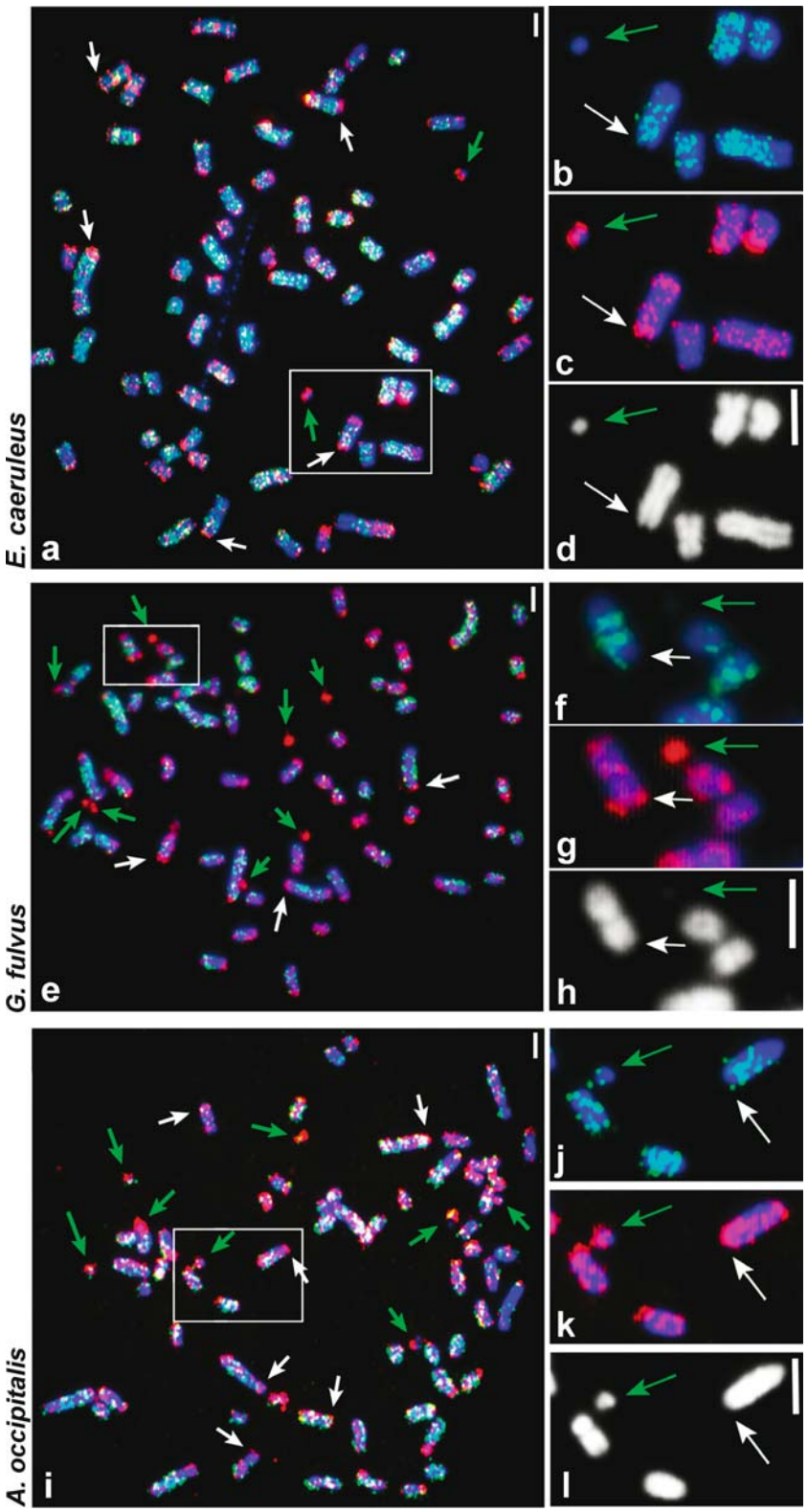
Compositional properties of the chromosomes of Accipitridae

The co-hybridization of the GC-richest and GC-poorest DNA fractions on chicken chromosomes clearly showed that the H3/H4 isochores are located on microchromosomes, in the telomeric regions of the macrochromosomes, and in some internal bands of the macrochromosomes. In contrast, the GC-poorest DNA were localized in internal regions of the macrochromosomes (Figure 1). The localization of the two DNA probes in chromosomes and in cell nuclei (see below) were in agreement with previous results (Andreozzi *et al*. 2001).

The chicken GC-richest and GC-poorest isochores were then hybridized on metaphase chromosomes of species from the Accipitridae family using low-stringency conditions. This was done in order to identify the chromosomal regions that showed the highest level of homology with the corresponding chicken DNA fractions. In the case of the chicken GC-richest isochores, this identified the chromosomal regions of Accipitridae that largely corresponded to the gene-dense chicken microchromosomes.

Elanus caeruleus has a karyotype with 2n = 68 chromosomes, with only one pair of dot-shaped microchromosomes. In this species, the results indicated a non-homogeneous distribution of the DNA sequences homologous to the GC-richest isochores from chicken (Figure 2). Indeed, hybridization signals were distributed on the large majority of the telomeric regions, including the single microchromosome pair that was almost completely covered by hybridization signals. In the case of the chicken GC-poorest isochores, hybridization signals were mainly observed in the internal bands of the chromosomes, and were absent in the telomeric regions that hybridized the chicken GC-richest isochores (Figure 2). Also the microchromosomes showed no hybridization with the L1 isochores. Thus, metaphase chromosomes from *E. caeruleus* showed a non-coincident localization of the GC-richest and the GC-poorest regions, as previously observed not only in chicken (Andreozzi *et al*. 2001), but also in mammalian chromosomes (Federico *et al*. 2000, Saccone *et al*. 2002, Federico *et al*. 2004). In fact, the GC-richest regions generally corresponded to the R(everse) bands, and the GC-poorest isochores to the G(iemsa) bands (data not shown).

Figure 2. The GC-richest and the GC-poorest chromosomal regions in *E. caeruleus*, *G. fulvus*, and *A. occipitalis*. (a, e, and i) Metaphase chromosomes (stained with DAPI, blue) from *E. caeruleus*, *G. fulvus*, and *A. occipitalis*, respectively, hybridized with the GC-richest (red signals) and the GC-poorest (green signals) isochores from the chicken genome. (b, c, d), (f, g, h), and (j, k, l) Enlargement of the squared area in a, e and i, respectively, showing the GC-poorest regions (green signals), the GC-richest regions (red signals), and the DAPI staining (printed in black and white), respectively. Green and white arrows indicate the microchromosomes and some representative GC-rich telomeric regions, respectively. The bar scales shown in the upper-right of panels **a**, **d**, **e**, **h**, **i** and **l** are 2 μm.


E. caeruleus
a
b
c
d
G. fulvus
e
f
g
h
A. occipitalis
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Similar results were also obtained on the two other species, *Gyps fulvus* and *Aegypius occipitalis* (Figure 2). These two species are characterized by a karyotype with a diploid number 2n = 66 chromosomes, including four microchromosome pairs that showed, as in *E. caeruleus*, a very high density of DNA homologous to the chicken GC-richest isochores. Moreover, also in these cases, the telomeric regions were enriched in DNA sequences with the highest level of homology with the chicken GC-richest isochores, whereas the internal regions showed homology with the chicken GC-poorest isochores.

Isochore distribution in the Accipitridae interphase cell nuclei

The chicken GC-richest and GC-poorest isochores were also hybridized on interphase nuclei of *E. caeruleus*, *G. fulvus* and *A. occipitalis* (Figure 3) in order to obtain information on the distribution of the gene-dense chromosomal regions that are also characterized, in other species, by a more open chromatin structure (Saccone *et al*. 2002, Federico *et al*. 2004). Our results showed that the chromosomal regions of Accipitridae with the highest level of homology with the GC-richest and the GC-poorest isochores from the chicken genome were observed in the interior and in the periphery of the nuclei, respectively. This is very evident in the case of *E. caeruleus* and *G. fulvus* cell nuclei but less so for *A. occipitalis* (see Figure 3). In fact, in this latter case, the GC-richest isochores from chicken showed hybridization signals more spread over all the nucleus if compared with that observed in the other two species. But, in any case, in *A. occipitalis*, the hybridization signals never showed a distribution like that obtained with the GC-poorest isochores. The above data and considerations justify our conclusion that, in the Accipitridae, the GC-richest and gene-richest regions are in the inner part of the cell nuclei.

Discussion

In the present work we investigated the compositional organization of the genome from diurnal raptors belonging to the Accipitridae family. The rationale to study these species concerns the special features of their karyotypes. In fact, diurnal raptors, at variance with other birds, are characterized by a karyotype with a very small number of microchromosomes, which are abundant in the karyotypes of other birds. Moreover, the karyotypes of these birds lack the largest macrochromosomes. Thus, the karyotypes of Black-winged kite, Griffon vulture, and White-headed vulture studied here, are composed of only middle-sized chromosomes plus one or four microchromosome pairs, respectively (De Boer 1976, De Boer & Sinoo 1984, Bed'Hom *et al*. 2003).

In the case of chicken, microchromosomes are endowed by a very high GC level and by all the structural and functional features related to the GC level, one of these being a very high gene concentration (McQueen *et al*. 1996, 1998, Smith *et al*. 2000, Andreozzi *et al*. 2001, ICGSC 2004). Moreover, this very high gene density was also demonstrated in almost all the telomeric regions of the macrochromosomes (Andreozzi *et al*. 2001, ICGSC 2004). At the interphase level, the gene-dense regions of the chicken genome were shown to be located in the inner part of the cell nucleus (Saccone *et al*. 2002).

The hybridization of the chicken GC-richest (and gene-richest) isochores on the chromosomes of the three birds studied here showed that the fluorescent signals are non-homogeneously distributed in the three karyotypes. More precisely, the chromosomal regions with the highest number of signals, and therefore endowed with the highest level of homology with the chicken GC-richest isochores, were essentially observed in the more telomeric bands. Considering the type of hybridization (heterologous) and the stringency conditions used, we can assume that the hybridized regions correspond to the GC-richest regions of the diurnal raptors' chromosomes, and to their gene-dense regions. Moreover, we can also assume that the hybridized sequences largely correspond to coding sequences, namely to sequences that are more conserved during the evolution of the genomes.

From an evolutionary point of view, if the classical avian karyotype, composed of few large macrochromosomes and many small microchromosomes, is considered as the ancestral one, then the chromosomes of the Accipitridae should derive from the fusion of microchromosomes and from size-reduction of the macrochromosomes, perhaps by a mechanism based on accumulation of reciprocal translocations and fusions in this evolutionary lineage, as expected by computer simulation (Bed'Hom 2000). In all cases the gene-dense regions are always located in the majority

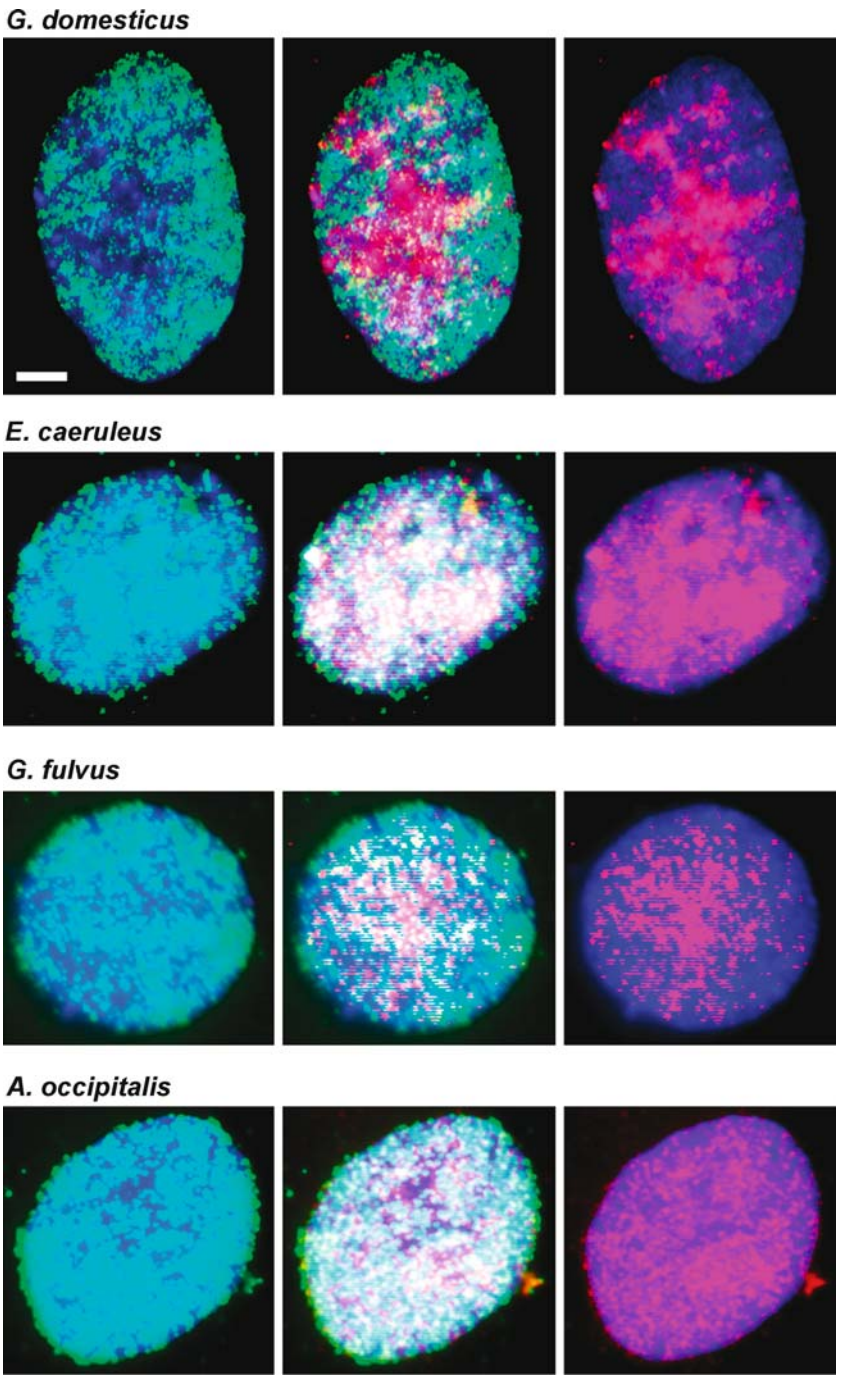

Figure 3. Compositional properties of interphase nuclei from Accipitridae. Interphase nuclei from *E. caeruleus*, *G. fulvus* and *A. occipitalis* were hybridized with the GC-richest (red signals) and the GC-poorest (green signals) isochores from the chicken genome. An interphase nucleus from chicken, hybridized with the same probes, is also shown for comparison. Nuclei in the middle of each line show the dual-colour *in-situ* hybridizations. The left and the right nuclei from each line are the same nuclei as in the middle showing the hybridization with the chicken GC-poorest and GC-richest isochores, respectively. Nuclei were stained with DAPI (blue). The bar in the upper-left panel is 2 μm.

of telomeric regions of macro-, micro- and middle-sized chromosomes.

The signal distribution in the mitotic chromosomes of Accipitridae resembles that of the GC-richest isochores observed in the human (Saccone *et al*. 1992, 1996, 1999), mouse (Saccone *et al*. 1997), pig (Federico *et al*. 2004) and chicken chromosomes (Andreozzi *et al*. 2001). It should be noted that the GC-richest bands observed in chicken microchromosomes should also be considered telomeric bands if we can take into account the small size of these chromosomes. Thus, one of the main conclusion of the present work is that the GC-richest and gene-richest regions of warm-blooded vertebrates, in spite of differences in the size and morphology of the chromosomes, are largely located in the more telomeric bands.

An important finding concerns the localization of the GC-richest and the GC-poorest chromosomal regions in the interior and in the periphery of nuclei, respectively. This indicates that the interior of the nuclei of the species investigated contains the DNA sequences having the highest level of homology with the chicken GC-richest and gene-richest isochores. These regions correspond to more open chromatin structures, as previously shown in both human and chicken (Saccone *et al*. 2002; and recently confirmed by Gilbert *et al*. 2004, Bolzer *et al*. 2005). In the case of GC-poor regions, it is most likely that they are, in fact, concentrated at the periphery of the nucleus (see Materials and methods). This indicates a general plan of organization of the avian and mammalian genomes at the nuclear level. Moreover, some very recent data on cold blooded vertebrates (Federico *et al*. 2005) indicate that the isochore organization in the cell nucleus and the corresponding chromatin architecture are the same in all vertebrates, with the gene-dense and the gene-poor regions located in the interior and in the periphery, respectively, of the interphase nucleus.

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