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Reciprocal chromosome painting between white hawk (*Leucopternis albicollis*) and chicken reveals extensive fusions and fissions during karyotype evolution of accipitridae (Aves, Falconiformes)

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Abstract Evolutionary cytogenetics can take confidence from methodological and analytical advances that promise to speed up data acquisition and analysis. Drastic chromosomal reshuffling has been documented in birds of prey by FISH. However, the available probes, derived from chicken, have the limitation of not being capable of determining if breakpoints are similar in different species: possible synapomorphies are based on the number of segments hybridized by each of chicken chromosome probes. Hence, we employed FACS to construct chromosome paint sets of the white hawk (*Leucopternis albicollis*), a Neotropical species of Accipitridae with 2n=66. FISH experiments enabled us to assign subchromosomal homologies between chicken and white hawk. In agreement with previous reports, we found the occurrence of fusions involving segments homologous to chicken microchromosomes and macrochromosomes. The use of these probes in other birds of prey can identify important chromosomal synapomorphies and clarify the phylogenetic position of different groups of Accipitridae.

Keywords *Leucopternis albicollis* · chromosome paints · *Gallus* · Accipitridae

Abbreviations

BAC	Bacterial artificial chromosome
DOP-PCR	Degenerate oligonucleotide-primed polymerase chain reaction
FACs	Fluorescence-activated chromosome sorting
FISH	Fluorescent in situ hybridization
GGA	*Gallus gallus*
LAL	*Leucopternis albicollis*

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Introduction

In recent years, several comparative mapping studies in birds have focused on individual chicken macro- and microchromosomes (Griffin et al. 1999; Crooijmans et al. 2001; Buitenhuis et al. 2002), expanding and refining the previously described synteny information between chicken, human, and mouse. Although data are still sparse, a few studies have suggested that the preservation of gene order along avian chromosomes may be highly conserved and substantially higher than in mammals (Shetty et al. 1999; Raudsepp et al. 2002; Guttenbach et al. 2003).

The conservation of chicken macrochromosome syntenic groups was found in species of birds belonging to a range of different orders or even in to a different subclass, such as the emu (*Dromais novaehollandiae*), a ratite with at least 80 million years of divergence from carinate birds (Shetty et al. 1999). This fact led to the assumption that the genomic organization found in chicken, with the exception of chromosome 4, which corresponds to two different elements in most species of birds, represents an ancestral characteristic. Based on this, a putative ancestral avian karyotype has been proposed, with a 2n=80 (Griffin et al. 2007) and atypical karyotypes in bird species must have derived from a complement similar to this one.

Some examples of drastic chromosomal reorganization were found among especially in birds of prey (Grützner et al. 2001; de Oliveira et al. 2005; Nanda et al. 2006). The processes that gave rise to these atypical chromosome complements observed in Accipitridae and Falconidae have just begun to be decoded, mostly by the use of chicken macro-chromosome paints and can be refined in the near future by the use of BAC-derived probes that identify microchromosomes and gene order. Hence, the new field of avian comparative cytogenetics can take confidence from methodological and analytical advances that promise to speed up data acquisition and analysis.

Examples from mammals clearly demonstrate the utility of karyological data for higher-level systematic studies (Ferguson-Smith and Trifonov 2007). However, in birds, the available probes have the limitation of not being capable of determining if breakpoints are similar in different species. Considering that the identification of synapomorphies is essential in phylogenetics, it would be important to have chromosome probes that correspond to region-specific paints in chicken, such as those derived from birds of prey. This scenario led us to employ FACS to construct chromosome paint sets of the white hawk (*Leucopternis albicollis* (LAL)), a Neotropical species of Accipitridae, usually included in the subfamily Buteoninae (Lerner and Mindell 2005). These probes were used in FISH experiments to reciprocally paint *Gallus gallus* (GGA) and white hawk metaphases and enabled us to assign subchromosomal homologies between these species and to obtain a better interpretation of the chromosome differences between *GGA* and Accipitridae. Our findings refine hypotheses about the genomic rearrangements that gave rise to the karyotypes of birds of prey.

Material and methods

Cell samples

Fibroblast cell lines derived from a female GGA embryo and from a female LAL feather pulp were grown according to Sasaki et al. (1968) with modifications. Cultures were initiated from this tissue using dissociated cells following incubation in collagenase for 1 h. Chromosomes were obtained by standard arrest with colcemid (Gibco), hypotonic treatment with 0.075 M KCl, and cell fixation in methanol/acetic acid (3:1) and kept in freezer for further experiments. Diploid number definition and karyotype ordering were performed in conventionally stained metaphases (Giemsa 5% in 0.07 M phosphate buffer, pH6.8).

Flow sorting and preparation of chromosome painting probes

Both GGA and LAL chromosome-specific probes were made by degenerate oligonucleotide-primed PCR (DOP-PCR) from flow-sorted chromosomes using PCR primers, amplification, and labeling conditions as previously described (Telenius et al. 1992; Rabbits et al. 1995; Griffin et al. 1999). Chromosome sorting was performed using a dual-laser cell sorter (MoFlo, DAKO, Glostrup, Denmark). This system yielded a bivariate analysis of the chromosomes by size and base-pair composition. About 400 chromosomes were sorted from each peak in the flow

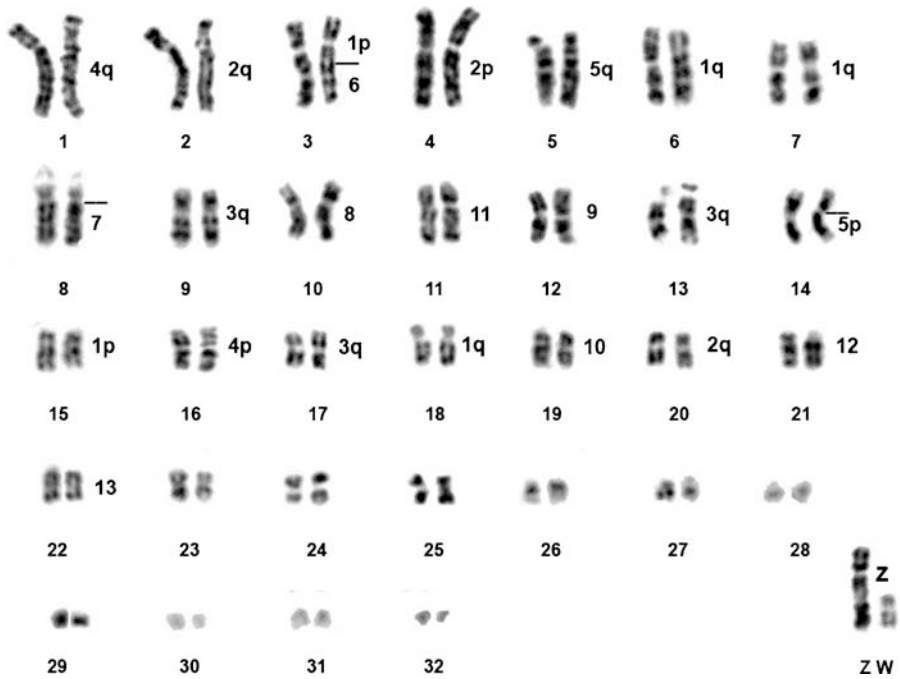

Fig. 1 G-banded karyotype of a female *L. albicollis*. The chromosomes are *numbered below*. Homology with chicken chromosomes is shown to the *right*

karyotype. Chromosomes were sorted directly into PCR tubes containing 30 ml distilled water; 6-MW primer (Rabbits et al. 1995) was used in the primary reaction and to label the chromosomal DNA in a secondary PCR.

Conventional staining, G-banding, in situ hybridization, and probe detection

Conventional staining was performed with Giemsa solution in phosphate buffer 0.06 M, pH6.8 during 15 min, and chromosome classification according to centromeric position used the nomenclature of Levan et al. (1964). G-banding with trypsin (Seabright 1971) prior to hybridization experiments was performed. After microscopic inspection and registration of the metaphase coordinates, slides were incubated in 4×SSC/0.2% Tween for 60 min, 70% ethanol for 15 min and methanol/acetic acid (3:1) for 15 min, followed by serial ethanol dehydration (70%, 90%, and 100%). In situ hybridization and probe detection were carried out exactly as described by de Oliveira et

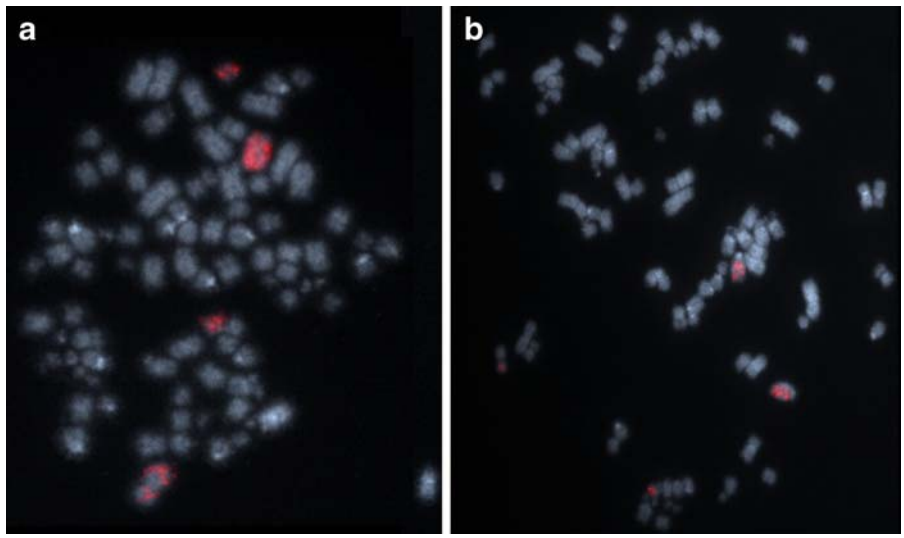

Fig. 2 Representative FISH experiments with chicken whole chromosome paints: GGA4 (**a**) and GGA 5 (**b**), each hybridizing to two different pairs of LAL chromosomes

al. (2005). Biotinylated DNA probes were detected by Avidin-Cy5 (Vector Laboratories). Chromosomes were counterstained with DAPI (40,6-diamidino-2-phenylindol, Sigma).

Image capture and processing

Metaphase images were captured using a cooled CCD camera (Photometrics NU200 equipped with a Kodak KAF1400 CCD chip) coupled to the microscope. Imaging software was SmartCapture (Digital Scientific, Cambridge, UK).

Results

White hawk karyotype: characterization and FISH with chicken chromosome paints

Conventional analysis of metaphases of the white hawk showed a karyotype of 2n=66 with 20 pairs of metacentric/submetacentric chromosomes (1–14, 16–18, 23–25), eight subtelocentric/telocentric pairs (15, 19–22, 26–28), and four pairs of microchromosomes (29–32). The *Z* is submetacentric, as large as pair 1, and the *W* is subtelocentric and about half the size of the *Z*. Paints specific to chicken macrochromosomes 1–10 and *Z* were used to paint white hawk metaphases (Fig. 1). The 11 chicken paints produced 21 clear signals on the white hawk karyotype. Paints GGA 1 to GGA 5 produced signals in multiple pairs in the white hawk karyotype, indicating the occurrence of fission events in the ancestral avian karyotype (Fig. 2). The only LAL chromosome painted by two different probes was LAL 2, which was hybridized by both GGA1 and GGA6.

Flow-sorting karyotype

The bivariate flow karyotype of the white hawk was resolved into 31 peaks. Flow sorting and DOP-PCR provided chromosome paints from each peak. These paints were then hybridized to white hawk metaphases in order to identify the chromosome content of each peak of the flow karyotype. Pairs 9–11 were contained in the same peak, as well as 29 with 30 and 31 with 32 (Fig. 3).

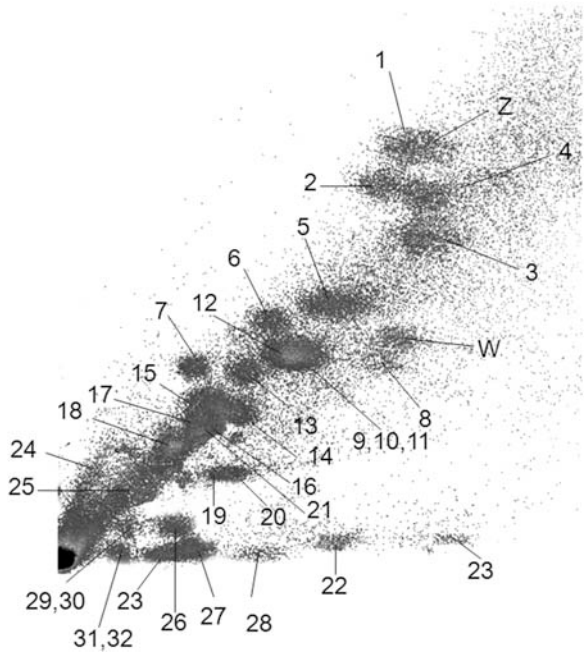

Fig. 3 The bivariate flow karyotype of the white hawk is shown. Chromosomes were sorted for DNA content and AT to GC base pair ratios into 33 peaks after staining with Hoechst 22358 (*vertical axis*) and chromomycin-A (*horizontal axis*). Highly pure sorts of single chromosomes were obtained for 30 pairs of white hawk chromosomes. Two peaks contain two chromosome pairs, and one peak contains three

White hawk chromosome paints on chicken metaphases

Paints of white hawk chromosomes were hybridized onto chicken metaphases (Fig. 4a–e). Chicken chromosomes 1 to 5 were painted by two (GGA 4 and 5), three (GGA 2), four (GGA 3), or five (GGA 1) different LAL probes. Moreover, LAL 3 whole-chromosome probe also painted chicken microchromosomes (Fig. 4a). Only one probe (LAL 2) painted two different macrochromosomes, revealing that LAL 2 arose by a rearrangement between GGA1p and GGA6. The comparative map of white hawk probes onto chicken macrochromosomes is shown in Fig. 5.

Discussion

Compared with a classical avian karyotype, characterized by the presence of a few large macrochromosomes and many microchromosomes, birds of prey have shown much derived karyotypes, usually dis-

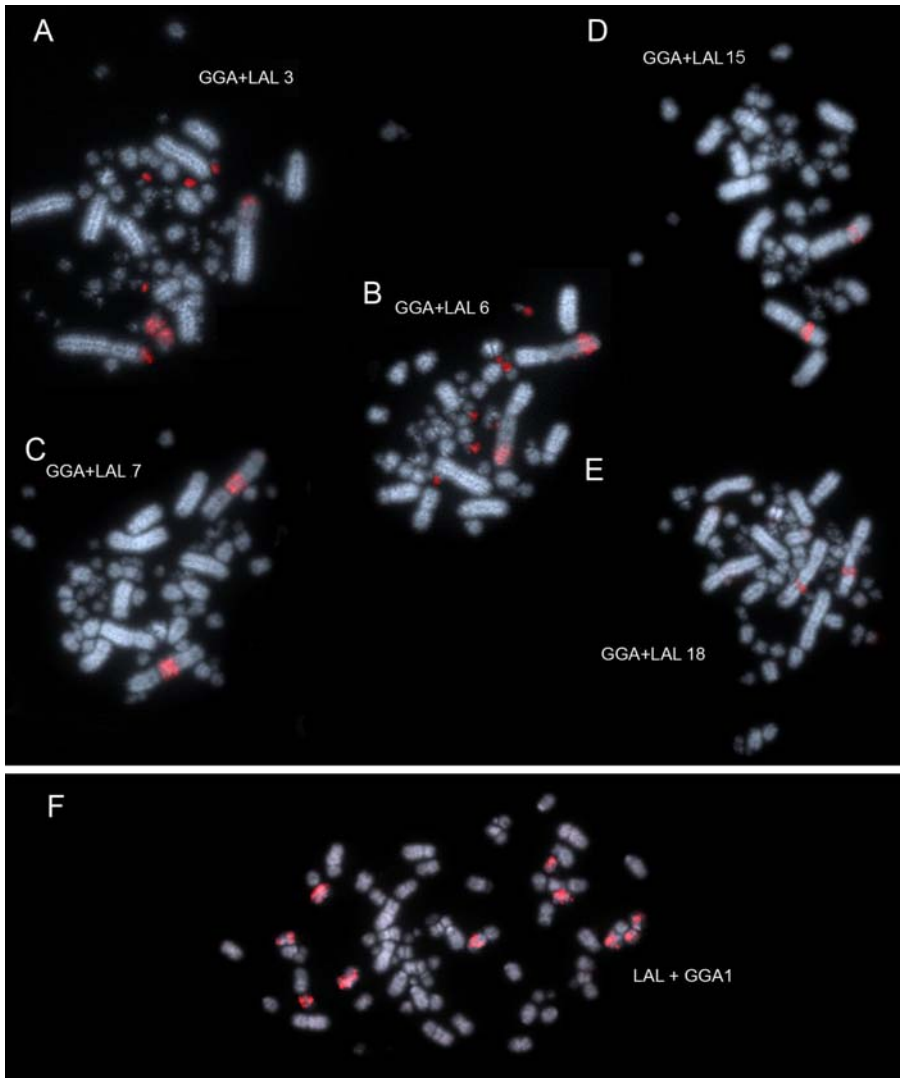

Fig. 4 Demonstration of how reciprocal painting provided additional data on the subregional homology between chicken and white hawk chromosomes. The paint from chicken chromosome 1 hybridizes five white hawk chromosomes (*F*), but the subregional homology was known only after these five white hawk chromosome probes were painted to chicken chromosome 1 (*A–E*)

playing a sharply different karyotypic organization, with numerous medium-sized chromosomes and only a few pairs of microchromosomes (Bed'Hom et al. 2003). For this reason, several species of Accipitridae and Falconidae have been subject of analysis with chromosome painting, but previous reports have used only GGA chromosome paints. In agreement with karyotypic data of other species of Buteoninae, with diploid number ranging from 2n=66 to 2n=70 (Amaral and Jorge 2003), *L. albicollis* showed a karyotype with 66 chromosomes, with a slight predominance of biarmed elements and only four pairs of microchromosomes. The establishment of fibroblast cultures from feather pulp allowed the obtention of whole chromosome probes by flow sorting, resolving the karyotype into 31 flow peaks, most of them containing copies of one pair of LAL chromosome.

Cross-species chromosome painting experiments in Accipitridae have shown the occurrence of fission events involving the largest pairs of macrochromosome found in the avian putative ancestor karyotype, as well as multiple fusions involving microchromosomes (de Oliveira et al. 2005; Nanda et al. 2006; Nishida et al. 2008). However, with unidirectional painting, the subchromosomal origin of each hybridization signal remained unknown. Considering that homology between chicken and many other different species of birds is highly conserved, and especially the homology between a species of turtle (*Pelodiscus sinensis*) and chicken chromosomes is also highly conserved, with the six largest chromosomes being

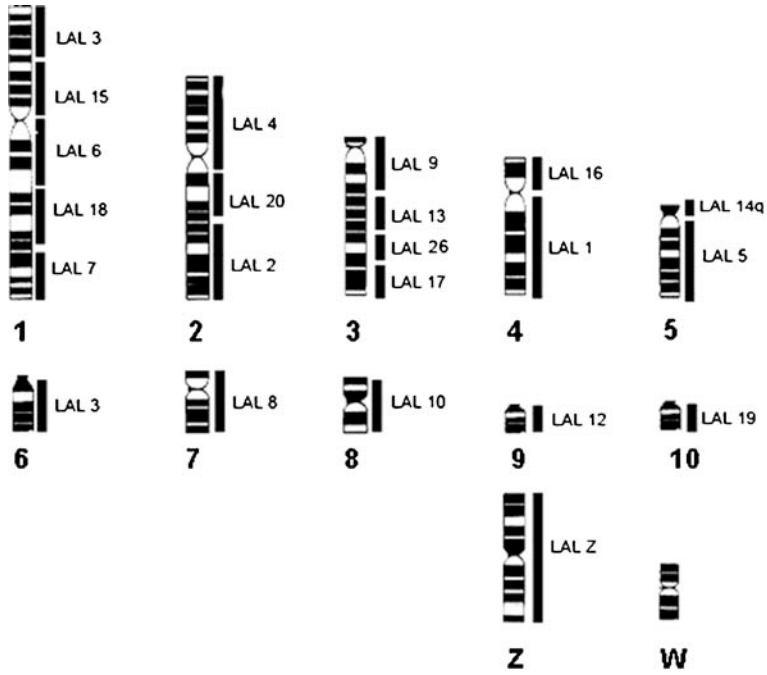

Fig. 5 Homology map between chicken macrochromosomes and white hawk paints

almost equivalent to each other, as well as the chicken Z chromosome, which correspond to a whole chromosome arm in the turtle (Matsuda et al. 2005), it is reasonable to conclude that Accipitrid karyotypes represent a derived lineage; hence, the fission events observed in chicken macrochromosomes occurred more recently in this group.

Reciprocal chromosome painting between GGA and LAL identified subchromosomal assignments for the cases in which GGA probes painted more than one pair in LAL karyotype. For instance, GGA 1 is highly rearranged in the LAL karyotype: five hybridization signals from five different LAL chromosomes were found on this chromosome (Fig. 4). Hence, the subchromosomal origins of LAL chromosomes from the putative ancestral avian karyotype were revealed by reciprocal chromosome painting to chicken chromosomes. The same was observed for other probes which painted two (GGA 4 and GGA 5), three (GGA 2), or four (GGA 3) distinct pairs in LAL karyotype.

The correct identification of the segments involved in rearrangements is crucial in the identification of homologies between different groups of birds of prey. So far, homology of rearrangements in different species of birds of prey has been proposed based on the number of pairs hybridized by each macrochromosome of GGA in their karyotype, without the confirmation of identical breakpoints (Nanda et al. 2006; Griffin et al. 2007). The use of both chicken and white hawk probes provided a more accurate method of identification of homologies and break points, as well as possible intrachromosomal rearrangements, such as inversions. The revaluation of homology maps between *Gallus* and other species of birds of prey using LAL chromosome-specific probes could reveal important information concerning phylogenetic relationships and karyotypic evolution in this group.

The most noticeable characteristics of the karyotypes of Accipitridae—lower diploid numbers and lower number of microchromosomes—occurred due to fusions involving microchromosomes, although the fragmentation of macrochromosomes should, by itself, increase the diploid number. The presence of karyotypes with 2n close to 66–70 in Accipitridae suggests that many of these fusion/fission events occurred very early in the history of this group. Even in Accipitridae with high diploid numbers, such as *Aquila alberti*, with 2n=82, the number of microchromosomes is lower than in other birds (only 12 pairs, while chicken shows at least 19 microchromosome pairs in a karyotype with 2n=78) (Padilla et al. 1999; Musa et al. 2005), suggesting that many pairs of microchromosomes have already fused with other segments, and the high diploid number must have

resulted from fission events involving macrochromosome, which are much smaller than the ones found in *Gallus* and other species with bimodal karyotypes.

Concluding, LAL probes could lead to more refined homology maps between different species of birds and chicken, clarifying the occurrence of possible recurrent breakpoint hotspots, synapomorphies, and chromosome rearrangements common to different avian lineages. A revaluation of homology maps among birds of prey would be valuable to further refine their chromosomal evolutionary history.

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