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RESEARCH ARTICLE

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Chromosome reshuffling in birds of prey: the karyotype of the world's largest eagle (Harpy eagle, *Harpia harpyja*) compared to that of the chicken (*Gallus gallus*)

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Abstract Like various other diurnal birds of prey, the world's largest eagle, the Harpy (*Harpia harpyja*), presents an atypical bird karyotype with 2*n*=58 chromosomes. There is little knowledge about the dramatic changes in the genomic reorganization of these species compared to other birds. Since recently, the chicken provides a "default map" for various birds including the first genomic DNA sequence of a bird species. Obviously, the gross division of the chicken genome into relatively gene-poor macrochromosomes and predominantly gene-rich microchromosomes has been conserved for more than 150 million years in most bird species. Here, we present classical features of the Harpy eagle karyotype but also chromosomal homologies between *H. harpyja* and the chicken by chromosome painting and comparison to the chicken genome map. We used two different sets of painting probes: (1) chicken chromosomes were divided into three size categories: (a) macrochromosomes 1–5 and Z, (b) medium-sized chromosomes 6–10, and (c) 19 microchromosomes; (2) combinatorially labeled chicken chromosome paints 1–6 and Z. Both probe sets were visualized on *H. harpyja* chromosomes by multicolor fluorescence in situ hybridization (FISH). Our data show how the organization into micro- and macrochromosomes has been lost in the Harpy eagle, seemingly without any preference or constraints.

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Introduction

The typical avian karyotype with 2*n*=78 chromosomes is widely conserved among very divergent orders of birds (Burt 2002; Schmid et al. 2000). It is composed of few large macrochromosomes that are comparable in size with mammalian chromosomes and numerous morphologically indistinguishable microchromosomes ranging in size from some 23 to less than 5 Mbp. Some birds of prey of the order Falconiformes, including hawks, buzzards, and eagles, however, show strikingly different karyotypes with lower numbers of mostly medium-sized chromosomes and only few chromosomes of the size of typical avian microchromosomes (Bed'Hom et al. 2003).

Because of its economic impact and its importance in biomedical research as a model organism for developmental biology and neurobiology, the genome of the domestic chicken *Gallus gallus domesticus* generally serves as the starting point and reference for the investigation of other avian genomes. Its physiology is well understood, and various genomic resources are available, including a recent first draft of the genome sequence (International Chicken Genome Sequencing Consortium 2004) (http://www.ensembl.org/Gallus_gallus). The chicken shares the typical avian karyotype with 2*n*=78 and micro- and macrochromosomes. Chicken macrochromosomes (1–9, Z, W) comprise about 75% of the genome, which mainly consists of AT-rich,

relatively gene-poor, and late replicating DNA, whereas microchromosomes show converse features (recently summarized in Schmid et al. 2000 and International Chicken Genome Sequencing Consortium 2004).

Lately, chromosome-specific painting probes for the chicken macrochromosomes 1–9, for several of the larger microchromosomes and for fractions of the smaller microchromosomes, became available (Griffin et al. 1999; Habermann et al. 2001). More recently, the entire chromosome set of the chicken was defined by fluorescence in situ hybridization (FISH) with DNA probes (Masabanda et al. 2004). Cross-species chromosome painting studies between the chicken and the emu (*Dromaius novaehollandiae*) demonstrated that macrochromosomes are well conserved for more than 80 million years of bird evolution (Shetty et al. 1999). These data suggest that the typical avian karyotype with macro- and microchromosomes may be the ancestral state for all birds. Further, microchromosomes are also present in some primitive amphibians and most reptiles, suggesting that some, if not most avian microchromosomes, represent ancestral vertebrate syntenies (Burt 2002).

Thus, in birds of prey, numerous chromosome rearrangements must have occurred, including fusions of microchromosomes and/or their translocation onto larger chromosomes (Bed'Hom 1999; de Lucca and Rocha 1992; Rodionov 1996; Tegelstrom et al. 1983). Like other raptors, the Harpy eagle (*Harpia harpyja*, Family Accipitridae), which inhabits neotropical rainforests from southern Mexico to northern Argentina, is a representative example for these atypical bird karyotypes. *H. harpyja* presents a karyotype of $2n$=58 (Hoffmann et al. 1976), consisting of largely medium-sized chromosomes that gradually decrease in size, and of which only four chromosome pairs are comparable in size to chicken microchromosomes.

We performed an initial characterization of the chromosome complement of the Harpy eagle employing differential staining techniques and FISH with a telomeric repeat probe conserved in vertebrates [$(TTAGGG)_n$; Meyne et al. 1989]. Further, we delineated chromosomal homologies between the Harpy eagle and the chicken by cross-species chromosome painting of various chicken chromosome-specific probes. These experiments provide initial insight into the extent and the evolutionary direction of chromosomal rearrangements that changed the karyotype of the Harpy eagle and probably those of other raptors.

Materials and methods

Cell samples

Blood samples were collected from two specimens (male and female) of Harpy eagle kept at the Zoológico Municipal de Curitiba (Curitiba, PR, Brazil). Blood cells were grown and harvested as described by Moorhead et al. (1960), with minor modifications. Metaphase preparations followed standard procedures. Chicken metaphase spreads were prepared from a fibroblast culture (Habermann et al. 2001).

Banding techniques

Metaphase chromosomes of *H. harpyja* were studied by C- and nucleolar organizer region (NOR) banding techniques according to Sumner (1972) and Howell and Black (1980). G-banding technique followed Seabright (1971) with minor modifications. 7-Aminoactinomycin-D (7-AAD; emission maximum 647 nm, red fluorescence) differential DNA staining was performed essentially according to Gill et al. (1975), but in combination with a subsequent 4′,6-diamidino-2-phenylindole (DAPI) counterstain (emission maximum 461 nm, blue fluorescence).

Probe composition, in situ hybridization, and detection

The biotinylated "All Human Telomere Probe" (ONCOR) was applied in FISH experiments according to the manufacturer's protocol. For multicolor cross-species chromosome painting, two independent experiments using different multiplex probe sets were performed. The probe sets were the same as previously described (Habermann et al. 2001): (1) chicken chromosome 1–6 and Z paint probes were combinatorially labeled with three fluorochromes and hybridized to eagle chromosomes; (2) chromosome paint probes of closely the entire chicken karyotype were divided into three differentially labeled probe pools according to their size: (a) macrochromosomes 1–5 and Z, (b) medium-sized chromosomes 6–10, and (c) 19 different microchromosomes. Both probe sets were labeled with biotin–dUTP, digoxigenin–dUTP, or TAMRA–dUTP, respectively, and mixed with tenfold excess of chicken Cot-1 DNA. Cross-species hybridizations to eagle chromosomes were performed for 72 h, followed by stringency washes for 2× 5 min in 50% formamide/2×SSC (37°C) and 2×5 min in 2×SSC (37°C). Biotinylated probes were detected with avidin-Cy3 or avidin-Cy5 (Amersham), digoxigenin-labeled probes with sheep anti-digoxigenin FITC coupled antibody (Roche). Chromosomes were counterstained with DAPI and embedded with Vectashield mounting medium (Vector Laboratories).

Microscopy and image analysis

Metaphase images were captured with a cooled CCD camera (Photometrics C250/A equipped with a Kodak KAF1400 chip) coupled to a Zeiss Axiophot microscope. Camera control and digital image acquisition was performed using SmartCapture VP software (Digital Scientific, Cambridge, UK).

Results

Classical cytogenetics, differential DNA staining, and telomeric repeats

The karyotype of *H. harpyja* (2*n*=58 chromosomes) has 18 biarmed and 10 acrocentric autosomal pairs. The sex chromosomes are ZZ in the male and ZW in the female. The Z is a large submetacentric, whereas the W is a medium-sized submetacentric chromosome. Constitutive heterochromatin blocks visualized by C-banding were located in the pericentromeric region of most chromosomes. The W chromosome showed a large heterochromatic block, corresponding to roughly two thirds of its total length (Fig. 1a). NORs were located by silver staining on chromosome 8 and on a microchromosome-sized pair, most likely chromosome 25 (Fig. 1b).

Differential DNA staining of chicken and *H. harpyja* metaphase chromosomes was performed using a combination of DAPI and 7-AAD (Fig. 1c, d). DAPI preferentially binds AT-rich DNA, whereas 7-AAD is a GC-selective DNA stain. GC-rich chicken microchromosomes showed intense 7-AAD fluorescence, compared to the macrochromosomes. In the Harpy eagle, bright 7-AAD fluorescence was observed on the microchromosomes but also in several blocks up to the size of chromosome arms on medium-sized and large chromosomes. In situ hybridization of a biotinylated telomere-specific $(TTAGGG)_n$ probe to metaphase chromosomes from *H. harpyja* (Fig. 1e) provided no evidence for the existence of interstitial telomeric sequences.

Cross-species chromosome painting

Chromosome painting with combinatorially labeled chicken (GGA) macrochromosome probes 1–6 and Z (Fig. 2a) revealed 16 homologous chromosome regions on 15 different autosome pairs of *H. harpyja* (HHA). The chicken Z painting probe hybridized to the entire *H. harpyja* Z chromosome and showed cross-hybridization to the entire W chromosome (Fig. 2b). The assignment of chicken homologous regions to G-banded *H. harpyja* chromosomes is summarized in Fig. 2d.

The set of pooled and differentially labeled chicken macrochromosomes 1–5, medium-sized chromosomes 6–10, and 19 different microchromosomes also delivered reproducible hybridization signals when hybridized to *H. harpyja* chromosomes (Fig. 2c). The probe pool of chicken macrochromosomes hybridized to 14 different *H. harpyja* autosomes (Fig. 2d). The probe pool representing the five medium-sized chicken chromosomes labeled five homologous chromosomes in *H. harpyja*. Finally, the probe pool representing 19 out of the 29 chicken microchromosomes delineated 16 regions on 15 different chromosomes of the Harpy eagle. Nine of these Harpy eagle chromosomes exclusively showed homologies to chicken microchromosomes. The remaining seven hybridization signals of the microchromosome probe pool were observed on segments of six different Harpy eagle chromosomes.

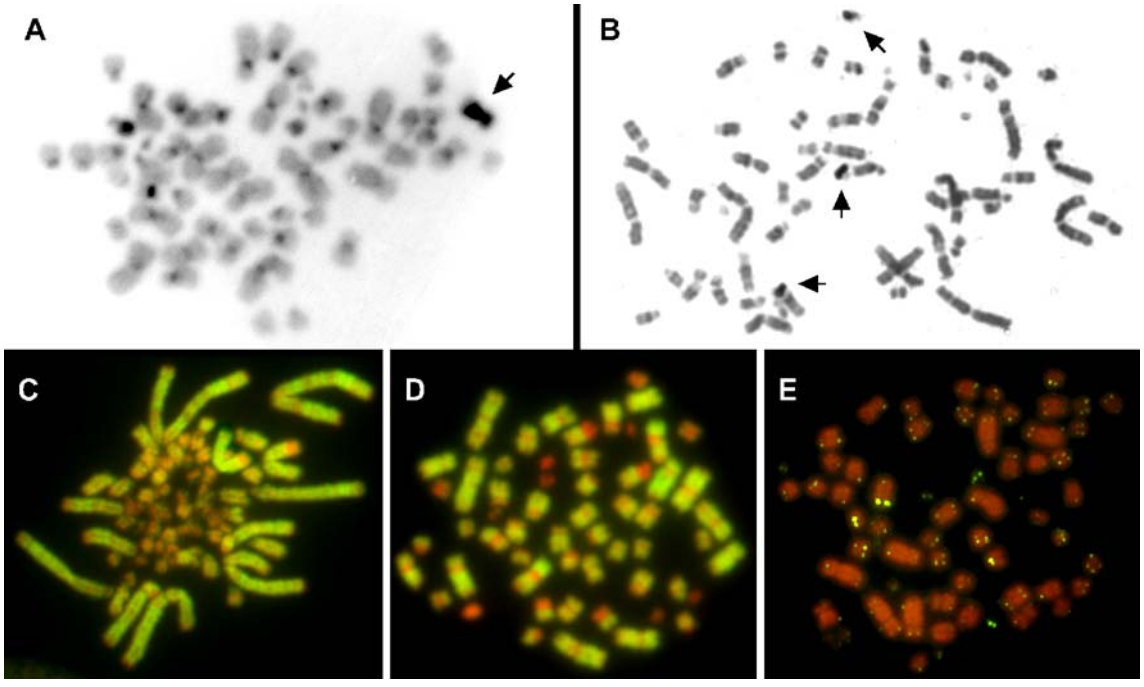

Fig. 1 **a** C-banded metaphase of a female *H. harpyja*. The *arrow* marks the W chromosome, of which a large region is heterochromatic. **b** Sequential G-banding and silver staining reveal *H. harpyja* NOR-bearing chromosomes 8 and 25. Only one of the two chromosomes 8 is stained in this metaphase. Differential DNA staining of **c** a chicken and **d** a *H. harpyja* metaphase with DAPI/7-AAD. GC-rich DNA is shown in *red*, AT-rich DNA in *green*. **e** In situ hybridization of a telomere-specific $(TTAGGG)_n$ probe (*green*) to a *H. harpyja* metaphase (*red*) produced hybridization signals on chromosome ends, but not in interstitial chromosome regions

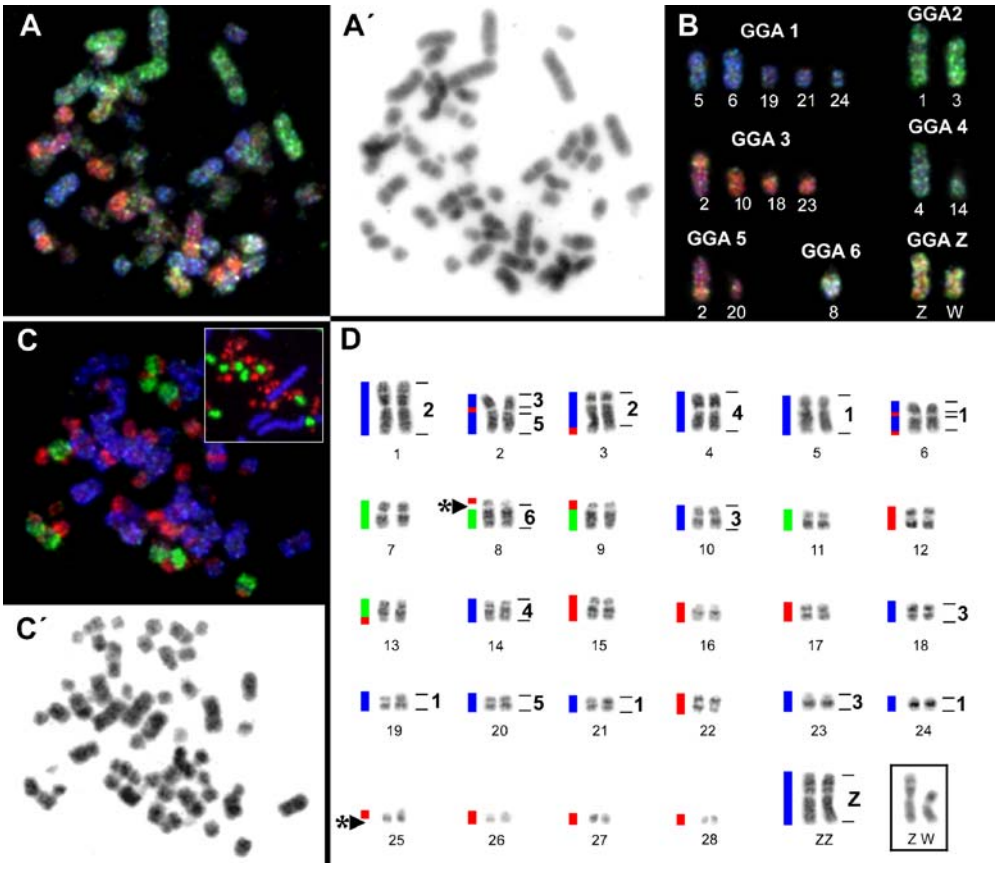

Fig. 2 **a** In situ hybridization of combinatorially labeled chicken (*GGA*) macrochromosome paint probes 1–6 and Z on a *H. harpyja* metaphase. *GGA 1* is shown in *blue*, *GGA 2* in *green*, *GGA 3* in *red*, *GGA 4* in *magenta*, *GGA 5* in *cyan*, *GGA 6* in *white*, and GGA Z in *yellow*. **a′** Chromosomal counterstain (*inverted DAPI*) of the metaphase depicted in **a**. **b** Summary of the hybridization results with chicken (*GGA*) chromosome 1–6 and Z paint probes on *H. harpyja* chromosomes. **c** Hybridization of pooled paint probes of chicken macrochromosomes 1–5 (*blue*), medium-sized chromosomes 6–10 (*green*), and 19 different microchromosomes (*red*) to *H. harpyja* metaphase chromosomes (*inset*: partial chicken metaphase hybridized with the same probe). **c′** Chromosomal counterstain (*inverted DAPI*) of the metaphase depicted in **c**. **d** G-banded karyotype of a male *H. harpyja* (*inset*: sex chromosome complement of a female individual), together with the assignment of chicken chromosome homologies delineated by cross-species chromosome painting. Homologies to individual chicken chromosomes 1–6 and Z (compare with Fig. 2a) are indicated by *numbers* to the right of each chromosome; homologies delineated by pooled chicken paint probes (compare with Fig. 2c) are depicted by *color-coded vertical bars* on the left (GGA 1–5 in *blue*, GGA 6–10 in *green*, and 19 different microchromosomes in *red*). *Asterisks* indicate NOR-bearing chromosomes

Discussion

To date, the karyotype of the Harpy eagle (*H. harpyja)* has only been analyzed by classical Giemsa staining (Hoffmann et al. 1976). The present study, using different banding techniques, has permitted the conventional characterization of the karyotype. The use of chromosome-specific probes from *G. gallus* in "painting" experiments, however, allowed the delineation of chromosomal homologies for an initial analysis of the mechanisms that led to the differentiation of Accipitridae karyotypes from the typical avian chromosome complement.

H. harpyja shows a karyotype of 2*n*=58 chromosomes with features similar to other Accipitridae species. C-banding demonstrated that most of the W chromosome is heterochromatic (Fig. 1a). Moreover, the chicken Z painting probe showed cross-hybridization to the entire *H. harpyja* W chromosome, indicating that similar repetitive DNA sequences are found in the W and Z.

The location of NORs on a pair of large chromosomes as found in the Harpy eagle is also shared by some other Accipitridae species. In addition, however, in *H. harpyja*, a pair of microchromosomes showed positive Ag–NORs, whereas in most other Accipitridae, either a pair of macro- or a pair of microchromosomes bear NORs (de Boer 1980). The most interesting observation from the classical cytogenetic characterization of the karyotype of the Harpy eagle compared to the chicken comes from DAPI/7-AAD staining (Fig. 1c, d). In the chicken, the staining pattern follows recent DNA sequencing results of the chicken genome (International Chicken Genome Sequencing Consortium 2004) (http://www.ensembl.org/Gallus_gallus). Microchromosomes were more intensively stained by the GC-selective fluorochrome 7-AAD, whereas macrochromosomes were highlighted by more AT-specific DAPI staining (Fig. 1c). This differentiation is lost in the Harpy eagle, where several medium-sized chromosomes show strong 7-AAD positive segments (Fig. 1d).

The chromosome painting analysis between the Harpy eagle and the chicken revealed that both species differ by at least 20 chromosome rearrangements (Fig. 3). The direction of change becomes evident when comparing karyotypes and genomes between the chicken and other birds, between birds and reptiles, and finally between the chicken and mammals. Recent cross-species chromosome painting studies suggest that between chicken and emu, all macrochromosomes are conserved, except for chicken chromosome 4 (Shetty et al. 1999). The comparison of chicken chromosome 4 and its homologs in other birds and mammals suggests its origin by fusion of an ancestral avian microchromosome (GGA 4p), with an ancestral avian macrochromosome (GGA 4q) (International Chicken Genome Sequencing Consortium 2004) (Fig. 3). Except for chromosome 4, the chicken appears to closely reflect the ancestral karyotype of Galliformes, Anseriformes, Columbiformes, Ciconiformes, Passeriformes, Strigiformes, and Struthioniformes, with chromosome numbers ranging from 2*n*=78 to 82 (Derjusheva et al. 2004; Guttenbach et al. 2003; Kasai et al. 2003; Raudsepp et al. 2002; Schmid et al. 2000; Shibusawa et al. 2004a,b). Moreover, recent gene mapping in reptiles identified the same gene order on reptile macrochromosomes as in birds (Matsuda et al. 2004), suggesting that both reptiles and birds share the same ancestral chromosome organization for more than 250 million years of evolution. As a consequence, the karyotype of the Harpy eagle should be considered as highly derived.

Further evidence in support of this conclusion is provided by the chicken–human genome alignment (http://www.ensembl.org). For example, chicken chromosome 3 is largely homologous to human 6p21.2-qter. Taking humans as the ultimate "outgroup" for birds, the contiguous human 6p21.2-qter and its chicken homolog should represent a shared ancestral chromosomal entity for both birds and mammals. In *H. harpyja*, however, this region is found fragmented into four homologous segments.

The homologs of chicken macrochromosomes 1–5 are split in *H. harpyja* as a consequence of ten fissions and one fusion (Fig. 3), whereas the five medium-sized chicken chromosomes 6–10 may have conserved large-scale homology between the two species. Most notably, a minimum of four fusions between homologs to chicken micro- and macrochromosomes, at least three further fusions between chicken microchromosome and medium-sized homologs, and another three fusions among microchromosomes occurred in the Harpy eagle (Fig. 3). Apparently, the homologs to chicken microchromosomes are fused to other chromosomes without any preference or constraints. A probe specific for telomeric DNA common to most vertebrates (Meyne et al. 1989), however, did not reveal any interstitial telomeric sites at fusion points of chromosomes (Fig. 1c).

As a consequence of this dramatic karyotype reorganization, the gross division of the genome into relatively gene-poor macrochromosomes and gene-rich microchro-

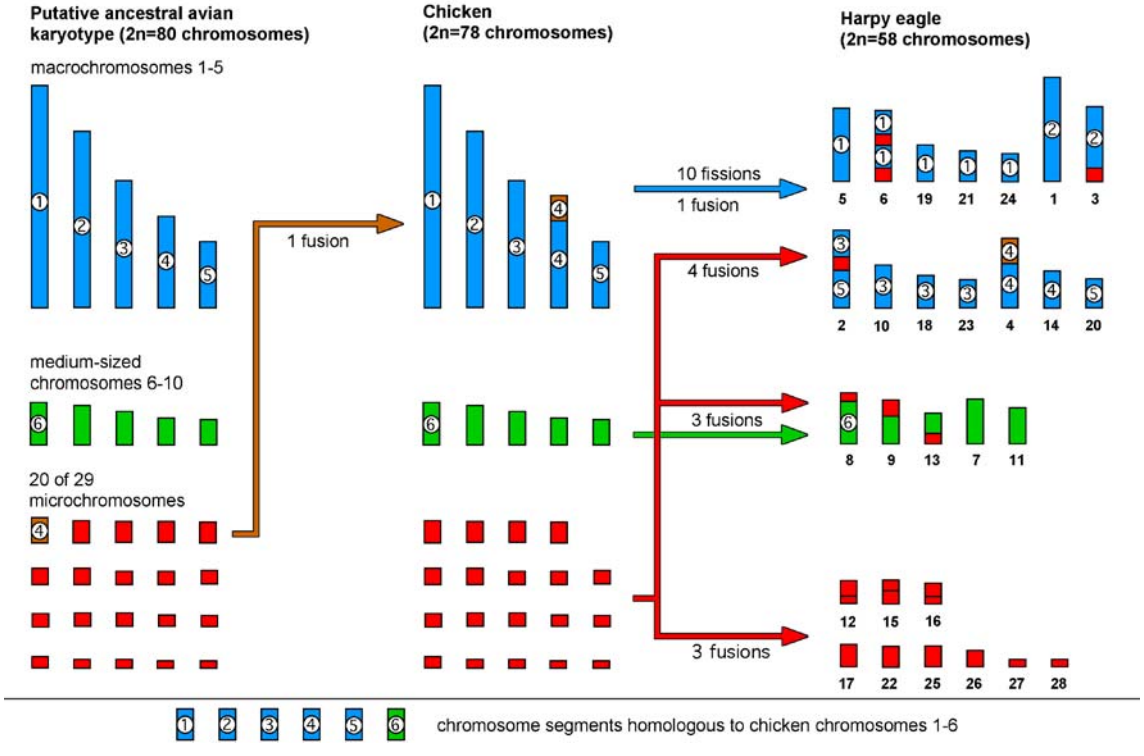

Fig. 3 Chromosomal rearrangements in the Harpy eagle. Compared to the putative ancestral avian karyotype, the chicken karyotype is derived by the fusion of a macrochromosome (*blue*) with a microchromosome (*brown*). In the Harpy eagle, chicken macrochromosome homologs are further derived by ten fissions and one fusion. Harpy eagle microchromosome homologs (*red*) were involved in at least 10 fusions (note: only 20 of 29 microchromosome homologs could be analyzed, because probes for the remaining nine chicken chromosomes were not available in this study)

mosomes, as observed in chicken and in many other bird species, has been lost in the Harpy eagle and most probably in other birds of prey as well. Traditional analyses of chromosomes already identified various highly conserved "flavors" of chromosomes or chromosome regions in evolution regarding gene density and replication timing (Holmquist 1992). This has been confirmed and dramatically extended by the initial results from the chicken genome project. In addition, a distinct arrangement of macro- and microchromosomes has been observed in three-dimensionally preserved chicken cell nuclei as well as in mitotic rosettes. Microchromosomes are preferentially clustered in the center of interphase nuclei and mitotic rosettes, whereas macrochromosomes are consistently located in the nuclear periphery (Habermann et al. 2001). Birds of prey may provide a model for cell biologists to unravel the gross genome organization and genomic "flavors" in an evolutionary framework.

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