# Understanding the Chromosomal Evolution in Cuckoos (Aves, Cuculiformes): A Journey Through Unusual Rearrangements

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**Abstract**

The Cuculiformes are a family of over 150 species that live in a range of habitats, such as forests, savannas, and deserts. Here, bacterial artificial chromosome (BAC) probes (75 from chicken and 14 from zebra finch macrochromosomes 1-10 +ZW and for microchromosomes 11-28 (except 16)) were used to investigate chromosome homologies between chicken and the squirrel cuckoo (*Piaya cayana*). In addition, repetitive DNA probes were applied to characterize the chromosome organization and to explore the role of these sequences in the karyotype evolution of *P. cayana*. We also applied BAC probes for chicken chromosome 17 and Z to the guira cuckoo (*Guira guira*) to test if this species has an unusual Robertsonian translocation between a microchromosome and the Z chromosome, recently described in the smooth-billed ani (*Crotophaga ani*). Our results revealed extensive chromosome reorganization with inter- and intrachromosomal rearrangements in *P. cayana*, including a conspicuous chromosome size and heterochromatin polymorphism on chromosome pair 20. Furthermore, we confirmed that the Z-autosome Robertsonian translocation found in *C. ani* is also found in *G. guira*, not *P. cayana*. These findings suggest that this translocation occurred prior to the divergence between *C. ani* and *G. guira*, but after the divergence with *P. cayana*.

**Keywords:** Birds, genome evolution, sex chromosomes, chromosomal rearrangements, heterochromatic polymorphism.

## Introduction

Cuculiformes is a group of birds commonly known as cuckoos exhibiting great diversity in morphology, ecology, and behavior (Shufeldt 1901; Payne 1997). There are ~150 species of cuckoos found worldwide (Gill et al. 2023), with a wide range of habitats, including forests, savannas, and deserts (Shufeldt 1901; Payne 1997). Five subfamilies are recognized among cuckoos, Crotophaginae, Neomorphinae, Centropodinae, Couinae, and Cuculinae (Sorenson and Payne 2005) (**Figure 1**). Cuckoos are essential components in many ecosystems, being both predators of insects and other tiny animals as well as food for other birds and mammals and have cultural significance since many traditions and civilizations value their distinctive calls. Little research has hitherto focused on chromosomal studies in these species and most of these used conventional karyotyping methods (Waldrigues and Ferrari 1982; Waldrigues et al. 1983). Despite this, these investigations showed a significant range of karyotypes, with diploid numbers ranging from 2n=64 in *Crotophaga major* (Crotophaginae)

(Waldrigues et al. 1983) to 2n=90 in *Piaya cayana* (Cuculinae) (dos Santos et al. 2020). Moreover, there have been many differences reported in chromosomal size and morphology, indicating various evolutionary chromosome rearrangements, including inversions, fusions, fissions, and translocations.

Molecular cytogenetic data in Cuculiformes are only available for *Guira guira* (Crotophaginae), *P. cayana*, and *Crotophaga ani* (dos Santos et al. 2020; Kretschmer et al. 2021). In *G. guira* and *P. cayana,* whole chromosomal painting probes derived from *Gallus gallus* (GGA) and *Leucopternis albicollis* were used to investigate the conservation of the syntenic groups corresponding to the avian ancestral macrochromosomes (GGA1-10) (dos Santos et al. 2020). This report highlighted fusion events in *G. guira,* bringing the ancestral diploid number down from 2n=80 to 2n=76 (dos Santos et al. 2020), however *P. cayana* had more fissions, leading to a higher diploid number (2n=90) (dos Santos et al. 2020). Moreover, bacterial artificial chromosome (BAC) probes derived from the macro- and microchromosomes of *G. gallus* were used to examine the karyotype of *C. ani* (Kretschmer et al. 2021). Several fusion events similar to those in *G. guira* were discovered in *C. ani* including a peculiar Robertsonian translocation involving the Z chromosome and the microchromosome pair homologous to *G. gallus* 17 (Kretschmer et al. 2021).

Since the discovery was made nearly a century ago, researchers have investigated the possible roles that chromosomal rearrangements may play in adaptation and speciation (Sturtevant 1926, 1938; Dobzhansky 1970; Bogart et al. 2022). Recent research demonstrated the importance of chromosomal rearrangements in promoting local adaptation in several studies (Faria et al. 2019; Wellenreuther et al. 2019; Cayuela et al. 2020). Low levels of recombination caused by chromosomal rearrangements within the affected genomic regions can result in independent evolution, even when the remaining portion of the genome experiences high levels of gene flow (Faria and Navarro 2010; Wellenreuther et al. 2019). Because of this independence, specific traits that are linked to local adaptation can be expressed (Mérot et al. 2018; Westram et al. 2018; Wellband et al. 2019; Cayuela et al. 2020).

Based on these findings, the initial goal of this work was to investigate the karyotype evolution of *P. cayana*, paying particular attention to intrachromosomal rearrangements in the macrochromosomes and the arrangement of the microchromosomes, aspects that were not explored by dos Santos et al. (2020). To achieve this goal, we mapped chicken BAC probes from chromosomes 1-28 +ZW, as well as probes corresponding to repetitive DNA sequences in the metaphase chromosomes of *P. cayana*. The results were compared with *G. guira* and *C. ani* to

highlight the evolutionary trends within cuckoos (or at least the species studied). We also mapped chicken BACs for chromosome 17 in *G. guira* to ask whether the Robertsonian translocation involving this chromosome and the Z chromosome found in *C. ani* is also present in *G. guira*.

## Material and Methods

### *Specimens and chromosome preparation*

This work examined three females *P. cayana* and one male *G. guira* (Table 1). The individuals were captured in their natural habitat between 2014 and 2022 using mist nets. More specifically, two *P. cayana* were obtained from municipality of Santana da Boa Vista, Rio Grande do Sul (RS) State, Brazil, and one from municipality of Porto Vera Cruz (RS, Brazil), while *G. guira* was captured in São Gabriel (RS, Brazil). All experiments performed here were in accordance with the protocols approved by the Ethics Committee on Animal Experimentation of Universidade Federal do Pampa (CEUA number 018/2014) and the System of Authorization and Information in Biodiversity (SISBIO, numbers 33860-1 and 44173-1). Metaphase chromosome spreads were obtained from fibroblast cell cultures, established from skin biopsies according to Furo et al. (2017), or bone marrow direct culture, following Garnero and Gunski (2000). Both methods included a colcemid treatment for an hour, a hypotonic solution (0.075 M KCl, for 15 min), and a fixation step using a 3:1 methanol/acetic acid solution. The cell line derived from the individual from Porto Vera Cruz was cultured up to the fourth passage. At each passage, the diploid number was examined to verify the maintenance of the original chromosome organization.

### *Diploid number, karyotype description, and chromosome banding*

The diploid number and chromosome morphology of *P. cayana* and *G. guira* were determined from the analysis of at least 20 metaphase chromosome spreads for each individual, conventionally stained with Giemsa 10% in 0.07 M phosphate buffer at pH 6.8. The chromosome morphology followed Guerra (1986). The G-banding patterns of *P. cayana* chromosomes were obtained with a combination of DAPI and propidium iodide (Joseph et

al. 2018). The distribution of constitutive heterochromatic blocks of *P. cayana* was analyzed by C-banding following Summer (1972).

***Fluorescence in situ hybridization (FISH)***

BAC probes from chicken autosomal chromosomes GGA1-28 (except GGA16) and Z and W sex chromosomes were applied to metaphases of *P. cayana* (**Table S1**). Two BAC probes were selected for chromosomes GGA6-28, while we applied more than two BACs for the first five macrochromosomes (GGA1-5) and the Z chromosome to detect intrachromosomal rearrangements, totaling 78 BAC clones. Only BAC probes from chicken chromosomes 17 and Z were used for *G. guira*. BAC clone isolation, amplification, labeling, and hybridization were performed following O'Connor et al. (2019). FISH results were confirmed by analyzing at least 10 metaphase spreads per experiment.

Concerning repetitive DNA probes, seven Oligonucleotides [$(CA)_{15}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, and $(GAG)_{10}$], directly labeled with Cy3 during synthesis (Sigma, St. Louis, MO, USA), were mapped to metaphases of *P. cayana*, according to Kubat et al. (2008). 18S rDNA fragments were obtained by polymerase chain reaction (PCR) as described in Cioffi et al. (2009) and labeled with Spectrum Green-dUTP (Vysis, Downers Grove, IL, USA) by nick translation, according to the manufacturer's recommendations (Roche, Mannheim, Germany). Results were confirmed by analyzing at least 10 metaphase spreads per experiment.

***Image acquisition and processing***

The BAC FISH images were acquired through a CCD camera paired with the SmartCapture system from Digital Scientific UK, coupled on an Olympus BX61 epifluorescence microscope. Meanwhile, the repetitive DNA FISH imagens were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan), equipped with CoolSNAP. Final image processing was performed using Adobe Photoshop 7.0.

## Results

Overall**,** our results confirmed the fissions previously found in *P. cayana* (PCA) by chromosome painting and revealed five intrachromosomal rearrangements in the first five macrochromosomes and in the Z chromosome. Regarding the microchromosomes, no evidence of interchromosomal rearrangements was found. In addition, we noticed a conspicuous chromosome size and heterochromatin polymorphism in one *P. cayana* individual from Santana da Boa Vista and one from Porto Vera Cruz involving the 20$^{th}$ microchromosome pair. The Robertsonian translocation involving the Z chromosome and the microchromosome homologous to GGA 17 found in *C. ani* was also confirmed in *G. guira*, indicating a common origin.

### *Diploid number, karyotype description and chromosome banding*

*P. cayana* had 2n = 90, with 13 macrochromosome pairs, including the Z and W sex chromosomes, and 32 microchromosome pairs (**Figure 2 A**). Pairs 1, 5, 6, and 10 are telocentric, 2 is submetacentric, 3, 4, and 11 are metacentric, and 7, 8, 9, and 12 are acrocentric. The morphology of the microchromosomes could not defined due their small size. The Z and W are submetacentric and acrocentric respectively. A large acrocentric chromosome, without a homologous chromosome at first glimpse and equivalent in size to pair 5 was found in one *P. cayana* individual from Santana da Boa Vista and one from Porto Vera Cruz. This chromosome was later identified by the FISH results as homologous to PCA chromosome 20 (see below).

C-banding revealed heterochromatin in a few autosomes and in the Z chromosome of *P. cayana*. Yet only the W chromosome and the large acrocentric chromosome of the 20$^{th}$ pair had substantial blocks of heterochromatin (**Figure 2 B**). Three potential patterns were established based on the C-banding patterns discovered in this pair: i) homomorphic for small heterochromatic block (**data not shown**), ii) homomorphic for large heterochromatic block (not seen in our data), and iii) heteromorphic condition (**Figure 2 B**).

### *Fluorescence in situ hybridization (FISH) of chicken and zebra finch BAC clones in P. cayana and G. guira*

When compared with chicken, three macrochromosomes are split in *P. cayana*: the ancestral avian chromosomes homologous to GGA 1 and 3 are split into two distinct pairs each (PCA1 and PCA6, PCA5 and 10, respectively),

while the homologous to GGA 2 split in three pairs (PCA2, PCA13, and PCA15). As compared to chicken, just one fusion involving GGA7 and an unknown microchromosome was found in *P. cayana* (**Figure 2 A**). **Figure 3** displays illustrative FISH pictures. The chromosome mapping of BACs from GGA 1 is shown in **Figure 4**, while the chromosome mapping of BACs from GGA 2-5 and Z is shown in **Figures S1–S5**. Besides, a total of five intrachromosomal rearrangements were found in the macrochromosomes 2, 3, 5, and Z of *P. cayana* (**Figures S1, S2, S4, and S5**).

As stated in the karyotype description, two *P. cayana* sampled individuals contained a large acrocentric chromosome that did not initially appear to have a homologous pair (**Figure 2 A**). Interestingly, the BAC probe from chicken chromosome 15 produced a signal in a microchromosome and the terminal region of this chromosome's long arms (**Figure 3 B-E**). In the individuals, without this large acrocentric chromosome, the BAC probe from chicken chromosome 15 produced a signal in a pair of microchromosomes (**Figure S6**).

In *G. guira*, the BAC probes from chicken chromosomes Z and 17 revealed the Z-autosome Robertsonian translocation (**Figure S7**), similar to previous findings in *C. ani*.

*Fluorescence in situ hybridization (FISH) of repetitive sequences in P. cayana*

One pair of *P. cayana's* macrochromosomes contained the 18S rDNA clusters (**Figure 5 A**). We mapped seven repeat motifs to comprehend better the size and heterochromatic polymorphism detected for chromosome 20 (**Figure 5 B-H and Table 2)**. The majority of these sequences were identified on chromosomes 1, 2, 3, and 5, and the sex chromosomes Z and W (**Figure 5 B-H and Table 2**). Except for the $GAG_{10}$, which generates signals scattered over all *P. cayana's* chromosomes, and, surprisingly, none of the repeat motifs employed was found accumulated in PCA20.

## Discussion

Previous cytogenetic studies on cuckoo species have revealed an interesting karyotype variation in chromosome number and morphology (Waldrigues and Ferrari 1982; Waldrigues et al. 1983; dos Santos et al. 2020), indicating that both inter- (fusion and fission events) and intrachromosomal rearrangements (pericentric

184 inversion and centromere repositions) have played an important role in the chromosome evolution of these
185 species. Our current results are a significant advance in those of dos Santos et al. (2020) who only used
186 macrochromosome paints 1-10+Z, thereby only detecting inter-chromosome rearrangements. Here we map intra-
187 chromosomal rearrangements as well as adding information on nearly three times as many chromosomes (W +
188 the microchromosomes to pair 28). *P. cayana* underwent substantial chromosome reorganization, including
189 intra- and interchromosomal rearrangements involving both macro and microchromosomes. Moreover, as
190 described earlier, pair 20 in two out of three *P. cayana* individuals analyzed here had a noticeable chromosome
191 size and heterochromatic polymorphism. We also described an unusual Z-autosome Robertsonian translocation
192 shared between *G. guira* and *C. ani*. Overall, if these species are reasonably representative, these results have
193 provided new insights into cuckoo species' karyotype and genome evolution.

194 The chromosome number (2n=90) and morphology of *P. cayana* are consistent with dos Santos et al. (2020),
195 except for the size and heterochromatic polymorphism found in autosome pair 20 of two individuals analyzed
196 here. Waldrigues et al. (1983) previously described the karyotype with 2n=76 in seven *P. cayana* specimens. The
197 number and morphology of macrochromosomes are the same, except for the lack of such a polymorphism. These
198 findings suggest that the variation in diploid number discovered by Waldrigues et al. (1983) corresponds to the
199 numbers of microchromosomes, most likely as a result of technical constraints. The fact that the polymorphism
200 discovered here was absent in the subjects of Waldrigues et al. (1983) analysis is a further conclusion.

201 On the W sex chromosome and one of the homologues of PCA 20$^{th}$ pair, heterochromatin accumulation is
202 observed. It is unusual for birds to exhibit heterochromatic polymorphism. For instance, heterochromatic
203 polymorphism has been identified in pair 7 of *C. aura*, where one of the chromosomes contains a larger block of
204 heterochromatin than its homologue (Tagliarini et al. 2009). Moreover, a male of *Cariama cristata* was found to
205 have an unpaired tiny acrocentric chromosome with prominent positive C-banding (Belterman and De Boer
206 1984). Nevertheless, these observations are often restricted to macrochromosomes since it is challenging to
207 detect variations in the amount of heterochromatin in microchromosomes unless the variations are quite large, as
208 in the case of *P. cayana*.

209 Chromosomal polymorphisms have played an important initial role in speciation forming gene flow barriers and
210 subsequent differentiation process (Faria and Navarro 2010; Dobigny et al. 2017; Satou et al. 2021; Galindo et
211 al. 2021). Among animals, several studies detected chromosomal polymorphisms, most of them due to centric

fusion/fission and inversions (reviewed in Dobigny et al. 2017). An alternate type of polymorphisms involves the amount, size, and chromosome position of heterochromatic material, such as that found in human chromosome groups D, F, and G, as well as pairs 1, 9, and 16 (Craig-Holmes and Shaw 1971). *P. cayana* appears to exhibit this distinct variety of polymorphism, which was not found in other cuckoo species studies so far (Waldrigues and Ferrari 1982; Waldrigues et al. 1983; dos Santos et al. 2020), including previous studies with *P. cayana* individuals (dos Santos et al. 2020). We suggest that chromosome 20's polymorphism and variable heterochromatin pattern is most likely caused by the addition of heterochromatic sequences since there is a clear increase in chromosomal size while the distal euchromatic region in the long arms has a size similar to its homolog **(Figure 2 A).**

As has previously been hypothesized for humans, the most plausible source of the heterochromatin variations is via uneven crossing-over in the tandemly repeated sequences (Craig-Holmes and Shaw 1971). Interestingly, the larger element of pair 20 did not contain any of the repeat motifs we employed in our experiments. According to Schueler and Sullivan (2006) and Eymery et al. (2009), constitutive heterochromatin often correlates to gene-poor regions that include tandem repeats of satellites, minisatellites, microsatellites, and transposable elements (Charlesworth et al. 1994; López-Flores and Garrido-Ramos 2012). Tandem repeating sequences are vital to the evolution of animal genomes, for instance, Ruiz-Herrera et al. (2006) provided evidence that chromosomal rearrangements have driven the evolution of the mammalian genome at fragile sites, composed of tandem repetitive sequences. Recently, we showed that heterochromatic chromosomes in birds, like the W sex chromosomes, feature microsatellites motifs that have been amplified significantly (Furo et al. 2017; Kretschmer et al. 2018; Gunski et al. 2019; de Souza et al. 2021). Many repetitive sequences, including transposable elements, are likely what invaded one of the chromosomes of the pair 20 of *P. cayana*. However, future studies are necessary to test this hypothesis.

Previously, whole chromosome painting with chicken and white hawk probes, have been carried out on the chromosomes of *G. guira* and *P. cayana* (dos Santos et al. 2020). According to this research, the karyotype evolution of these groups has been significantly influenced by chromosomal rearrangements involving both macro- and microchromosomes (dos Santos et al. 2020). Using BAC-FISH, we were able to corroborate the fissions previously discovered in *P. cayana* (dos Santos et al. 2020). Regarding the microchromosomes, there was no evidence of chromosomal rearrangement involving the chicken microchromosome pairs 11-28 (except

16) in *P. cayana*. Moreover, the gap observed here and by dos Santos et al. (2020) in pair 7 of *P. cayana* was not covered by the microchromosomes tested in our analysis, indicating that any of the chicken microchromosomes not used in this study (pairs 29-38 or 16) may have fused to this chromosome.

Cuckoo species exhibit uncommon rearrangements, such as a translocation between the Z chromosome and microchromosome 17 in *C. ani* (Kretschmer et al. 2021). In *G. guira*, this translocation was also discovered. Nevertheless, it had not been identified in *P. cayana.* Our findings suggested that this rearrangement happened, at the very least, in the two species' most recent common ancestor. As these species share the subfamily Crotophaginae (Sorenson and Payne 2005), it is likely that other members of this subfamily also exhibit this translocation. Recent studies also described the occurrence of Z-autosome translocation in some species of Sylvioidea, a songbird group that includes the warblers, thrushes, and babblers (Pala et al. 2012; Sigeman et al. 2019; Sigeman et al. 2020; Dierickx et al. 2020; Sigeman et al. 2022), and in parrots (Huang et al. 2021). Hence, contrary to what was presumed, the avian ZW sex chromosome system is not exceptionally stable (Nanda and Schmid 2002; Nanda et al. 2008).

Taken together, the results of our cytogenetic analysis show that the mechanism of chromosomal evolution in cuckoo species involved fissions, fusions, inversions, and accumulation of repetitive sequences, which resulted in unusual rearrangements like Z-autosome Robertsonian translocation and a substantial amount of heterochromatic polymorphism.

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**Data availability**

All data generated or analyzed during this study are included in this published article or supplementary files.

## Author contribution

Conceptualization: RK, DKG

Data curation: RK, MSS, GAT

Formal analysis: RK, MSS, GAT

Funding acquisition: RK, DKG, ADG, RJG, MBC

Investigation: RK, EZ, TROF, EHCO, MSS, GAT, ADG, RJG, MBC

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Project administration: RK, DKG

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Supervision: DKG

Validation: RK, MSS, GAT

Visualization: RK, MSS, GAT

Writing – original draft: RK

Writing – review & editing: RK, MSS, GAT, EZ, TROF, ADVG, RJG, MBC, EHCO, DKG

## Competing interests

The authors declare no conflict of interest.

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## Ethics approval

The experiments followed protocols approved by the ethics committee from Universidade Federal do Pampa (no. 018/2014). The specimens were collected with permissions from Sistema de Autorização e Informação em Biodiversidade (SISBIO 33860-1 and 44173-1).

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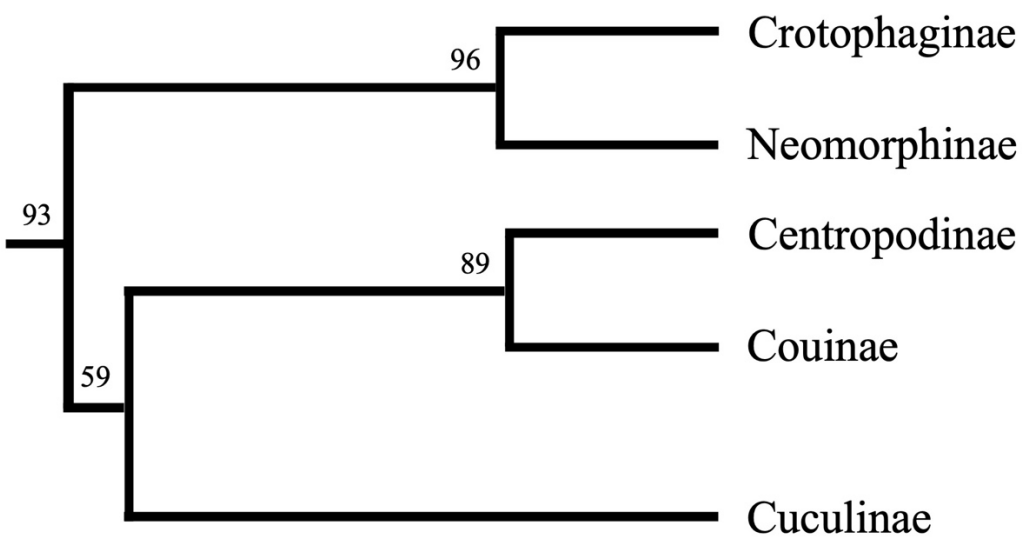
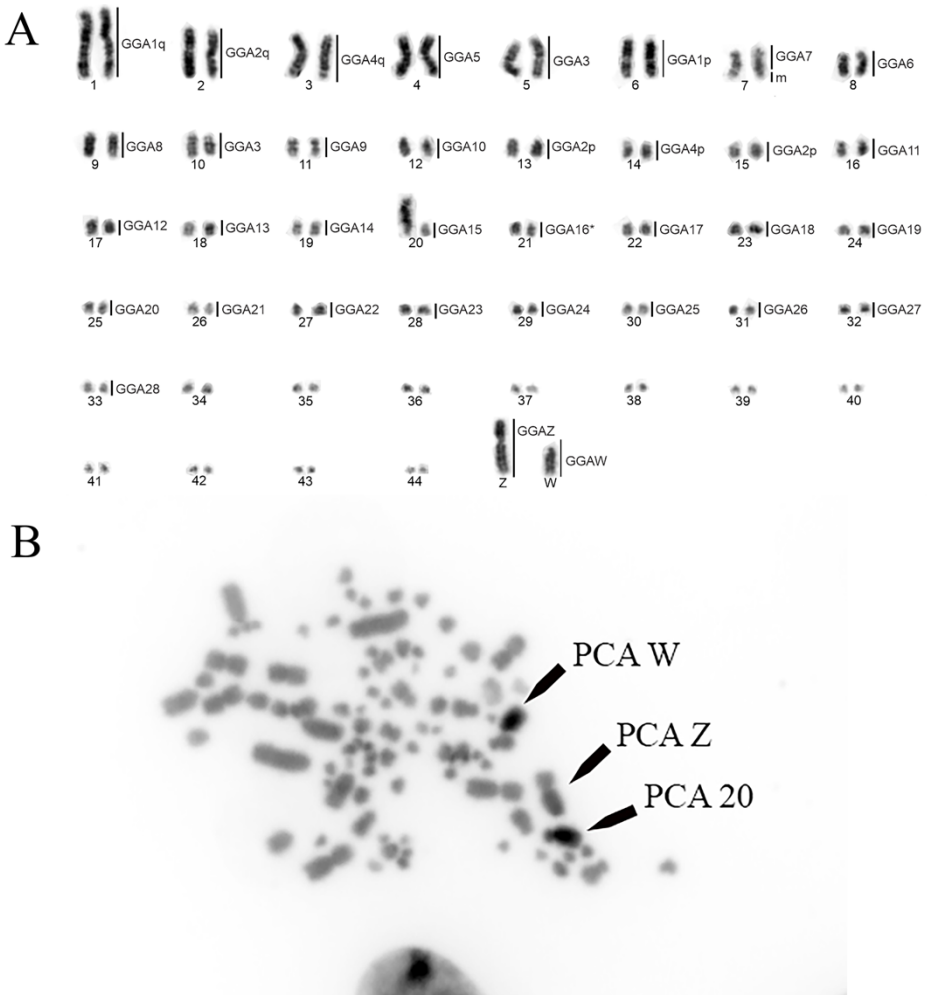



415

416 **Figure 1** – Phylogeny of the five subfamilies among cuckoos. Bootstrap values are shown above each node. The

417 phylogeny was adapted from Sorenson and Payne (2005).




418

419 **Figure 2** – Characterization of a female *Piaya cayana* (PCA) karyotype by classical cytogenetics: A) G-banded
420 karyotype with polymorphism in the 20th autosome pair (PCA 20). On the right of each chromosome pair is a
421 representation of the homology maps with *Gallus gallus* (GGA); B) C-banded metaphase. The PCA 20, the Z
422 and W sex chromosomes are indicated by arrows. Using C-banding, the homologous microchromosome 20
423 could not be located.

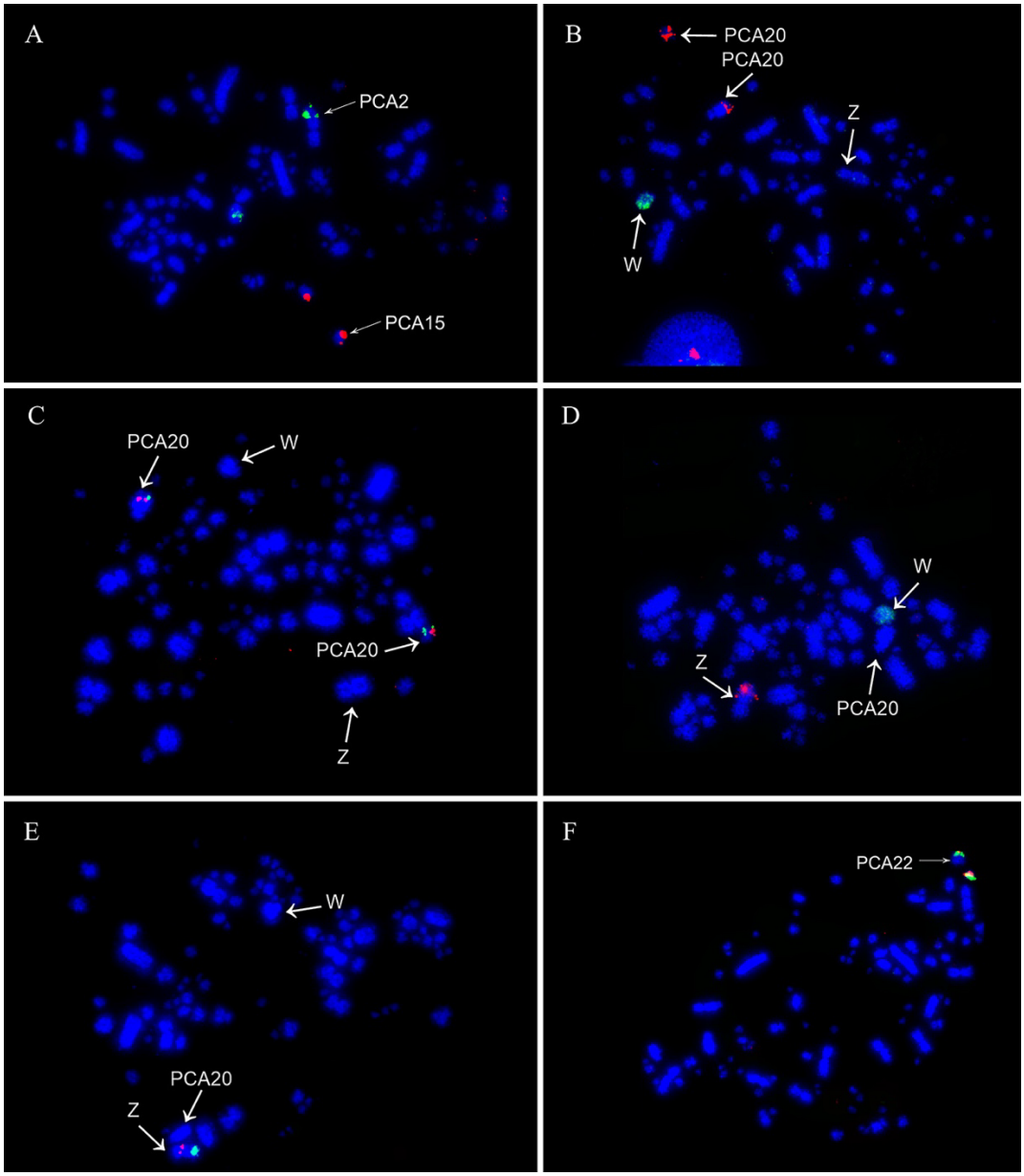



**Figure 3 -** Examples of FISH investigations in *Piaya Cayana* (PCA) using chicken (CH261) or zebra finch (TGMCBA) BAC probes: A) Macrochromosome 2 CH261-123O22 Texas Red and CH261-44H14 FITC; B) chicken microchromosome 15 CH261-90P23 Texas Red and chromosome W CH261- 94E12 FITC; C) chicken microchromosome 15 CH261-90P23 Texas Red and TGMCBA-266G23 FITC; D) chicken chromosome Z CH261-129A16 Texas Red and chromosome W CH261- 94E12 FITC; E) chicken chromosome Z CH261-129A16 FITC and CH261-133M4 Texas Red; F) Microchromosome 17 TGMCBA-375I5 Texas Red and CH261-42P16 FITC.

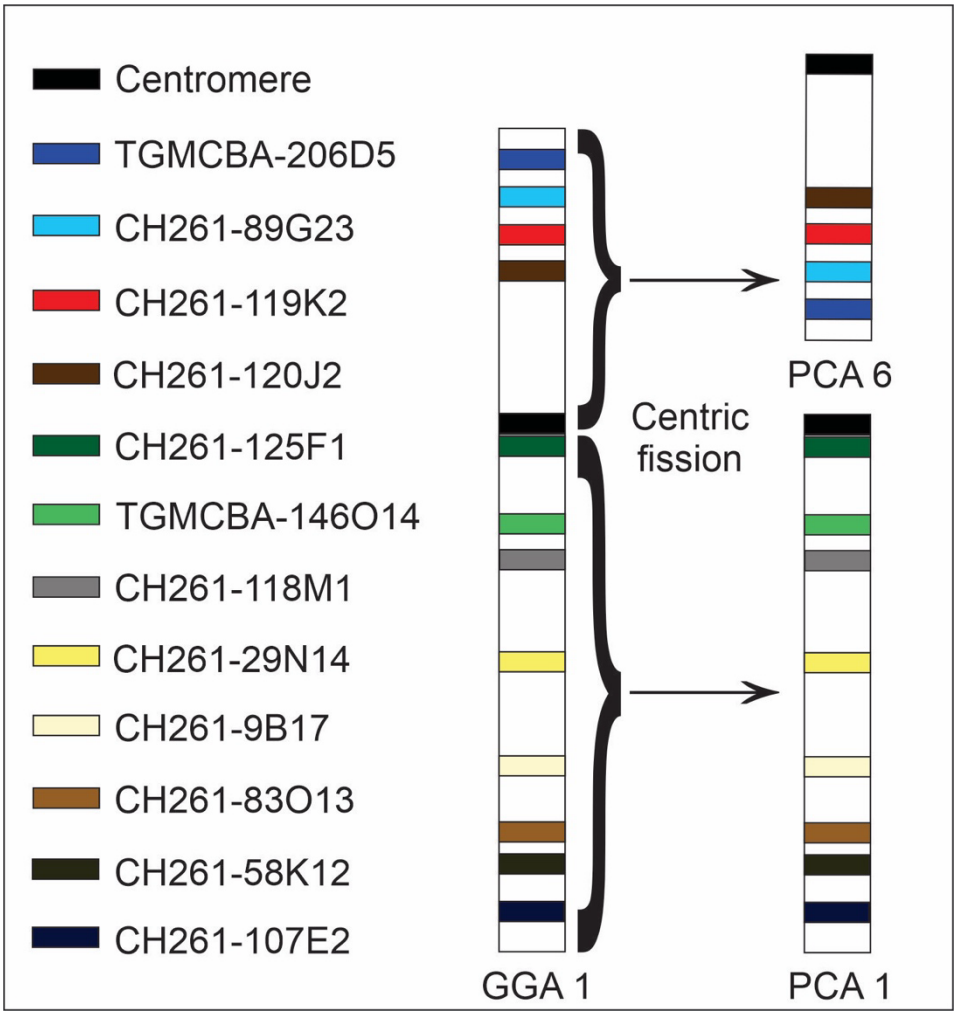
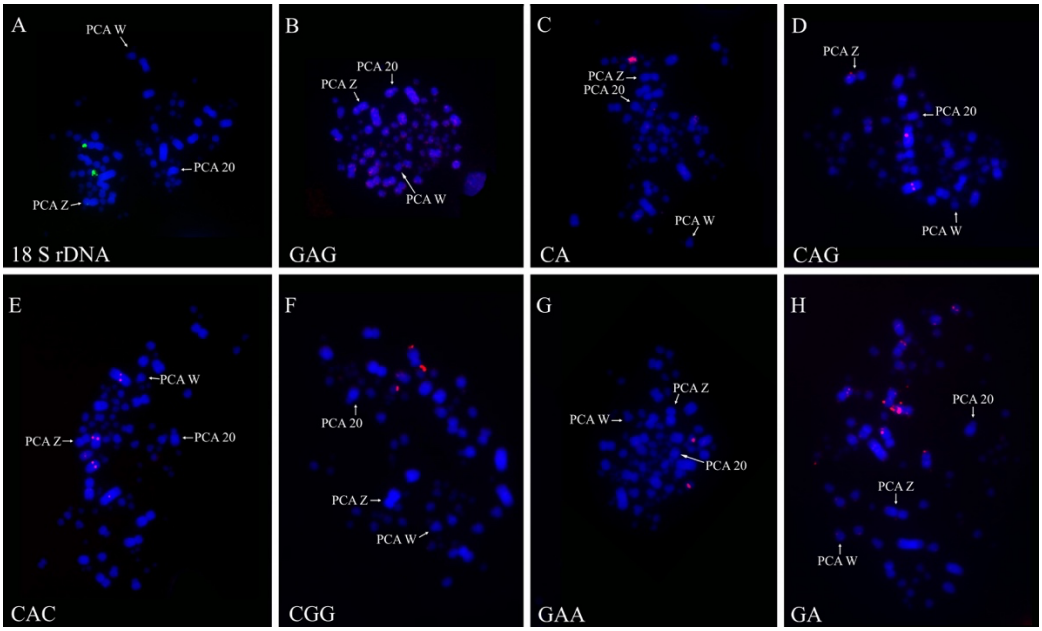



**Figure 4 –** Schematization of the chromosomal localization of the zebra finch (TGMCBA) and chicken (CH261) BACs that are homologous to chicken chromosome 1 (GGA1) employed in our investigation. The colors represent the selected BACs and centromeres. The detected chromosomal rearrangements are indicated by the brackets. PCA1 and PCA 6 were produced by centric fission in *Piaya cayana* (PCA).




437

438 **Figure 5 -** Representative examples of FISH experiments using different repeat motif in *P. cayana* (PCA). The

439 arrows point to Z and W sex chromosomes and chromosome 20 with a large accumulation of heterochromatin.

440

441 Table 1 - Specimen information and chromosome preparation protocols used in this study.

| Individuals | Sex | Location | Chromosome preparation protocol |
|---|---|---|---|
| *Piaya cayana* 1 | Female | Porto Vera Cruz - RS, Brazil | Fibroblast cell culture |
| *Piaya cayana* 2 | Female | Santana da Boa Vista - RS, Brazil | Bone marrow direct culture |
| *Piaya cayana* 3 | Female | Santana da Boa Vista - RS, Brazil | Bone marrow direct culture |
| *Guira guira* | Male | São Gabriel - RS, Brazil | Bone marrow direct culture |

442 RS = Rio Grande do Sul State.

443

444 **Table 2 -** Hybridization of microsatellite sequences in *Piaya cayana*.

| **Repeat motif** | **Pattern of Hybridization** |
|---|---|
| $(CA)_{15}$ | Zq and Wq, weak signals in some microchromosomes |
| $(CAC)_{10}$ | Interstitial region in 1q, 5q, weak in Zq and Wq |
| $(CAG)_{10}$ | Interstitial region in 1q and in Zq |

| | |
|---|---|
| $(CGG)_{10}$ | One pair of microchromosomes and terminal region in Zq |
| $(GA)_{15}$ | Interstitial and telomeric region in 2q, telomeric region of 2, and telomeric region of 3p, weak signals in some microchromosomes |
| $(GAA)_{10}$ | Centromeric region of chromosome 11 |
| $(GAG)_{10}$ | Dispersed in all chromosomes |

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