Comparative Chromosome Painting in the Black-Hawk-Eagle (Spizaetus tyrannus) and Gallus gallus with the use of Macro and Microchromosome Probes.

Abstract:
Although most birds show karyotypes with diploid number around 2n=80, with few macrochromosomes and many microchromosomes pairs, some groups, such as the Accipitriformes, are characterized by a large karyotypic reorganization, which resulted in complements with low diploid numbers, and a smaller number of microchromosomal pairs when compared to other birds. Among Accipitriformes, the Accipitridae family is the most diverse and includes the subfamily Aquilinae, composed of medium to large sized species. The Black-Hawk-Eagle (Spizaetus tyrannus - STY), found in South America, is a member of this subfamily. Available chromosome data for this species includes only conventional staining. Hence, in order to provide additional information on karyotype evolution process within this group, we performed comparative chromosome painting between S. tyrannus and Gallus gallus (GGA). Our results revealed that at least 29 fission-fusion events were observed in the STY karyotype, based on homology with GGA. Fissions occurred mainly in syntenic groups homologous to GGA1-GGA5. On the other hand, the majority of the microchromosomes were found fused to other chromosomal elements in STY, indicating these rearrangements had an important role in the reduction of the diploid number to 2n=68. Comparison with hybridization pattern of the Japanese-Mountain-Eagle, the only Aquilinae analyzed by comparative chromosome painting previously, did not reveal any synapomorphy that could represent a chromosome signature to this subfamily. Therefore, conclusions about karyotype evolution in Aquilinae require additional painting studies.
brings new pieces of information for the big puzzle that avian karyotype evolution represents. Below I indicate some points to be adjusted and put special attention to one suggestion for discussion: The work would profit too much if the analyses were done under a phylogenetic context. An updated phylogeny with the special focus on the phylogenetic position and relation between Chicken and Spizaetus would bring more light to the general results found.

A-From the cytotaxonomic point of view, Gallus gallus has a basal karyotype, very similar to the avian putative ancestor. As this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. However, except for the use of other probes, which could reveal intrachromosomal rearrangements, a phylogenetic analysis of GGA and birds of prey is not very informative, except as outgroup

Abstract

2- I missed some introduction about BIRDs cytogenetic in the beginning of Abstract. The authors go too direct about Accipitriformes and most readers do not have such phylogenetic information to which group we are dealing with.
A- We agree, and added an introduction to the abstract "Although most birds show karyotypes with diploid number around 2n=80, with few macrochromosomes and many microchromosomes pairs, some groups, such as the Accipitriformes, are characterized by a large karyotypic reorganization, which resulted in complements with low diploid numbers, and a smaller number of microchromosomal pairs when compared to other birds"

3- Why are the authors aiming to investigate homologies with chicken?
A- Because this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. We added a small explanation in the introduction “In general, studies focusing on chromosome evolution in birds are based in comparative chromosome painting using chicken whole chromosome probes (Gallus gallus – GGA, 2n=78), due to the similarity of the karyotype of this species with the PAK [5].”

4- Abstract also brings some previous data on NNI that were not originated from this work. I suggest removing.
A- We removed it

5- The terms STY and NNI are used without any previous information/explanation about, causing misunderstandings.
A- We corrected it

6- A final conclusion is missing in the abstract.
A- We added a conclusion in the abstract: “Comparison with hybridization pattern of the Japanese-Mountain-Eagle, the only Aquilinae analyzed by comparative chromosome painting previously, did not reveal any synapomorphy that could represent a chromosome signature to this subfamily. Therefore, conclusions about karyotype evolution in Aquilinae require additional painting studies”.

Introduction

7- Page3 line3: Present an unusual (delete with)
A- We deleted it.

8- Page 3 line 12: The accurate identification of the chromosomal pairs involved…. if we aim to identify synapomorphies….
A- We corrected it

9- Introduction miss a clear motivation for the study.
A- We improved it: “Among them, the subfamily Aquilinae includes medium and large species, distributed globally, usually known as booted eagle. Usually, ten genera are found within Aquilinae. Cytogenetically, the only information concerning Aquilinae is the definition of the diploid number of six species (four genera), ranging from 2n=66 to 82 [19].”

10- Why using GGA probes?
A- Because this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. We added a small explanation in the introduction.
11- I missed some info (Figure would be better) about the phylogenetic position and relation between Chicken and Spizaetus.
A-The authors agree that for this study are not necessary introduce some information about the phylogenetic position between Gallus gallus and S. tyrannus, however short information of phylogenetic relationship of the S.tyrannus within the Aquilinae Subfamily were added in the introduction aiming improve the reading of this paper.
12- Moreover, inform here as well than STY corresponds to Spizaetus tyrannus on its first mention.
A-This was corrected.
Results
13- Subtitle Karyotypes before the first paragraph.
A-We added it.
Figures
14- Figure 3: I did not understand why using the metaphases present in right column? The signals and chromosomal morphology are clearly visible in the FISH images. Scale bars are missing in all figures.
A- We removed the right column and added the scale bars in all figures.
Reviewer #2:
In this study, the authors used comparative chromosome painting to reveal homology of chromosomal segments between Spizaetus tyrannus (the Black-Hawk-Eagle) and Gallus gallus. The study is conceptually and methodically motivated. The karyotype of S. tyrannus was previously studied only using conventional chromosome staining technique that showed 2n=68 (32m/sm+8st+16a+8m+ZW), and this happened for the first time that the karyotype of this bird of prey was studied by comparative chromosome painting. As a result, the study provides a novel insight into the cytogenetics of the Black-Hawk-Eagle. Both whole chromosome-specific G. gallus probes of the 1st-10th pairs and chromosome-specific G. gallus BAC probes from 11 pairs of microchromosomes were used. The study evidenced 29 evolutionary fission-fusion events that happened in the evolution of S. tyrannus and identified the particular chromosome pairs in the referenced G. gallus karyotype, which have undergone restructuring or have remained unchanged. Another sufficient result concerns the comparison between S. tyrannus and Nisaetus nipalensis orientalis, which is its only close relative studied so far by comparative chromosome painting. The comparison showed both similarities and differences between the species. The MS is well illustrated by tables and pictures of good quality.
In general, the work is interesting and deserves publication in the journal. However, there are some shortcomings in the work. Main disadvantages are (1) almost complete absence of basic data on the karyotypes of the discussed species, which makes it difficult to adequately assess the results obtained, and (2) ignorance of the taxonomic component.
A- We try to improve it.
All other comments that I have are mainly suggestions for improving the article (unfortunately, there is no line numbering in the MS, which makes it difficult for the reviewer to work).

1-Running title. It is too long, should be shorter, e.g., “Comparative Chromosome Painting in Spizaetus tyrannus and Gallus gallus”.
A-W corrected it.
2. Abstract. Please, decipher GGA, should be Gallus gallus (GGA), as done above for Spizaetus tyrannus (STY)
A- We corrected it.

Introduction.

3- Page 3. Paragraph 1. Please, provide a putative avian ancestral karyotype, with 2n and the number of microchromosomes.
A-We provided this information in the introduction of the paper.
4- Page 3. Paragraph 1. Here (or further, in Discussion), provide the G. gallus karyotype to make further reasoning clearer.
A-We did it.

5- Page 3. Paragraph 3. Unify way of citing – in other places you use numbering.
A-We corrected it.

6- Page 4. Paragraph 4. Please, specify the species studied, Spizaetus tyrannus
A-We corrected it.

Discussion.

7- Page 6. Paragraph 1. Please, expand the statement "We report slight differences in chromosome morphology…." by clarifying what Tagliarini et al. (2007) have reported. Describe the differences, give for comparison one and the other karyotypes (otherwise, you only have a declaration).

A- In order to clarify the difference in chromosome morphology described between this study and the Tagliarini et al., (2007), we made a comparative table that was introduced in the results.

8- Page 7. Paragraph 3. The Japanese mountain hawk-eagle Nisaetus nipalensis orientalis (Temminck & Schlegel, 1844), not the Hodgson’s hawk-eagle Nisaetus nipalensis Hodgson, 1836.
A- We corrected it.

9- Other remarks can be found in the MS attached.
A-We followed all the instructions of attached.

Reviewer #3:
This study provides a cytogenetic mapping of the Black-Hawk-Eagle (Spizaetus tyrannus) using whole-chromosome paints and BAC probes of Gallus gallus. Using this cytogenetic mapping, the authors investigate the chromosome homologies between Spizaetus tyrannus and Gallus gallus. Overall the manuscript presents an advance in cytogenetic of Accipitriformes. The manuscript is written in understandable English, but some procedures require more details. Please see below my comments that could help to further enhance the quality of the study.

2) Abstract: “chicken (Gallus gallus – GGA)”
A- We corrected it.

3) Introduction and discussion: The manuscript is written in a manner suitable for a more specialized journal such as Cytogenetic and Genome Research or Comparative Cytogenetics, but not ideally for a general journal such as PlosOne. The introduction and discussion are rather limited to the focus of the analyses. This is a shame as I think the authors did a good job to gather nice cytogenetic results. Thus, the authors should consider redrafting these sections on a broad context.

The introduction could be more informative about the study as a whole. For example, the authors can give information about the contribution of this kind of work to karyotypic evolution and chromosomal organization of Accipitriformes. In this line, the last paragraph of introduction can be better written showing the importance of this study, and not merely comment “This study presents the cytogenetic mapping of a species….”. Moreover, other intriguing topic is about the origin of microchromosomes. And maybe the authors could comment a little bit about this topic in the introduction and also discussion. Below is a reference related to this topic.

- Waters, P. D., Patel, H. R., Ruiz-Herrera, A., Alvarez-Gonzalez, L., Lister, N. C., Simakov, O., ... & Graves, J. A. M. (2021). Microchromosomes are building blocks of bird, reptile and mammal chromosomes. bioRxiv.
A-The introduction was rewritten and several new information were added to improve the understanding of the manuscript. Also, some information from the reference recommended by the reviewer were considerate in this topic.

4) Results, paragraph 1: The authors commented that they detected “four pairs of microchromosomes” but they indicated five (29, 30, 31, 32, and 33). Considering the Figure 1B the pair 29 apparently is not a microchromosome.
A-We reviewed the Karyotype of S.tyrannus and considered only four pairs of microchromosomes (Pairs: 30, 31, 32 and 33). Also, we corrected it throughout of the
5) Results, paragraph 2: “The most extreme examples are the fission of GGA1 into six pairs in STY, and GGA3 into three distinct pairs”. It seems that for GGA3 are four pairs (13, 16, 19, and 20) (Figure 4), right?

A-The reviewer is right. The GGA3 probe correspond to four pairs (13,16,19 and 20). We changed it.

6) Discussion, paragraph 3: “…karyotype of STY when compared to Gallus”; “…karyotype of STY when compared to Gallus gallus”.

A-We corrected it.

7) Discussion, paragraph 4: “…microchromosomes (STY4, 7 and 9; NNI2, 4 and 9), none of them”; “microchromosomes (STY: pairs 4, 7 and 9; NNI: pairs 2, 4 and 9), none of them”.

A-We corrected it.

8) Discussion, paragraph 5: “These results show that they are morphologically similar species that until the last decade were part of the same genus [14].”. I think the authors results did not show an association of chromosomal data with morphological similarity and this should be corrected.

A-We removed it.

9) Conclusion: “…of S.tyrannus should be…”; “…of S. tyrannus should be…”.

A-We corrected it.

10) Methods, paragraph 1: What the name and country of the Zoos?

A-The information was added in the Methods.

11) Methods, paragraph 2: “…and labelled directly by FTIC”; “and labelled directly by fluorescein isothiocyanate (FITC)”.

A-We changed it.

12) Methods: the results of this study are based on FISH with GGA probes. However, FISH procedure is not sufficiently described. I would appreciate if at least main/important steps of the FISH procedure are described. This would also avoid any doubt about the accuracy of the results.

A- We provided the main steps of the FISH procedure.

13) Figure 3: “FITC”.

A- We corrected it.

14) Please, give a scale bar information for all figures. It is very important, specially considering the presence of macrochromosomes and microchromosomes.

A-We added the scale bar in all figures.

**Additional Information:**

**Question**

**Response**

**Financial Disclosure**

Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research

This research was partially funded by a grant to EHCO from CNPq (307382/2019-2) and to MAFS from the Wellcome Trust in support of the Cambridge Resource Centre for Comparative Genomics and by the Biotechnology and Biological Sciences Research Council (BB/K008161/1) to the University of Kent.
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Comparative Chromosome Painting in the Black-Hawk-Eagle 
(Spizaetus tyrannus) and Gallus gallus with the use of Macro and Microchromosome Probes.

Short Title: Comparative Chromosome Painting in Spizaetus tyrannus and Gallus gallus

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Abstract

Although most birds show karyotypes with diploid number around 2n=80, with few macrochromosomes and many microchromosomes pairs, some groups, such as the Accipitriformes, are characterized by a large karyotypic reorganization, which resulted in complements with low diploid numbers, and a smaller number of microchromosomal pairs when compared to other birds. Among Accipitriformes, the Accipitridae family is the most diverse and includes the subfamily Aquilinae, composed of medium to large sized species. The Black-Hawk-Eagle (Spizaetus tyrannus - STY), found in South America, is a member of this subfamily. Available chromosome data for this species includes only conventional staining. Hence, in order to provide additional information on karyotype evolution process within this group, we performed comparative chromosome painting between S. tyrannus and Gallus gallus (GGA). Our results revealed that at least 29 fission-fusion events were observed in the STY karyotype, based on homology with GGA. Fissions occurred mainly in syntenic groups homologous to GGA1-GGA5. On the other hand, the majority of the microchromosomes were found fused to other chromosomal elements in STY, indicating these rearrangements had an important role in the reduction of the diploid number to 2n=68. Comparison with hybridization pattern of the Japanese-Mountain-Eagle, the only Aquilinae analyzed by comparative chromosome painting previously, did not reveal any synapomorphy that could represent a chromosome signature to this subfamily. Therefore, conclusions about karyotype evolution in Aquilinae require additional painting studies.

Key Words: Aquilinae, Chromosome painting, BACs, Microchromosomes
Introduction

Usually, bird genome is organized in karyotypes consisting of few macrochromosomes and many tiny microchromosomes [1]. However, there are some exceptions. For instance, excluding the New World vultures (Cathartidae), which show similar karyotypes to the putative avian ancestral karyotype (PAK) with diploid number around 2n=60, including 10 pairs of macrochromosomes and 30 pairs of microchromosomes [1], species belonging to the Order Accipitriformes present an interesting chromosomal diversity. They have lower diploid numbers, 2n = 54-68, and a reduction of microchromosomes to between 4 and 8 pairs, due mainly to fusions involving these small elements, occurred during their divergence [2-4].

In general, studies focusing on chromosome evolution in birds are based in comparative chromosome painting using chicken whole chromosome probes (Gallus gallus – GGA, 2n=78), due to the similarity of the karyotype of this species with the PAK [5]. The use of this methodology in species of birds of prey has revealed that, despite the lower diploid numbers observed in this group, the large karyotype reorganization in Accipitriformes included multiple fissions in the macrochromosome pairs homologous to GGA1-GGA5. The reduction of the chromosome number would be due to the concomitant occurrence of several fusion events involving microchromosomes [6-11].

Microchromosomes are gene rich elements, and genome comparative analyses have shown their conservation as syntenic groups among distantly related bird groups [12-13]. In fact, rearrangements involving microchromosomes were detected in few orders: Psittaciformes, Cuculiformes, Suliformes, Caprimulgiformes and the Accipitriformes [13-15]. Due to difficulties of the isolation of individual microchromosome pairs by flow cytometry for specific probe production, most data concerning microchromosomes were obtained by the use of pools of microchromosomes,
i.e., chromosome paints that recognize more than one pair. Therefore, improved identification of chromosome pairs involved in rearrangements is a priority if we are to achieve a more definitive analysis and identify synapomorphies based on chromosome characters [16-17].

Currently, the order Accipitriformes is composed of four families, of which Accipitridae is the most diverse, with approximately 230 species distributed in 14 subfamilies [18]. Among them, the subfamily Aquilinae includes medium and large species, distributed globally, usually known as booted eagle. Usually, ten genera are found within Aquilinae. Cytogenetically, the only information concerning Aquilinae is the definition of the diploid number of six species (four genera), ranging from 2n=66 to 82 [19].

The Black-Hawk-Eagle (Spizaetus tyrannus-STY) is a representative of this subfamily, found in South and Central Americas, from southern Mexico down to Argentina [18]. Considering that the only chromosomal analysis of S. tyrannus to date was based on conventional staining, revealing a karyotype within the Aquilinae standard, with 2n=68 [1], the aim of this study was to present the cytogenetic mapping of S. tyrannus by comparative painting. In addition to whole-chromosome paints of Gallus gallus (GGA), we used BAC probes from GGA clones that identified 11 individual pairs of microchromosomes. The results were compared to Nisaetus nipalensis orientalis-NNI (2n=66) [10], also from the subfamily Aquilinae, in order to identify chromosomal rearrangements related to karyotype evolution in this group.
Results

Karyotype description

The karyotype of *Spizaetus tyrannus* presented a diploid number 2n=68, consisting of 21 met or submetacentric pairs (pairs 1-4, 6-7, 9-10, 12, 14, 16-17, 19-22, 24-29 and the sex chromosome), seven acrocentric (pairs 5, 8, 11, 13, 15, 18 and 23), and four pairs of microchromosomes (pairs 30-33). The Z chromosome is a large metacentric, with size between pairs 3 or 4, while the W chromosome is an average submetacentric, similar in size to pairs 8 or 9 (Figure 1). In Table 1, we reported some differences in chromosome morphology of *S. tyrannus* described by Tagliarini et al., [1] (Table 1).

| Pairs  | This study | Tagliarini et al., 2007 | Pairs  | This study | Tagliarini et al., 2007 |
|--------|------------|------------------------|--------|------------|------------------------|
| Chr 1. | SM         | SM                     | Chr 18.| AC         | AC                     |
| Chr 2. | SM         | SM                     | Chr 19.| SM         | SM                     |
| Chr 3. | SM         | SM                     | Chr 20.| SM         | SM                     |
| Chr 4. | SM         | SM                     | Chr 21.| SM         | SM                     |
| Chr 5. | AC         | SM                     | Chr 22.| SM         | SM                     |
| Chr 6. | SM         | ST                     | Chr 23.| AC         | AC                     |
| Chr 7. | SM         | ST                     | Chr 24.| SM         | AC                     |
| Chr 8. | AC         | ST                     | Chr 25.| SM         | AC                     |
| Chr 9. | SM         | SM                     | Chr 26.| SM         | AC                     |
| Chr 10.| SM         | ST                     | Chr 27.| SM         | SM                     |
| Chr 11.| AC         | SM                     | Chr 28.| SM         | AC                     |
| Chr 12.| SM         | SM                     | Chr 29.| SM         | SM                     |
| Chr 13.| AC         | SM                     | Chr 30.| Micro      | Micro                  |
| Chr 14.| SM         | AC                     | Chr 31.| Micro      | Micro                  |
| Chr 15.| AC         | AC                     | Chr 32.| Micro      | Micro                  |
| Chr 16.| SM         | ST                     | Chr 33.| Micro      | Micro                  |
| Chr 17.| SM         | ST                     | Chr ZW.| M and SM   | M and SM               |
**Comparative Chromosome painting**

*G. gallus* probes used in the fluorescent *in situ* hybridization (FISH) experiments produced reproducible results. Hybridizations with chromosome-specific probes for the first ten pairs of GGA produced 22 signals, with the first five pairs producing multiple signals, ranging from 2 to 6 signals (Figure 2). For instance, GGA1 probe painted six distinct pairs in the *S. tyrannus* karyotype (pairs 5, 6, 12, 14, 18, and 25), while the probes GGA6-10 pairs showed only one signal each. Table 2 details the distribution of the signals produced by GGA whole specific probes in the karyotype of *S. tyrannus*.

### Table 2- Results of hybridizations with *G. gallus* probes showing the homology between GGA probes in the karyotype of *S. tyrannus* (STY).

| Probes | STY Chromosomes | Probes | STY Chromosomes |
|--------|-----------------|--------|-----------------|
| GGA1   | (5, 6, 12, 14, 18, 25) | GGA6   | 9               |
| GGA2   | (1, 3q, 21)     | GGA7   | 8               |
| GGA3   | (13, 16q, 19, 20) | GGA8   | 7               |
| GGA4   | (2, 17)         | GGA9   | 11q             |
| GGA5   | (4, 15q)        | GGA10  | 10q             |

A total of 19 out of 22 *G. gallus* BAC clones produced results. Both BACs from the GGA22 chromosome did not produce any detectable signal, as well as one of the BACs from GGA21. Among the 19 probes that gave good quality results, both proximal (BACP) and distal (BACd) referring to 8 pairs, were found in the same segment in the STY karyotype. However, BACs corresponding to GGA17 hybridized to two different pairs - BAC17p marked STY 9q, while BAC17d marked STY 24q. (Figure 3). All Chicken BACs and their respective homology in the karyotype of *S. tyrannus* are summarized in Table 3.
Table 3- Summary of the results of experiments using GGA BACs on the karyotype of *S. tyrannus*

(*BACs marking the same segment in STY karyotype; p=proximal region; d=distal region*)

| GGA  | BAC ID       | STY | GGA  | BAC ID       | STY |
|------|--------------|-----|------|--------------|-----|
| 17p  | CH261-113A7  | 23d | 23d  | CH261-90K11  | 23p*|
| 17d  | CH261-42P16  | 24q | 24p  | CH261-103F4  | 15p*|
| 18p  | CH261-60N6   | 19p*| 24d  | CH261-65O4   | 15p*|
| 18d  | CH261-72B18  | 19p*| 25p  | CH261-59C21  | 20p*|
| 19p  | CH261-10F1   | 13p*| 25d  | CH261-127K7  | 20p*|
| 19d  | CH261-50H12  | 13p*| 26p  | CH261-186M13 | 27p*|
| 21p  | CH261-83I20  | No signal | 26d | CH261-170L23 | 27p*|
| 21d  | CH261-122K8  | 4p  | 27p  | CH261-66M16  | 16p*|
| 22p  | CH261-40J9   | No signal | 27d | CH261-28L10  | 16p*|
| 22d  | CH261-18G17  | No signal | 28p | CH261-64A15  | 11p*|
| 23p  | CH261-191G17 | 23p*| 28d  | CH261-72A10  | 11p*|

Homologies obtained both by whole chromosome painting and BAC probes are shown in figure 4.

**Discussion**

The karyotype of *S. tyrannus* obtained herein presented 2n=68, confirming data from a previous report [1]. We report slight differences in chromosome morphology however, due to the higher number of biarmed pairs (Table 1).

The results of comparative chromosome painting with whole chromosome probes of *G. gallus* showed a similar pattern to other birds of prey in the family Accipitridae, with a large reorganization of the syntenic groups homologous to the first five pairs of *G. gallus*. That is, each probe (GGA1 - GGA5) corresponded to at least two distinct pairs (Figure 3). The most extreme examples are the fission of GGA1 into six pairs in STY, and GGA3 into four distinct pairs. These results are congruent with other birds of prey,
considering that GGA1 can reveal syntenic segments in four pairs (*Gypaetus barbatus*, 2n=60) to seven pairs (*Nisaetus nipalensis orientalis* - NNI), while GGA3 is hybridized to four pairs in all species analyzed in this family. The exception is NNI where it hybridizes to only 2 pairs [11]. On the other hand, GGA6 - GGA10 are conserved syntenies, with only one signal for each pair.

All associations observed in the karyotype of STY based in its homology with *G. gallus* are represented in Figure 4. In general, 16 fissions and 13 fusions were detected, totaling 29 rearrangements in the karyotype of STY when compared to *G. gallus*, with fissions occurring mainly in relation to the first five pairs of macrochromosomes and fusions involving mainly the microchromosomes. In the microchromosomes, chicken BAC probes showed that their syntenies were not disrupted by fission events as probes for proximal and distal regions were found hybridizing to the same pair in STY, except for GGA17, which produced signals in STY9 and STY24. However, all the identified BAC signals showed that each GGA microchromosome was fused to a STY segment homologous to either a GGA macro or microchromosome. This indicates that chromosomal fusions had an important role in reducing the diploid number in STY and other Accipitriformes. It is important to note that not all GGA microchromosomes are represented by chicken BACs, and hence other fusions must have occurred in this species to maintain 2n = 68.

The closest subspecies to *Spizaetus tyrannus* with chromosome painting data is the Japanese-Eagle (*Nisaetus nipalensis* orientalis), with 2n=66 [10]. Although geographically separated, they are morphologically similar, and until the last decade were classified as part of the same genus. Despite now being separated into distinct genera, molecular data support their close phylogenetic relationship [20]. Nevertheless, the comparative chromosome painting detects many differences. For instance, GGA1-9
probes produced signals in 21 pairs in STY, and 22 in NNI; the difference was due to an extra fission of GGA1 in NNI. Despite both species presenting three fusions involving the first nine pairs with microchromosomes (STY: pairs 4, 7 and 9; NNI: pairs 2, 4 and 9), none of them share the same GGA syntenic groups, and microchromosomes involved in NNI were not identified. Additionally, in both species GGA3 hybridizes to 4 pairs, however in STY all these segments are fused with microchromosomes (GGA: pairs 18, 19, 24 and 25), whereas in NNI only one segment of GGA3 is fused with a microchromosome (unidentified pair) [10].

Regarding the phylogenetic relationship of Aquilinae with other subfamilies within Accipitridae, although STY and NNI present some karyotypic similarities common to diurnal birds of prey, such as recurrent breakpoints mainly in relation to the GGA1-GGA5 pairs [10,11], we did not identify any synapomorphic associations which could represent ancestral characteristics for the Aquilinae [21-22]. Hence, while other subfamilies, such as Buteoninae and Harpiinae present well-established chromosomal signatures that allow the elaboration of their putative ancestral karyotypes [7], the available chromosome data indicate absence of chromosomal signatures between STY and NNI, which can be explained by their significant geographic isolation, inhabiting opposite regions in the globe.

**Conclusion**

The present work is the first comparative chromosome mapping of a species in the gen**S. tyrannus** and has revealed substantial karyotypic reorganization common to birds of prey of the family Accipitridae. Together with **G. gallus** chromosome-specific probes for the larger pairs, chicken BACs were able to provide a more comprehensive
result with additional information on the organization of the *S. tyrannus* karyotype. There are many similarities with the *N. nipalensis orientalis*, including numerous fissions of the first five pairs homologous to GGA with only one less in STY (21 events against 22 in NNI), and three fusions involving homologues of GGA1-GGA9 chromosomes and microchromosomes, but with breakpoints that are not shared between these two species. For a broader analysis at the phylogenetic level, it would be necessary to have comparative mapping of other species of the genus *Spizaetus* so that an ancestral karyotype of this genus could be traced.

**Methods**

*Samples and Chromosome Preparations*

The experiments followed the standards approved by the Ethics Committee for the Use of Animals in Research (CEPAE-UFPA under number 170-13). We performed fibroblast cell cultures from skin biopsies and feather pulp of *Spizaetus tyrannus* (STY) obtained from two female individuals maintained in Zoos (Criadouro Gavião Real, Capitão Poço, Brazil), following the protocol of Sasaki et al. [23] with modifications. After tissue cleavage in Petri dishes, the samples were incubated with 1% type 1 collagenase (GIBCO) for 1 hour at 37°C for tissue dissociation. Metaphase chromosomes were obtained after incubation for one hour with Colcemid (0.05 µg/mL), hypotonic solution (KCl at 0.075 M) for 20 minutes and fixation in methanol/acetic acid (3:1). Karyotype analysis was performed using conventional staining with 5% Giemsa in 0.07 M phosphate buffer (pH 6.8) for 5 minutes, slides were analyzed using a 100× objective (Leica, CO, USA) and GenASIs software (ADS Biotec, Omaha, NE, USA).
Two types of *Gallus gallus* probes were used: whole-chromosome-specific probes of the first 10 pairs, and bacterial artificial chromosomes (BACs) probes from 11 microchromosome pairs. Whole chromosome paints were developed and provided by the Cambridge Resource Center for Comparative Genomics (Cambridge, UK) using the Fluorescent Activated Cell Sorting (FACS) technique and labeled with biotin, fluorescein and/or digoxigenin (Roche Diagnostics, Mannheim, Germany), and detected with the addition of avidin-Cy3 (or Cy5) or anti-digoxigenin-rhodamin (Vector Laboratories, Burlingame, CA, USA). BAC clones ranged from 150,000 kb to 210,000 kb in size were selected from the CHORI-261 Chicken BAC library (Children's Hospital Oakland Research Institute, Oakland-USA), corresponding to sequences from the proximal and distal regions of the microchromosomes (each pair represented by two BACs, in a total of 22 BACs covering pairs 17 to 28, except for pair 20). Clones were produced following the protocol of the mini prep kit (Qiagen, Hilden, Germany) and labelled directly by fluorescein isothiocyanate (FITC) (green) or Texas Red (red) through Nick Translation (Roche, Mannheim, Germany).

Hybridization experiments followed standard procedures [7, 12]. Probes (1 µL labeled probe in 14 µL hybridization buffer) were denatured at 70°C for 10 min and preannealed for 30 min at 37°C. Hybridization mix was added on slides with chromosome preparations previously denatured at 70% formamide for 1 min and 20 s and dehydrated by serial ethanol dehydration (70%, 90% and 100%). Detection was performed using Avidin-Cy5 or anti-digoxigenin (Vector Laboratories, Burlingame, CA, USA). Slides were analyzed with an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera and appropriate filters. Images were captured using SmartCapture3 (Digital Scientific UK).
Declarations

Ethics Approval: The experiments were carried out according to the ethical protocols approved by an ethics committee (CEUA—Federal University of Pará) under no. 170/2013 and SISBIO 68443-1.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article

Author Contributions: Conceptualization, C.C.A.L, I.O.F. and E.H.C.O.; Data curation and formal analysis, C.C.A.L., I.O.F., J.P. and E.H.C.O.; Investigation, C.C.A.L., I.O.F., Methodology, I.O.F., P.C.M.O., and J.P.; Project administration, E.H.C.O.; Funding acquisition, M.A.F.S., D.K.G. and E.H.C.O.; Validation, C.C.A.L., I.O.F. and E.H.C.O.; Writing (original draft), C.C.A.L., I.O.F. and E.H.C.O.; Writing (review and editing), D.K.G., P.C.M.O. and M.A.F.S.

Acknowledgements: We would like to thank all the staff of the Laboratório de Citogenômica e Mutagênese Ambiental (SAMAM, IEC) and Cambridge Resource Centre for Comparative Genomics for their technical support.

Conflict of interest: The authors declare that they have no conflict of interest
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**Figure Legends**

**Figure 1** - Metaphase (A) and karyotype (B) of *S. tyrannus* with diploid number 2n = 68, obtained with Giemsa conventional staining. The red arrows in (A) indicate the sex chromosomes. Scale bar: 5µm

**Figure 2** - Representative results of FISH experiments using *G. gallus* chromosome-specific probes corresponding to pairs GGA1 to GGA5 in *S. tyrannus* karyotype. Red and green signals represent probes labelled with Cy3 or FITC, respectively. Scale bar: 5µm

**Figure 3** - Representative results of hybridizations with some *G. gallus* BACs probes in the karyotype of *S. tyrannus*. (A1 and A1.1) Chicken BAC17 was the only one to hybridize
different chromosomes. Red signals represent probes labeled with Cy3, corresponding to the proximal region (p); Green signals represent probes labeled with FITC, corresponding to the distal region (d). Arrows indicate the signals. Scale bar: 5µm

**Figure 4**- Idiogram representing the homology between the *S. tyrannus* chromosomes and the macrochromosome chromosome-specific probes and microchromosomes BAC clones from *G. gallus*. Empty boxes mean no signal detected in those chromosomes with the set of probes used. BACs corresponding to pairs 20 and 22 (*) was not used or did not produce any detectable signals, respectively.
Comparative Chromosome Painting in the Black-Hawk-Eagle
(*Spizaetus tyranus*) and *Gallus gallus* with the use of Macro
and Microchromosome Probes.

**Short Title:** Comparative Chromosome Painting in *Spizaetus tyranus* and *Gallus gallus*

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Abstract

Although most birds show karyotypes with diploid number around 2n=80, consisting of few macrochromosomes and many microchromosome pairs, however some groups, such as the Accipitriformes, are characterized by a large karyotypic reorganization, which resulted in complements with low diploid numbers, and a smaller number of microchromosomal pairs when compared to the karyotypes of most other birds, with diploid numbers around 2n=80 and many pairs of microchromosomes. Among Accipitriformes, the Accipitridae family is the most diverse and currently divided into 14 subfamilies, including the subfamily Aquilinae, composed of medium to large sized species. An example is the Black-Hawk-Eagle (Spizaetus tyrannus - STY), found in South America, is a member of this subfamily. Available chromosome data for this species includes only conventional staining. Hence, in order to provide additional information on re-aiming understanding the aspects of karyotypic evolution process within this group, we presenting in this study the performed comparative chromosome mapping – chromosome homologies between S. tyrannus this species and the chicken Gallus gallus (GGA) using comparative chromosome painting. Our results revealed that at least 29 fission-fusion events were observed in the STY karyotype, based on homology with GGA. The majority of fissions occurred mainly in segments syntetic groups homologous to GGA1-GGA5, whereas the majority of the GGA microchromosomes remained conserved.
and were found fused to other chromosomal elements in STY. **Comparison with** When compared to the hybridization pattern of the Japanese-Mountain-Eagle (*Nisaetus nipalensis orientalis*), the only close relative of *S. tyrannus* analyzed by comparative-chromosome painting so far, we observed that there are **showed many-some similarities, but also-and differences** between these species that, including numerous fissions and fusions events of the first five pairs homologous to GGA. Therefore, conclusions about karyotype evolution in Aquilinae require additional painting studies with only one less in STY (21 events against 22 in NNI), and three fusions involving homologues of GGA1-GGA9 chromosomes and microchromosomes, but with breakpoints that are not shared between these two species. However, for a phylogenetic analyze within of the subfamily Aquilinae would be necessary comparative mapping of other species of the genus *Spizaetus* so that an ancestral karyotype of this subfamily could be traced.

**Key Words:** Aquilinae, Chromosome painting, BACs, Microchromosomes

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**Introduction**

**This** Usually, bird genome is organized in karyotypes of birds, consisting of few macrochromosomes and many tiny microchromosomes. However, there are some exceptions. For instance, excluding the New World vultures (Cathartidae), which show
similar karyotypes to the putative avian ancestral karyotype with diploid number around $2n=80$, including 10 pairs of macrochromosomes and 30 pairs of microchromosomes [1]. Species belonging to the Order Accipitriformes present with an unusual chromosomal diversity. They have smaller--lower diploid numbers, $2n=54-68$, and a reduction of microchromosomes to between 4 and 8 pairs, due mainly to several chromosomes fusions involving these small elements rearrangements that occurred during their divergence [2-4].

In generally, the studies about focusing on the chromosome evolution in birds are Accipitriformes were mainly based in comparative chromosome painting using chicken whole chromosome paints probes of the chicken- (*Gallus gallus* - GGA, $2n=78$), the most common chromosome probe used in birds, due to the similarity of the genome organization karyotype of this species with the proposed putative avian ancestral karyotype (PAK) of this species as the ancestral of Aves [5]. According to these studies, the use of this methodology in species of birds of prey has revealed that, despite the lower diploid numbers observed in this group, comparative chromosome painting studies using whole chromosome paints of the chicken (*Gallus gallus* - GGA), this the large karyotype reorganization in Accipitriformes included several many multiple fissions in the macrochromosome-macrochromosome pairs homologous to GGA1-GGA5. The reduction of the chromosome number would be due to the concomitant occurrence of and large several fusions events involving, while microchromosomes were largely involved in fusion events [6-19].

The microchromosomes in birds karyotype are gene rich elements, and and highly-conserved. Some genome comparative analyses have shown considerable their conservation as syntenic groups among distantly related bird groups [12-13]. In fact, rearrangements involving microchromosomes were detected only in a few species order.
such as Psittaciformes, Cuculiformes and the Accipitriformes were detected rearrangements involving these elements [13-15].

Due to difficulties involving the isolation of individual microchromosome pairs by flow cytometry for specific probe production, most data concerning microchromosomes were obtained using by the use of pools of microchromosomes, i.e., chromosome paints that recognize more than one pair. Therefore, improved identification of chromosome pairs involved in rearrangements is a priority if we are to achieve a more definitive analysis and identify synapomorphies based on chromosome characters [16-17].

Currently, the order Accipitriformes is composed of four families, of which the Accipitridae family being the most diverse, with approximately 230 species distributed in 14 subfamilies in quantity of species, within this family is the subfamily Aquilinae [18]. Among them, the subfamily Aquilinae includes medium and large species, distributed globally, usually known as booted eagle. Usually, ten genera are found within Aquilinae, of which only four have their diploid numbers defined, ranging from 2n=66 to 68 [19].

The Black-Hawk-Eagle (Spizaetus tyrannus, STY) is a representative of this subfamily, belongs to this subfamily. This species belongs to the subfamily Aquilinae (Accipitriformes, Accipitridae), and is found in South and Central America, from southern Mexico down to Argentina [189, Sick, 1997; Mikich & Bernils, 2004]. Considering that the only chromosomal analysis of S. tyrannus to date was based on conventional staining, revealing a karyotype within the Accipitridae-Aquilinae standard, with 2n=68 and only four pairs of microchromosomes [1], the aim of this study was to present—the cytogenetic mapping of S. tyrannus by comparative painting. In addition to
whole-chromosome paints of *Gallus gallus* (GGA), we used BAC probes from GGA clones that identified 11 individual pairs of microchromosomes. The results were compared to *Nisaetus nipalensis orientalis* - NNI (2n=66) [10], also from the subfamily Aquilinae, in order to identify chromosomal rearrangements related to karyotype evolution in this group.

This study aimed at understanding the karyotype evolution within the subfamily Aquilinae, we presented the cytogenetic mapping of *S. tyrannus* species of the genus *Spizaetus* by comparative painting. We also compared this species to the *Nisaetus nipalensis orientalis* - NNI (2n=66) [10], the only close relative. Additionally, to whole chromosome paints of *Gallus gallus* (GGA), we used bacterial artificial chromosomes (BACs). BAC probes from GGA G. gallus clones that identified corresponded to 11 individual pairs of microchromosomes were used aiming to identify possible rearrangements that could help to understand the role of microchromosomes in the karyotype evolution of *S. tyrannus*.

Results

*Karyotype description*

The karyotype of *Spizaetus tyrannus* presented a diploid number 2n=68, consisting of 21 meta or submetacentric pairs (pairs 1-4, 6-7, 9-10, 12, 14, 16-17, 19-22, 24-29 and the sex chromosomes), 7 seven acrocentric (pairs 5, 8, 11, 13, 15, 18 and 23), and 4 four pairs of microchromosomes (pairs 29-30-33). The Z chromosome is a large
metacentric, with size between pairs 3 or 4, while the W chromosome is an average submetacentric, similar in size to pairs 8 or 9 (Figure 1). In Table 1, we reported some differences in chromosome morphology of *S. tyrannus* described by Tagliarini et al., [1] (Table 1).

**Table 1.** Karyotype of *S. tyrannus* described by Tagliarini et al., 2006 and at this study. (Metacentric: M; Submetacentric: SM; Subtelocentric: ST; Acrocentric: AC)

| Pairs | This study | Tagliarini et al., 2007 | Pairs | This study | Tagliarini et al., 2007 |
|-------|------------|-------------------------|-------|------------|-------------------------|
| Chr 1 | SM         | SM                      | Chr 18| AC         | AC                      |
| Chr 2 | SM         | SM                      | Chr 19| SM         | SM                      |
| Chr 3 | SM         | SM                      | Chr 20| SM         | SM                      |
| Chr 4 | SM         | SM                      | Chr 21| SM         | SM                      |
| Chr 5 | AC         | SM                      | Chr 22| SM         | SM                      |
| Chr 6 | SM         | ST                      | Chr 23| AC         | AC                      |
| Chr 7 | SM         | SM                      | Chr 24| SM         | AC                      |
| Chr 8 | AC         | ST                      | Chr 25| SM         | AC                      |
| Chr 9 | SM         | SM                      | Chr 26| SM         | AC                      |
| Chr 10| SM         | ST                      | Chr 27| SM         | SM                      |
| Chr 11| AC         | SM                      | Chr 28| SM         | AC                      |
| Chr 12| SM         | SM                      | Chr 29| SM         | SM                      |
| Chr 13| AC         | SM                      | Chr 30| Micro      | Micro                   |
| Chr 14| SM         | AC                      | Chr 31| Micro      | Micro                   |
| Chr 15| AC         | AC                      | Chr 32| Micro      | Micro                   |
| Chr 16| SM         | ST                      | Chr 33| Micro      | Micro                   |
| Chr 17| SM         | ST                      | Chr ZW| M and SM   | M and SM                |

*Comparative Chromosome painting*
**GGA-G. gallus** probes used in the fluorescent *in situ* hybridization (FISH) experiments produced reproducible results. Hybridizations with chromosome-specific probes for the first ten pairs of GGA produced 22 signals, with the first five pairs producing multiple signals, ranging from 2 to 6 signals (Figure 2). For instance, GGA1 probe painted six distinct pairs in the *S. tyrannus* STY karyotype (pairs 5, 6, 12, 14, 18, 25), while the probes GGA6-10 pairs showed only one signal each. Table 24 details the distribution of the signals produced by GGA whole specific probes in the karyotype of *Spizaetus S. tyrannus*.

| Probe | STY Chromosomes | Probe | STY Chromosomes |
|-------|-----------------|-------|-----------------|
| GGA1  | (5, 6, 12, 14, 18, 25) | GGA6  | 9 |
| GGA2  | (1, 3q, 21) | GGA7  | 8 |
| GGA3  | (13, 16q, 19, 20) | GGA8  | 7 |

Table 24: Results of hybridizations with *Gallus G. gallus* probes showing the homology between GGA probes in the karyotype of *Spizaetus S. tyrannus* (STY).

A total of 19 out of 22 *Gallus G. gallus* BAC clones produced results. Both BACs from the GGA22 chromosome did not produce any detectable signal, as well as one of the BACs from GGA21. Among the 19 probes that gave good quality results, both proximal (BACp) and distal (BACd) referring to 8 pairs, were found in the same segment.
in the STY karyotype. However, BACs corresponding to GGA17 hybridized to two different pairs - BAC17p marked STY 9q, while BAC17d marked STY 24q. (Figure 3).

All Chicken BACs and their respective homology in the karyotype of *Spizaetus tyranus* are summarized in Table 3.

**Table 3**: Summary of the results of experiments using GGA BACs on the karyotype of *Spizaetus tyranus* (*=BACs marking the same segment in STY karyotype; p=proximal region; d=distal region).  

| GGA | BAC ID     | STY | GGA | BAC ID     | STY |
|-----|------------|-----|-----|------------|-----|
| 17p | CH261-113A7| 9q  | 23d | CH261-90K11| 23p*|
| 17d | CH261-42P16| 24q | 24p | CH261-103F4| 15p*|
| 18p | CH261-60N6  | 19p*| 24d | CH261-65O4  | 15p*|
| 18d | CH261-72B18 | 19p*| 25p | CH261-59C21 | 20p*|
| 19p | CH261-10F1  | 13p*| 25d | CH261-127K7 | 20p*|
| 19d | CH261-50H12 | 13p*| 26p | CH261-186M13| 27p*|
| 21p | CH261-83I20 | No signal | 26d | CH261-170L23 | 27p*|
| 21d | CH261-122K8 | 4p  | 27p | CH261-66M16 | 16p*|
| 22p | CH261-40J9  | No signal | 27d | CH261-28L10 | 16p*|
| 22d | CH261-18G17 | No signal | 28p | CH261-64A15 | 11p*|
| 23p | CH261-191G17| 23p*| 28d | CH261-72A10 | 11p*|

Homologies obtained both by whole chromosome painting and BAC probes are shown in figure 4.

**Discussion**

The karyotype of *Spizaetus tyranus* obtained herein presented 2n=68, confirming data from a previous report [1]. We report slight differences in chromosome morphology however, due to the higher number of biarmed pairs (Table 1).
The results of comparative chromosome painting with whole chromosome probes of *Gallus* *G. gallus* showed a similar pattern to other birds of prey in the family Accipitridae, with a large reorganization of the syntenic groups homologous to the first five pairs of *G. gallus*.*galus*. That is, each probe (GGA1 - GGA5) corresponded to at least two distinct pairs (Figure 3). The most extreme examples are the fission of GGA1 into six pairs in STY, and GGA3 into three-four distinct pairs. These results are congruent with other birds of prey, considering that GGA1 can reveal syntenic segments in four pairs (*Gypaetus barbatus, 2n=60*) to seven pairs (*Nisaetus nipalensis orientalis - NNI*), while GGA3 is hybridized to four pairs in all species analyzed in this family. The exception is NNI where it hybridizes to only 2 pairs [114]. On the other hand, GGA6 - GGA10 are conserved syntenies, with only one signal for each pair.

All associations observed in the karyotype of STY based in its homology with *Gallus* *G. gallus* are represented in Figure 4. In general, 16 fissions and 13 fusions were detected, totaling 29 rearrangements in the karyotype of STY when compared to *Gallus*, with fissions occurring mainly in relation to the first five pairs of macrochromosomes and fusions involving mainly the microchromosomes. In the microchromosomes, chicken BAC probes showed that their syntenies were not disrupted by fission events as probes for proximal and distal regions were found hybridizing to the same pair in STY. However, all the identified BAC signals showed that each GGA microchromosome was fused to a STY segment homologous to either a GGA macro or microchromosome. This indicates that chromosomal fusion led to the lower diploid number in STY and other Accipitriformes. Despite the numerous fissions observed, it is important to note that not all GGA microchromosomes are represented by chicken BACs, and hence other fusions must have occurred in this species to maintain a 2n = 68.
The closest subspecies to *Spizaetus tyrannus* with chromosome painting data is the Japanese-Eagle (*Nisaetus nipalensis orientalis*) [910]. Although geographically separated, they are morphologically similar, and until the last decade were classified as part of the same genus. Despite now being separated into distinct genera, molecular data support their close phylogenetic relationship [1420]. Nevertheless, the comparative chromosome painting detects many differences. For instance, GGA1-9 probes produced signals in 21 pairs in STY, and 22 in NNI; the difference was due to an extra fission of GGA1 in NNI. Despite both species presenting three fusions involving the first nine pairs with microchromosomes (STY: pairs 4, 7 and 9; NNI: pairs 2, 4 and 9), none of them share the same GGA syntenic groups, and microchromosomes involved in NNI were not identified. Additionally, in both species GGA3 hybridizes to 4 pairs, however in STY all these segments are fused with microchromosomes (GGA: pairs 18, 19, 24 and 25), whereas in NNI only one segment of GGA3 is fused with a microchromosome (unidentified pair) [910].

Another point relates to the difference in diploid number of *S. tyrannus* and *N. nipalensis orientalis* (2n = 66 in NNI and 2n = 68 in STY). This can be explained by a centric fusion of two pairs of microchromosomes (pairs not identified) in the NNI7 chromosome resulting in one pair of microchromosomes less in the karyotype of *N. nipalensis orientalis* [109]. These results show that they are morphologically similar species that until the last decade were part of the same genus [14].

Moreover, despite STY and NNI presenting some karyotypic similarities common to diurnal birds of prey such as recurrent breakpoints mainly in relation to the GGA1-GGA5 pairs [109,110], we did not identify any synapomorphic associations which could represent ancestral characteristics for the Aquilinae [16,1621-22]. Hence, while other
subfamilies, such as Buteoninae and Harpiinae present well-established chromosomal signatures that allow the elaboration of their putative ancestral karyotypes [26], the available chromosome data indicate an absence of chromosomal signatures between STY and NNI, which can be explained by their significant geographic isolation, inhabiting opposite regions in the globe.
Conclusion

The present work is the first comparative chromosome mapping of a species in the genus *Spizaetus*—*S. tyrannus*—and has revealed substantial karyotypic reorganization common to birds of prey of the family Accipitridae. Together with *Gallus G. gallus* chromosome-specific probes for the larger pairs, chicken BACs were able to provide a more comprehensive result with additional information on the organization of the *S. tyrannus* Black Hawk Eagle karyotype. There are many similarities with the *N. nipalensis orientalis* Japanese Eagle, including numerous fissions of the first five pairs homologous to GGA with only one less in STY (21 events against 22 in NNI), and three fusions involving homologues of GGA1-GGA9 chromosomes and microchromosomes, but with breakpoints that are not shared between these two species. For a broader analysis at the phylogenetic level, it would be necessary to have comparative mapping of other species of the genus *Spizaetus* so that an ancestral karyotype of this genus could be traced. Furthermore, the new set of chromosome probes of *S. tyrannus* should be helpful in further studies of the phylogenetic relationships in Accipitriformes.
Methods

Samples and Chromosome Preparations

The experiments followed the standards approved by the Ethics Committee for the Use of Animals in Research (CEPAE-UFPA under number 170-13). We performed fibroblast cultures from skin biopsies and feather pulp of Spizaetus tyrannus (STY) obtained from two female individuals maintained in Zoos (Criadouro Gavião Real, Capitão Poço, Brazil), following the protocol of Sasaki et al. [213] with modifications. After tissue cleavage in Petri dishes, the samples were incubated with 1% type 1 collagenase (GIBCO) for 1 hour at 37°C for tissue disassociation. Metaphase chromosomes were obtained after incubation for one hour with Colcemid (0.05 µg/mL), hypotonic solution (KCl at 0.075 M) for 30 minutes and fixation in methanol/acetic acid (3:1). Karyotype analysis was performed using conventional staining with 5% Giemsa in 0.07 M phosphate buffer (pH 6.8) for 5 minutes, slides were analyzed using a 100x objective (Leica, CO, USA) and GenASIs software (ADS Biotec, Omaha, NE, USA).

GGA Probes and FISH Experiments

Two types of Gallus gallus probes were used: whole-chromosome-specific probes of the first 10 pairs, and chromosome-specific bacterial artificial chromosomes (BACs) probes from 11 microchromosome pairs. Whole chromosome paints were developed and provided by the Cambridge Resource Center for Comparative Genomics (Cambridge, UK) using the Fluorescent Activated Cell Sorting (FACS) technique and labeled with biotin, fluorescein and/or digoxigenin (Roche Diagnostics, Mannheim, Germany), and detected with the addition of avidin-Cy3 (or Cy5) or anti-digoxigenin-
rhodamin (Vector Laboratories, Burlingame, CA, USA). BAC clones ranged from 150,000 kb to 210,000 kb in size were selected from the CHORI-261 Chicken BAC library (Children's Hospital Oakland Research Institute, Oakland-USA), corresponding to sequences from the proximal and distal regions of the microchromosomes (each pair represented by two BACs, in a total of 22 BACs covering 11 chromosome pairs). Clones were produced following the protocol of the mini prep kit (Qiagen, Hilden, Germany) and labelled directly by fluorescein isothiocyanate (FITC) (green) or Texas Red (red) through Nick Translation (Roche, Mannheim, Germany).

Hybridization experiments followed standard procedures (de Oliveira et al., 2010[7]; O'Connor et al., 2018). The probes (1 µL labeled probe in 14 µL hybridization buffer) were denatured in 70°C for 10 min and preannealed for 30 min at 37°C, then the hybridization solution was added on slides with chromosome preparations previously desnatured at 70% formamide for 1 min and 20 s and dehydrated by serial ethanol dehydration (70%, 90% and 100%). The detection by Avidin-Cy5 or antidigoxigenin (Vector Laboratories, Burlingame, CA, USA). Slides were analyzed with an Olympus BX-61 epifluorescence microscope equipped with a cooled CCD camera and appropriate filters. Images were captured using SmartCapture3 (Digital Scientific UK).
Declarations

Ethics Approval: The experiments were carried out according to the ethical protocols approved by an ethics committee (CEUA—Federal University of Pará) under no. 170/2013 and SISBIO 68443-1.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Author Contributions: Conceptualization, C.C.A.L, I.O.F. and E.H.C.O.; Data curation and formal analysis, C.C.A.L, I.O.F., J.P. and E.H.C.O.; Investigation, C.C.A.L, I.O.F., Methodology, I.O.F., P.C.M.O., and J.P.; Project administration, E.H.C.O.; Funding acquisition, M.A.F.S., D.K.G. and E.H.C.O.; Validation, C.C.A.L, I.O.F. and E.H.C.O.; Writing (original draft), C.C.A.L, I.O.F. and E.H.C.O.; Writing (review and editing), D.K.G., P.C.M.O. and M.A.F.S.

Funding: This research was partially funded by a grant to EHCO from CNPq (307382/2019-2) and to MAFS from the Wellcome Trust in support of the Cambridge Resource Centre for Comparative Genomics and by the Biotechnology and Biological
Acknowledgements: We would like to thank all the staff of the Laboratório de Cultura de Tecidos e Citogenética (SAMAM, IEC) and Cambridge Resource Centre for Comparative Genomics for their technical support.

Conflict of interest: The authors declare that they have no conflict of interest

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Figure Legends

**Figure 1**- Metaphase (A) and karyotype (B) of *Spizaetus tyrannus* with diploid number 2n = 68, obtained with Giemsa conventional staining. The red arrows in (A) indicate the sex chromosomes. *Scale bar: 5 μm*

**Figure 2**- Representative results of FISH experiments using *Gallus gallus* chromosome-specific probes corresponding to pairs GGA1 to GGA5 in *Spizaetus tyrannus* karyotype. Red and green signals represent probes labelled with Cy3 or FITC, respectively. *Scale bar: 5 μm*

**Figure 3**- Representative results of hybridizations with some *Gallus gallus* BACs probes in the karyotype of *Spizaetus tyrannus*. (A1 and A1.1) Chicken BAC17 was the only one to hybridize on different chromosomes. Red signals represent probes labeled with Cy3, corresponding to the proximal region (p); Green signals represent probes labeled with FITC, corresponding to the distal region (d). Arrows indicate the signals. *Scale bar: 5 μm*

**Figure 4**- Idiogram representing the homology between the *Spizaetus tyrannus* chromosomes and the macrochromosome chromosome-specific probes and microchromosomes BAC clones from *Gallus gallus*. Empty boxes mean no signal detected in those chromosomes with the set of probes used. BACs corresponding to pairs 20 and 22 (*) did not produced any detectable signals.
Dear Reviewers,

We are thankful for the constructive reviews that we received; they certainly helped us to improve the manuscript. Please find below our responses to each of your comments.

Reviewer #1:

1- The work from Carvalho et al brings and interesting analysis in the in the Black-Hawk-Eagle (*Spizaetus tyrannus*) using BAC and WCP experiments, both derived from *Gallus gallus*. The results are very well represented in high-quality figures and the and brings new pieces of information for the big puzzle that avian karyotype evolution represents. Below I indicate some points to be adjusted and put special attention to one suggestion for discussion: The work would profit too much if the analyses were done under a phylogenetic context. An updated phylogeny with the special focus on the phylogenetic position and relation between Chicken and *Spizaetus* would bring more light to the general results found.

A- From the cytotaxonomic point of view, *Gallus gallus* has a basal karyotype, very similar to the avian putative ancestor. As this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. However, except for the use
of other probes, which could reveal intrachromosomal rearrangements, a phylogenetic analysis of GGA and birds of prey is not very informative, except as outgroup

Abstract

2- I missed some introduction about BIRDS cytogenetic in the beginning of Abstract. The authors go too direct about Accipitriformes and most readers do not have such phylogenetic information to which group we are dealing with.

A- We agree, and added an introduction to the abstract “Although most birds show karyotypes with diploid number around 2n=80, with few macrochromosomes and many microchromosomes pairs, some groups, such as the Accipitriformes, are characterized by a large karyotypic reorganization, which resulted in complements with low diploid numbers, and a smaller number of microchromosomal pairs when compared to other birds”

3- Why are the authors aiming to investigate homologies with chicken?

A- Because this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. We added a small explanation in the introduction “In general, studies focusing on chromosome evolution in birds are based in comparative chromosome painting using chicken whole chromosome probes (Gallus gallus – GGA, 2n=78), due to the similarity of the karyotype of this species with the PAK [5].”

4- Abstract also brings some previous data on NNI that were not originated from this work. I suggest removing.

A- We removed it

5- The terms STY and NNI are used without any previous information/explanation about, causing misunderstandings.

A- We corrected it
6- A final conclusion is missing in the abstract.

A- We added a conclusion in the abstract: “Comparison with hybridization pattern of the Japanese-Mountain-Eagle, the only Aquilinae analyzed by comparative chromosome painting previously, did not reveal any synapomorphy that could represent a chromosome signature to this subfamily. Therefore, conclusions about karyotype evolution in Aquilinae require additional painting studies”.

9- Introduction miss a clear motivation for the study.

A- We improved it: “Among them, the subfamily Aquilinae includes medium and large species, distributed globally, usually known as booted eagle. Usually, ten genera are found within Aquilinae. Cytogenetically, the only information concerning Aquilinae is the definition of the diploid number of six species (four genera), ranging from 2n=66 to 82 [19]”.

10- Why using GGA probes?

A- Because this species is an important biological model, and because it also retained a plesiomorphic karyotype, GGA probes are used as a standard for chromosomal studies in birds. We added a small explanation in the introduction.

11- I missed some info (Figure would be better) about the phylogenetic position and relation between Chicken and Spizaetus.
A- The authors agree that for this study are not necessary introduce some information about the phylogenetic position between *Gallus gallus* and *S. tyrannus*, however short information of phylogenetic relationship of the *S.tyrannus* within the Aquilinae Subfamily were added in the introduction aiming improve the reading of this paper.

12- Moreover, inform here as well that STY corresponds to *Spizaetus tyrannus* on its first mention.

A- This was corrected.

Results

13- Subtitle Karyotypes before the first paragraph.

A- We added it.

Figures

14- Figure 3: I did not understand why using the metaphases present in right column? The signals and chromosomal morphology are clearly visible in the FISH images. Scale bars are missing in all figures.

A- We removed the right column and added the scale bars in all figures.

Reviewer #2:

In this study, the authors used comparative chromosome painting to reveal homology of chromosomal segments between *Spizaetus tyrannus* (the Black-Hawk-Eagle) and *Gallus gallus*. The study is conceptually and methodically motivated. The karyotype of *S. tyrannus* was previously studied only using conventional chromosome staining technique that showed 2n=68 (32m/sm+8st+18a+8m+ZW), and this happened for the first time that the karyotype of this bird of prey was studied by comparative chromosome painting. As a result, the study provides a novel insight into the cytogenetics of the Black-Hawk-Eagle. Both whole chromosome-specific *G. gallus* probes of the 1st-10th pairs and chromosome-specific *G. gallus* BAC probes from 11 pairs of microchromosomes were used. The study evidenced 29 evolutionary fission-fusion events that happened in the evolution of *S. tyrannus* and identified the particular chromosome pairs in the referenced *G. gallus*
karyotype, which have undergone restructuring or have remained unchanged. Another sufficient result concerns the comparison between *S. tyrannus* and *Nisaetus nipalensis orientalis*, which is its only close relative studied so far by comparative chromosome painting. The comparison showed both similarities and differences between the species. The MS is well illustrated by tables and pictures of good quality. In general, the work is interesting and deserves publication in the journal. However, there are some shortcomings in the work. Main disadvantages are (1) almost complete absence of basic data on the karyotypes of the discussed species, which makes it difficult to adequately assess the results obtained, and (2) ignorance of the taxonomic component.

A- We try to improve it.

All other comments that I have are mainly suggestions for improving the article (unfortunately, there is no line numbering in the MS, which makes it difficult for the reviewer to work).

1-Running title. It is too long, should be shorter, e.g., “Comparative Chromosome Painting in *Spizaetus tyrannus* and *Gallus gallus*”.

A- W corrected it.

2. Abstract. Please, decipher GGA, should be *Gallus gallus* (GGA), as done above for *Spizaetus tyrannus* (STY)

A- We corrected it.

Introduction.

3- Page 3. Paragraph 1. Please, provide a putative avian ancestral karyotype, with 2n and the number of microchromosomes.

A- We provided this information in the introduction of the paper.

4- Page 3. Paragraph 1. Here (or further, in Discussion), provide the *G. gallus* karyotype to make further reasoning clearer.
A- We did it.

5- Page 3. Paragraph 3. Unify way of citing – in other places you use numbering.
   A- We corrected it.

6- Page 4. Paragraph 4. Please, specify the species studied, *Spizaetus tyrannus*
   A- We corrected it.

Discussion.

7- Page 6, Paragraph 1. Please, expand the statement “We report slight differences in chromosome morphology…” by clarifying what Tagliarini et al. (2007) have reported. Describe the differences, give for comparison one and the other karyotypes (otherwise, you only have a declaration).

   A- In order to clarify the difference in chromosome morphology described between this study and the Tagliarini et al., (2007), we made a comparative table that was introduce in the results.

8- Page 7. Paragraph 3. The Japanese mountain hawk-eagle *Nisaetus nipalensis orientalis* (Temminck & Schlegel, 1844), not the Hodgson's hawk-eagle *Nisaetus nipalensis* Hodgson, 1836.

   A- We corrected it.

9- Other remarks can be found in the MS attached.

   A- We followed all the instructions of attached.

Reviewer #3:

This study provides a cytogenetic mapping of the Black-Hawk-Eagle (*Spizaetus tyrannus*) using whole-chromosome paints and BAC probes of *Gallus gallus*. Using this
cytogenetic mapping, the authors investigate the chromosome homologies between *Spizaetus tyrannus* and *Gallus gallus*. Overall the manuscript presents an advance in cytogenetic of Accipitriformes. The manuscript is written in understandable English, but some procedures require more details. Please see below my comments that could help to further enhance the quality of the study.

2) Abstract: “chicken (*Gallus gallus* – GGA)”.

A- We corrected it.

3) Introduction and discussion: The manuscript is written in a manner suitable for a more specialized journal such as Cytogenetic and Genome Research or Comparative Cytogenetics, but not ideally for a general journal such as PlosOne. The introduction and discussion are rather limited to the focus of the analyses. This is a shame as I think the authors did a good job to gather nice cytogenetic results. Thus, the authors should consider redrafting these sections on a broad context.

The introduction could be more informative about the study as a whole. For example, the authors can give information about the contribution of this kind of work to karyotypic evolution and chromosomal organization of Accipitriformes. In this line, the last paragraph of introduction can be better written showing the importance of this study, and not merely comment “This study presents the cytogenetic mapping of a species…”. Moreover, other intriguing topic is about the origin of microchromosomes. And maybe the authors could comment a little bit about this topic in the introduction and also discussion. Below is a reference related to this topic.

- Waters, P. D., Patel, H. R., Ruíz-Herrera, A., Álvarez-González, L., Lister, N. C., Simakov, O., ... & Graves, J. A. M. (2021). Microchromosomes are building blocks of bird, reptile and mammal chromosomes. bioRxiv.

A- The introduction was rewritten and several new information were added to improve the understanding of the manuscript. Also, some information from the reference recommended by the reviewer were considerate in this topic.
4) Results, paragraph 1: The authors commented that they detected “four pairs of microchromosomes” but they indicated five (29, 30, 31, 32, and 33). Considering the Figure 1B the pair 29 apparently is not a microchromosome.

A- We reviewed the Karyotype of *S.tyrannus* and considered only four pairs of microchromosomes (Pairs: 30, 31, 32 and 33). Also, we corrected it throughout of the text.

5) Results, paragraph 2: “The most extreme examples are the fission of GGA1 into six pairs in STY, and GGA3 into three distinct pairs”. It seems that for GGA3 are four pairs (13, 16, 19, and 20) (Figure 4), right?

A- The reviewer is right. The GGA3 probe correspond to four pairs (13,16,19 and 20).
We changed it.

6) Discussion, paragraph 3: “…karyotype of STY when compared to *Gallus*”; “…karyotype of STY when compared to *Gallus gallus*”.

A- We corrected it.

7) Discussion, paragraph 4: “…microchromosomes (STY4, 7 and 9; NNI2, 4 and 9), none of them”; “microchromosomes (STY: pairs 4, 7 and 9; NNI: pairs 2, 4 and 9), none of them”.

“…with microchromosomes (GGA: pairs 18, 19, 24 and 25)”.

A- We corrected it.

8) Discussion, paragraph 5: “These results show that they are morphologically similar species that until the last decade were part of the same genus [14].”. I think the authors results did not show an association of chromosomal data with morphological similarity and this should be corrected.

A- We removed it.
9) Conclusion: “…of S.tyrannus should be…”; “…of S. tyrannus should be…”.

A- We corrected it.

10) Methods, paragraph 1: What the name and country of the Zoos?

A- The information was added in the Methods.

11) Methods, paragraph 2: “…and labelled directly by FTIC”; “and labelled directly by fluorescein isothiocyanate (FITC)”.

A- We changed it.

12) Methods: the results of this study are based on FISH with GGA probes. However, FISH procedure is not sufficiently described. I would appreciate if at least main/important steps of the FISH procedure are described. This would also avoid any doubt about the accuracy of the results.

A- We provided the main steps of the FISH procedure.

13) Figure 3: “FITC”.

A- We corrected it.

14) Please, give a scale bar information for all figures. It is very important, specially considering the presence of macrochromosomes and microchromosomes.

A- We added the scale bar in all figures.