Understanding Microchromosomal Organization and Evolution in Four Representative Woodpeckers (Picidae, Piciformes) through BAC-FISH Analysis

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Abstract

The genome organization of woodpeckers has several distinctive features e.g. an uncommon accumulation of repetitive sequences, enlarged Z chromosomes and atypical diploid numbers. Despite the large diversity of species, there is a paucity of detailed cytogenomic studies for this group and we thus aimed to rectify this. Genome organization patterns and hence evolutionary change in the microchromosome formation of four species (*Colaptes campestris*, *Veniliornis spilogaster, Melanerpes candidus* and *Picumnus nebulosus)* was established through fluorescence *in situ* hybridization (FISH) using Bacterial Artificial Chromosomes (BACs) originally derived from *Gallus gallus* and *Taeniopygia guttata*. Findings suggest that *P. nebulosus* (2n=110), which was described for the first time, had the most basal karyotype among species of Picidae studied here, and probably arose as a result of fissions of avian ancestral macrochromosomes. We defined a new chromosomal number for *V. spilogaster* (2n=88) and demonstrated microchromosomal rearrangements involving *C. campestris* plus a single, unique, hitherto undescribed rearrangement in *V. spilogaster*. This comprised an inversion after a fusion involving the ancestral microchromosome 12 (homologous to chicken microchromosome 12). We also determined that the low diploid number of *M. candidus* is related to microchromosome fusions. Woodpeckers thus exhibit significantly rearranged karyotypes compared to the putative ancestral karyotype (PAK).

Keywords: Cytogenetics; Bacterial Artificial Chromosomes; Rearrangements; Chromosome Evolution.

Introduction

The placement of the family Picidae (woodpeckers) in the bird phylogeny, along with its nearest relatives such as Indicatoridae and Capitonidae, is well-supported according previous to studies (Hackett et al. 2008; Jarvis et al. 2014; Prum et al. 2015). The specific evolutionary relationships however among various taxa within the family Picidae still lack clarity. For many years, several efforts have been devoted to elucidating the evolutionary relationships within the family Picidae; the study of Shakya et al. (2017) is an example. This family is typically categorized into three subfamilies. Among them, Jynginae, is suggested to be the closest relative to all other woodpeckers (Benz et al. 2006; De Filippis and Moore 2000; Dufort 2015; Webb and Moore 2005; Winkler et al. 2014). A second subfamily, Picumninae, consists of 29 species distributed among three genera: *Verreauxia*, *Sasia*, and *Picumnus* (Winkler and Christie 2002). The third subfamily, Picinae, otherwise known as conventional woodpeckers, comprises 176 species distributed among 29 genera. After some discussion and disagreements in previous studies, Picinae was divided into five tribes: Nesoctitini, Hemicercini, Campephilini, Picini, and Melanerpini (Dickinson and Remsen 2013; Dufort 2015). Despite recent advances in phylogenetic studies however, there are some uncertain relationships in the Picidae tree, especially in the subfamily Picumninae. Here, the genus *Picumnus* represents rare species with a localized distribution and many species have been omitted from molecular phylogenetic investigations. The task of establishing relationships in these birds is further complicated by significant instances of hybridization among these species (Dickinson and Remsen 2013; Dufort 2016; Shakya et al. 2017).

66 The family Picidae is highly diverse, containing more than 230 species distributed
67 around the world, playing a vital role in ecosystems and offering several ecological
68 benefits. These include serving as natural insect controllers, targeting wood-boring
69 insects that can harm trees and thereby contributing to overall forest health
70 (Winkler et al. 2014). Despite their association with decaying trees, the cavities
71 woodpeckers create become habitats for various wildlife, thus enhancing
72 biodiversity, and woodpeckers also aid in seed dispersal by consuming fruits and
73 berries. Monitoring their populations can provide insights into ecosystem health
74 (Robles and Pasinelli 2014; Bi et al. 2019; Wiley and Miller 2020) and they are
75 models of study in a range of fields, such as phylogeography, macroecology, and
76 biogeography. Furthermore, they are key models in the fields of anatomy and
77 physiology when investigating mechanisms that protect against head injury (May et
78 al. 1976; Farah et al. 2018; Smoliga and Wang, 2019).
79
80 From a cytogenetic point of view, woodpeckers show a wide variation in diploid
81 number (2n), from 64 in *Melanerpes candidus* (Picinae) (de Oliveira et al. 2017) to
82 more than 100 in species from the genus *Dendrocopos* (Shields 1982). They have an
83 enlarged Z (sex) chromosome, which is the largest element of the karyotype, unlike
84 in other birds, where it typically ranks as the fourth to sixth largest (Shields 1982;
85 Rutkowska et al. 2012; de Oliveira et al. 2017; Bertocchi et al. 2018). Woodpeckers
86 show morphological variety of the macrochromosomes when compared to the
87 putative avian ancestral karyotype (PAK). The microchromosomes, minute elements
88 nearly indistinguishable from one another from a cytogenetic point of view
89 (Kretschmer et al. 2018), are also poorly described in this group.

In species of the family Picidae, some pairs of macrochromosomes correspond to those observed in the PAK: The first two pairs observed in *Colaptes campestris*, *Colaptes melanochloros*, and *Melanerpes candidus* are submetacentric (de Oliveira et al. 2017), similar to that observed in *Gallus gallus* and in the PAK (Griffin et al. 2007). So far, 20 woodpeckers have been karyotyped (albeit partially, focussing on the macrochromosomes) and analysed by classical cytogenetics. Of these, only *Colaptes campestris*, *Colaptes melanochloros*, *Veniliornis spilogaster* and *Melanerpes candidus* have been analysed by molecular techniques such as FISH using microsatellites probes (de Oliveira et al. 2017; Bertocchi et al. 2018). Multiple inter- and intrachromosomal rearrangements are therefore likely but, hitherto, have been relatively undefined (de Oliveira et al. 2017).

Members of this family have interesting genomic features compared to other bird species. Regarding repetitive DNA content, the family Picidae shows the largest proportion of these elements in the genome, for instance, containing more than 25.8% in *Melanerpes aurifrons* (Zhang et al. 2014; Wiley and Miller 2020). These birds have also experienced the largest amount of DNA loss (424 Mb) over the past ~70 million years, resulting in a decrease of their genome size (Kapusta et al. 2019). Furthermore, they present an uncommon accumulation of repetitive sequences on the Z chromosome (de Oliveira et al. 2017; Bertocchi et al. 2018). Despite presenting with the same accumulation pattern in the Z sex chromosome, other representatives of the Piciformes, Toucans, present with a low amount of repetitive sequences in their genomic content compared to woodpeckers (Kretschmer et al. 2020).

The presence of microchromosomes is a universal feature in all bird species. With advances in genomic organization studies, it is now well established that these tiny chromosomes play important functions. They contain approximately 50% of the genes (McQueen et al. 1998; Smith et al. 2000; Habermann et al. 2001; Burt 2002; Waters et al. 2021) and have a recombination rate almost five times higher than mammalian chromosomes (Burt 2002). Additional analyses are needed in order to understand the functioning of these tiny elements and how they change in the context of genome organization and evolution (Graves and Shetty 2000; Ellegren 2010).

Reptile and bird micro- and macrochromosomes exhibit a remarkable degree of conservation in avian, turtle, and squamate lineages (Mengden and Stock 1980; Srikulnath et al. 2021; Waters et al. 2021; O'Connor et al. 2018, 2019). Current data analysis suggests that microchromosomes from the vertebrate ancestor display strong homology with specific bird macrochromosomal regions, reflecting paralogous sequences generated during early vertebrate evolution (O'Connor et al. 2019). Furthermore, microchromosomes retain a gene-rich content and low sequence repetition, potentially safeguarding them from rearrangements and repetitive element insertions; their longer subtelomeric regions, spatial isolation, and increased interaction of chromatin fibers may also play a part (Fulton et al. 2004, Warren et al. 2017, O'Connor et al. 2019). Consequently, avian microchromosomes represent remnants of the original building blocks of the vertebrate genome, distinguished by conserved features in reptilian and avian lineages (Waters et al.

2021). In contrast, mammalian genomes deviate from this pattern, undergoing extensive chromosomal rearrangements that result in diverse fusions and fissions.

Fluorescence *in-situ* hybridization (FISH) using whole chromosome painting (probes derived from flow-sorted individual chromosomes and microdissection) or bacterial artificial chromosomes (BACs) for individual genomic loci is a useful molecular tool to investigate chromosomal organization and evolution in birds and other species (Shetty et al. 1999; Guttenbach et al. 2003; Masabanda et al. 2003; Derjusheva et al. 2004; Griffin et al. 2007; Damas et al. 2017; O'Connor et al. 2024, among others). Such comparative cytogenomic analysis permits the identification of regions of homology that were likely present in the ancestral genome. It allows the exploration of interchromosomal (e.g. fusions, fissions, and translocations) and intrachromosomal rearrangements (e.g. inversions) during evolution (Griffin et al., 2007; Kretschmer et al., 2018; O'Connor et al. 2024). The application of reliable cross-species BAC-FISH based on conserved sequence-selected clones (Damas et al. 2018; O'Connor et al 2019) has permitted the identification of microchromosome rearrangements, which were historically limited by the paucity of suitable tools and protocols (O'Connor et al. 2024).

The notion that microchromosomal organization is highly conserved, with little in the way of chromosomal rearrangements (O'Connor et al. 2019; Waters et al. 2021; O'Connor et al. 2024) is supported by the study of nine bird orders (Lithgow et al. 2014; Damas et al. 2017; O'Connor et al. 2019; Kretschmer et al. 2021a). Only Falconiformes, Psittaciformes, Caprimulgiformes, Cuculiformes, Suliformes,

Charadriiformes, and a small proportion of Passeriformes have presented with chromosomal fusions involving them (Joseph et al. 2018; O'Connor et al. 2018; O'Connor et al. 2019; Kretschmer et al. 2021a; Kretschmer et al. 2021b; dos Santos et al. 2022).

Given the limited research on cross-species chromosome mapping in Piciformes, and none of them pertaining to microchromosome organization in Picidae members, this investigation aims to address this gap in our knowledge. The primary objective of this work was thus to identify microchromosome rearrangements involved in the karyotype evolution of four representative woodpecker species: *Melanerpes candidus* (Picinae), *Colaptes campestris* (Picinae), *Veniliornis spilogast*er (Picinae), and *Picumnus nebulosus* (Picumnidae). To achieve this, we performed BAC-FISH using chicken and zebra finch microchromosome probes in the selected woodpecker species, simultaneously describing the karyotype of *Picumnus nebulosus* for the first time.

Materials and Methods

Ethics Statements

All experiments followed protocols approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of the Pampa (010/2018) and Biodiversity Authorization and Information System (33860–1, 44173–1 and 61047-4). We followed the Guide for the Care and Use of laboratory Animals.

186 ***Bird samples***

187 Nine individuals belonging to distinct Picidae species (subfamilies Picinae and
188 Picumnidae) were sampled: one male of *Melanerpes candidus* (Melanerpini), one
189 female of *Colaptes campestris* (Picinae), three females and one male of *Veniliornis*
190 *spilogaster* (Melanerpini), and three males of *Picumnus nebulosus* (Picumninae).
191 Woodpeckers were collected with mist nets in their natural environment in two
192 cities: Porto Vera Cruz and São Gabriel, in South Brazil.

193

194 ***Animal care statement***

195 A euthanasia method a lethal dose of Ketamine 5% (300mg/kg)/ Xylazine 2% (50
196 mg/kg) was administered intravenously.

197

198 ***Chromosome harvesting***

199 Mitotic chromosomes were obtained by two protocols**:** fibroblast cell culture (Sasaki
200 et al. 1968) and bone marrow direct preparation (Garnero and Gunski 2000).
201 Fibroblast cell culture derived from skin biopsies was conducted through the
202 implementation of the subsequent procedures: cells were cultured in flasks ($25cm^2$)
203 with Dulbecco's Modified Eagle's medium (Sigma-Aldrich, MO, USA), enriched with
204 15% fetal bovine serum (GIBCO/Thermo Fisher Scientific, USA), antibiotics 1%
205 penicillin (10.000 units/mL) and streptomycin (10.000 μg/mL) (Sigma-Aldrich, St.
206 Louis, MO, USA), and Amphotericin B (2.50 μg/mL) (GIBCO/Thermo Fisher Scientific,
207 USA). Then, it was incubated at 37°C until the chromosome harvesting stage, where
208 the cells were exposed to colchicine 0.01% (Sigma-Aldrich, MO, USA) for 1h at 37°C,
209 and hypotonic (0.075M KCl) treatment for 15min at 37°C, followed by a fixation step

210 with methanol: acetic acid (3:1).

211

212 For direct preparation, we extracted the bone marrow in RPMI 1640 medium
213 (GIBCO/Thermo Fisher Scientific, USA) at 37°C with colchicine for 1h, followed by
214 hypotonic treatment with 0.075M KCl for 20min and methanol–acetic acid (3:1)
215 were used for cell fixation. All cells (direct and cultured) were immobilized on glass
216 slides for cytogenetic and FISH analysis.

217

218 ***Cytogenetics: Classical analyses***

219 Metaphases were stained with Giemsa 5% in a phosphate buffer at pH6.8. At least
220 20 metaphase spreads per individual were analyzed to confirm the chromosomal
221 morphology and diploid number; karyograms were assembled according to Guerra
222 (2004).

223

224 ***BAC-FISH experiments***

225 For the identification of chromosomal homologies, FISH analyses were performed
226 using 36 BAC probes (chromosomes 10-28, except chromosome 16) from chicken
227 (*Gallus gallus*, CHORI-261 Chicken BAC Library) or zebra finch (*Taeniopygia guttata,*
228 TGMCBA). We used two BACs per microchromosome, positioned as distantly as
229 possible on each chicken or zebra finch chromosome. The majority of BAC probes
230 utilized originated from chicken, however, for certain chromosomes, chicken BACs
231 proved ineffective across all bird species (Damas et al. 2017). In such instances, we
232 opted for BAC probes sourced from the zebra finch. We called the zebra finch BAC
233 probes by the name of their chicken homolog for clarity (please see supplementary

Table 1). BAC clone isolation, amplification, and labeling were performed according to the protocol previously described by O'Connor et al. (2018; 2019).

Potential microchromosome rearrangements such as fusions and fissions were detected by the following criteria: "conserved" if FISH signals of both BAC probes were observed on the same microchromosome with a compatible size regarding tiny element; "fusion" if a microchromosome probe hybridizes to a chromosome (macro or micro); and "fission" if both BAC probes show FISH signals on distinct sides of a macrochromosome or if it presents positive signals in more than one microchromosome pair (Fig. S1) (de Souza et al. 2022).

Results

The woodpeckers in this study presented the following chromosome numbers: 2n = 84 in *C. campestris* (Fig. 1A), 2n = 88 in *V. spilogaster* (Fig. 1B), 2n = 64 in *M. candidus* (Fig.1C), and 2n = 110 in *P. nebulosus* (Fig. 1D). From the 18th pair onwards, all remaining chromosomes showed telocentric morphology in *C. campestris*, *V. spilogaster*, and *M. candidus*. *P. nebulosus* presented acrocentric morphology from the 19th to the 30th chromosome pair, except in the 20th and 22nd pairs. From the 31st pair to the 54th, all chromosomes are telocentric. The Z chromosome presented with the largest size among the complement in all four woodpecker species. The whole set of BAC probes displayed hybridization signals in the four woodpecker species (Fig. 2).

257 Interchromosomal rearrangements involving microchromosomes were observed in
258 *C. campestris*, *V. spilogaster*, and *M*. *candidus*, as summarized in Table 1).
259 Specifically, in *C. campestris*, GGA14 was shown to be homologous to p-arm of
260 chromosome 2 (Fig. 2A), while no evidence of rearrangements was found in relation
261 to the remaining microchromosomes. *V. spilogaster* displayed three fusions between
262 macro and microchromosomes. The interchromosomal rearrangements involved a
263 fusion between the ancestral chromosome homolog to the GGA12 and the 2nd pair
264 of *V. spilogaster*, followed by an inversion (Fig. 3A-B). Additionally, GGA13 was
265 shown to be homologous to 1st pair of macrochromosomes (p arm), whereas GGA19
266 seems to be homologous to a pair of macrochromosomes (q arm), which could not
267 be identified (Fig. 2B). In addition to fusions between macro and
268 microchromosomes, *V. spilogaster* also showed fusions between the ancestral
269 chromosome homolog to GGA23 and a microchromosome pair. *M*. *candidus*
270 presented a total of 10 fusions, including one involving the ancestral chromosome
271 homolog to GGA13 and the p arm of the third largest chromosome pair (Fig. 2C). The
272 nine remaining fusions occurred between microchromosomes. In contrast, *P*.
273 *nebulosus* did not show any fusion involving these elements (Fig. 2D).
274
275 **Discussion**
276 The results of the present study, related to the diploid number and chromosomal
277 morphology of the species *C. campestris* and *M. candidus*, corroborate with those of
278 de Oliveira et al. (2017) and Bertocchi et al. (2018). We found a new chromosomal
279 number for *V. spilogaster,* however, which was previously described with 2n=80
280 (Bertocchi et al. 2018). In our study, *V. spilogaster* presented four additional

281 microchromosome pairs, totaling 88 chromosomes (44 pairs). This miscounting in
282 earlier studies could have happened due to the large number of microchromosomes
283 in this species and technical limitations. We have sampled four *V. spilogaster*
284 individuals, and all of them had 2n=88, however, FISH experiments were performed
285 in only one specimen. Thus, there does not seem to be an inter-populational
286 polymorphism in chromosome number in this species. Here, the karyotype of *P.*
287 *nebulosus* 2n=110 was described for the first time. This species presented many
288 pairs of acrocentric and telocentric chromosomes, only the Z chromosome was
289 metacentric. In addition, there is a reduction in the size of macrochromosomes in
290 comparison with the other three species. Woodpeckers thus have a wide variety of
291 chromosome numbers from as low as 64 up to more than 100 chromosomes, as in
292 the case of two species of the genus *Dendrocopos* (2n=108) (Shields 1982; de
293 Oliveira et al. 2017).
294
295 The Z chromosome is highly conserved throughout avian lineages, and this seemed
296 to be confirmed by comparative FISH mapping (Stiglec et al. 2007). This sex
297 chromosome usually ranges between fourth and sixth in size among all the
298 chromosomes, which is not the case in woodpeckers and other species of Piciformes
299 (Nanda et al. 2008; de Oliveira et al. 2017; Kretschmer et al. 2020; Kretschmer et al.
300 2021a). In our work, the Z chromosome of the four woodpeckers was the largest
301 element in the karyotype, an expected result, considering that this is the most
302 striking characteristic of the chromosomes of Piciformes. There are several possible
303 explanations why this chromosome has become the largest element of the
304 chromosomal complement, including the accumulation of repetitive sequences

and/or chromosomal rearrangements, such as macrochromosomal fissions or microchromosome fusions (de Oliveira et al. 2017; Kretschmer et al. 2020; Kretschmer et al. 2021a). The accumulation of repetitive sequences on the Z chromosome, despite being very uncommon in birds, has been reported in some species of the family Rhampastidae (Piciformes) that also showed macrochromosomal fissions (Kretschmer et al. 2020). Using chicken and zebra finch microchromosome probes in *C. campestris*, *V. spilogaster*, *M. candidus*, and *P. nebulosus*, it was possible to suggest there is no translocations observed and test the hypothesis where Z chromosome enlargement could have occurred through fusions between this chromosome and microchromosomes (from 10-28 pairs except 16). However, a fusion could have occurred with other microchromosome that were not examined in this study (GGA16, 29-38).

In contrast to previous analyses that demonstrated a high degree of microchromosome conservation in birds (O'Connor et al. 2019; Waters et al. 2021), equivalent cytogenomic studies on the metaphases of four species of woodpeckers showed that some of these elements are not conserved. Only the species *P. nebulosus* presented a conserved pattern, whereas the three remaining woodpecker species illustrated different rearrangements involving microchromosomes.

M. candidus, which has the lowest diploid number among the analyzed woodpecker species, showed a large number of fusions involving microchromosomes. Although rare in nature, rearrangements involving avian microchromosomes can occur, as described in seven bird orders (O'Connor et al. 2019; Kretschmer et al. 2021a;

329 Kretschmer et al. 2021b; de Souza et al. 2022; O'Connor et al. 2024). The low 2n is,
330 most likely, related to fusions involving macrochromosome or microchromosome as
331 observed in Falconiformes (Joseph et al. 2018; O'Connor et al. 2019). These present
332 as highly rearranged karyotypes with a relatively low number of chromosomes;
333 *Tolmomyias sulphurescens* 2n=60 (Passeriformes) is another example (Kretschmer et
334 al. 2021b).
335
336 Analyzing the karyotype of *C. campestris* we found only one fusion between the
337 ancestral microchromosome corresponding to GGA14 and the second largest
338 chromosome. No fusions of microchromosomes were observed in pairs 1 and 3, as
339 suggested by de Oliveira et al (2017). Therefore, the decrease in the diploid number
340 of this species in relation to the most ancestral species, *Colaptes auratus* (2n=90),
341 did not occur due to fusions of microchromosomes with the largest
342 macrochromosome pairs. It is important, however, to emphasize that it was not
343 possible to detect potential fusions between microchromosomes corresponding to
344 GGA16, GGA29-38 due to the absence of chromosomal probes for these pairs. In
345 addition, complementary analysis using probes of chicken macrochromosomes (1-9)
346 is indicated to find out if there are other rearrangements involving
347 macrochromosomes in this species, as suggested by Oliveira et al. (2017). These
348 authors proposed that the accumulation of interstitial telomeric sequences in the
349 centromeric regions of the first 3 pairs of macrochromosomes in *C. campestris* could
350 be an indicative of microchromosomal fusions.
351

352 *V. spilogaster* presented with an interchromosomal rearrangement that had not
353 previously been described - a fusion between an ancestral microchromosome
354 (GGA12 homologue) and a macrochromosome (VSP2), followed by an inversion. This
355 phenomenon was derived from observations of FISH experiments where the two
356 GGA12 BAC probes hybridized separately at the end of one of the largest
357 macrochromosomes. Chromosomal inversions comprise a more common type of
358 rearrangement that can act on the mechanisms of polymorphism, sex chromosome
359 evolution, supergene formation, and also on reproductive isolation (Hooper and
360 Price 2017). This type of rearrangement has been described in several bird species,
361 such as *Elaenia spectabilis* and two species of the genus *Turdus*, where it was
362 characterized as an apomorphy (Kretschmer et al. 2015; Kretschmer et al., 2014).
363 Although rare, fusions between microchromosomes and macrochromosomes have
364 been reported in some species, especially in Falconiformes, however,
365 microchromosomes remained intact after the fusion events and retain many of their
366 original properties (O'Connor et al. 2019). A possible explanation for this would be
367 the high percentage of microchromosome genes and the few breakpoints. Unlike
368 other birds, the family Picidae has a large number of repetitive sequences in their
369 genomes (Zhang et al. 2014), which could have facilitated the occurrence of this
370 unusual rearrangement. To summarize, *V. spilogaster* showed an atypical
371 chromosomal reorganization, evidencing the important role of rearrangement
372 mechanisms in the karyotypic evolution of this species. Another hypothesis for the
373 large amount of rearrangements found in the family Picidae is the accumulation of
374 repetitive sequences such as microsatellites and transposable elements, which could
375 have led to chromosomal modifications (Zhang et al. 2014; de Oliveira et al. 2017;

376 Bertocchi et al. 2018). Interesting evidence for these events is that Ramphastidae
377 species (Piciformes), although they are close to the woodpeckers, do not present
378 with any microchromosomal rearrangements, perhaps due to the lower proportion
379 of repetitive elements compared to woodpeckers (Kretschmer et al. 2020).
380
381 Among the interchromosomal rearrangements observed in woodpeckers, only the
382 fusion of ancestral microchromosome corresponding to GGA13 occurred in three of
383 the four species, suggesting that this event was present in the common ancestor.
384 Furthermore, GGA13 is more prone to undergo chromosomal rearrangements than
385 the other microchromosomes (Kretschmer et al. 2021a). This greater propensity for
386 rearrangements involving this microchromosome was also observed in species of the
387 orders Psittaciformes, Falconiformes, Passeriformes, Caprimulgiformes, and
388 Suliformes (Joseph et al. 2018; O'Connor et al. 2018; O'Connor et al. 2019;
389 Kretschmer et al. 2021b). In a study conducted by Waters et al. (2021), sequencing
390 and alignment of microchromosomes from birds, turtles, and humans revealed that
391 these chromosomes were the same across all bird and reptile species. Even more
392 surprisingly, they were identical to the small chromosomes of amphioxus, a spineless
393 fish-like animal that shared a common ancestor with vertebrates 684 million years
394 ago. In the present study, it was demonstrated that microchromosomes underwent
395 fusion followed by inversion, further highlighting that microchromosomes are
396 undergoing structural loss and generating new chromosomes in birds.
397
398 According to the phylogeny described by Shakya et al. (2017), *P. nebulosus* is the
399 most basal, followed by *C. campestris*, *V. spilogaster*, and *M. candidus* is the most

derived. Based on this information, we can observe that the family Picidae possibly had an ancestor with higher diploid number than the PAK (2n=80, Griffin et al., 2007), given that *Jynix torquila* (2n=90), a more basal species than *P. nebulosus*, also presents this characteristic. An example of a basal species that does not have a karyotype similar to the PAK (2n=80) is *Casuarius casuarius* (Struthioniformes), a Paleognathae bird with 92 chromosomes. One possible explanation for the high diploid number of this species would be the occurrence of fissions involving microchromosomes (Kretschmer et al. 2018; Kretschmer et al. 2020). However, in the species *P. nebulosus*, this type of rearrangement was not identified, suggesting that fissions of ancestral macrochromosomes may have occurred due to the high number of microchromosomes and the reduced size of macrochromosomes. Additionally, as seen in the phylogenetic tree (Fig. 5), it can be inferred that Piciformes ancestor possibly had a similar pattern of high diploid number, as also observed in the family Ramphastidae (Kretschmer et al. 2021a).

Our studies revealed distinct microchromosomal evolutionary histories among four woodpecker species: *C*. *campestris* exhibited a single rearrangement, *M. candidus* presented 10 chromosome fusions, while *P*. *nebulosus* showed no signs of any rearrangements. In contrast, *V*. *spilogaster* displayed four fusions, one of which involved the ancestral chromosome 12 (GGA12), followed by an inversion that disrupted the chromosomal region of the ancestral microchromosome. Our study thus provides the first evidence of a break in an avian microchromosome.

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Competing interests

The authors declare there are no competing interests.

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Data availability statement

Data is not available.

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568

569 **Tables**

570

571 Table 1 – Microchromosome organization patterns in four woodpecker species.

BAC Clones	Chromosome	*Colaptes campestris*	*Veniliornis spilogaster*	*Melanerpes candidus*	*Picumnu nebulosu*
CH261-115G24 and CH261-71G18	10	micro	micro	Fusion (Micro)	micro
CH261-154H1 and CH261-121N21	11	micro	micro	micro	micro
CH261-60P3 and CH261-4M5	12	micro	Fusion (Macro)	micro	micro
TGMCBA-321B13 and CH261-115I12	13	micro	Fusion (Macro)	Fusion (Macro)	micro
CH261-122H14 and CH261-69D20	14	Fusion (Macro)	micro	micro	micro
CH261-90P23 and TGMCBA-266G23	15	micro	micro	Fusion (Micro)	micro
TGMCBA-375I5 and CH261-42P16	17	micro	micro	micro	micro
CH261-60N6 and CH261-72B18	18	micro	micro	micro	micro

CH261-10F1 and CH261-50H12	19	micro	Fusion (Macro)	Fusion (Micro)	micro
TGMCBA-250E3 and TGMCBA-341F20	20	micro	micro	micro	micro
CH261-83I20 and CH261-122K8	21	micro	micro	Fusion (Micro)	micro
CH261-40J9 and CH261-18G17	22	micro	micro	Fusion (Micro)	micro
CH261-191G17 and CH261-90K11	23	micro	Fusion (Micro)	Fusion (Micro)	micro
CH261-103F4 and CH261-65O4	24	micro	micro	Fusion (Micro)	micro
CH261-59C21 and CH261-127K7	25	micro	micro	micro	micro
CH261-186M13 and CH261-170L23	26	micro	micro	micro	micro
CH261-66M16 and CH261-28L10	27	micro	micro	Fusion (Micro)	micro
CH261-72A10 and CH261-64A15	28	micro	micro	Fusion (Micro)	micro

BACs= Bacterial Artificial Chromosomes; GGA = *Gallus gallus*; Macro= Macrochromosomes; Micro= Microchromossomes.

Figure Captions

Figure 1 – Conventionally stained karyotypes (Giemsa 5%) of *Colaptes campestris* (A), *Veniliornis spilogaster* (B), *Melanerpes candidus* (C) and *Picumnus nebulosus* (D).

Figure 2 – BAC-FISH experiments in *Colaptes campestris* - CCA (A), *Veniliornis spilogaster* - VSP (B), *Melanerpes candidus* - MCA (C) and *Picumnus nebulosus* PNE (D). GGA14 69D20 FITC and 122H14 Texas Red (A); GGA19 50H12 FITC and 10F1 Texas Red (B); GGA13 115I12 FITC and 321B13 Texas Red (C); GGA12 4M5 FITC and 60P3 Texas Red (D); FITC= Fluorescein isothiocyanate. GGA = *Gallus gallus*.

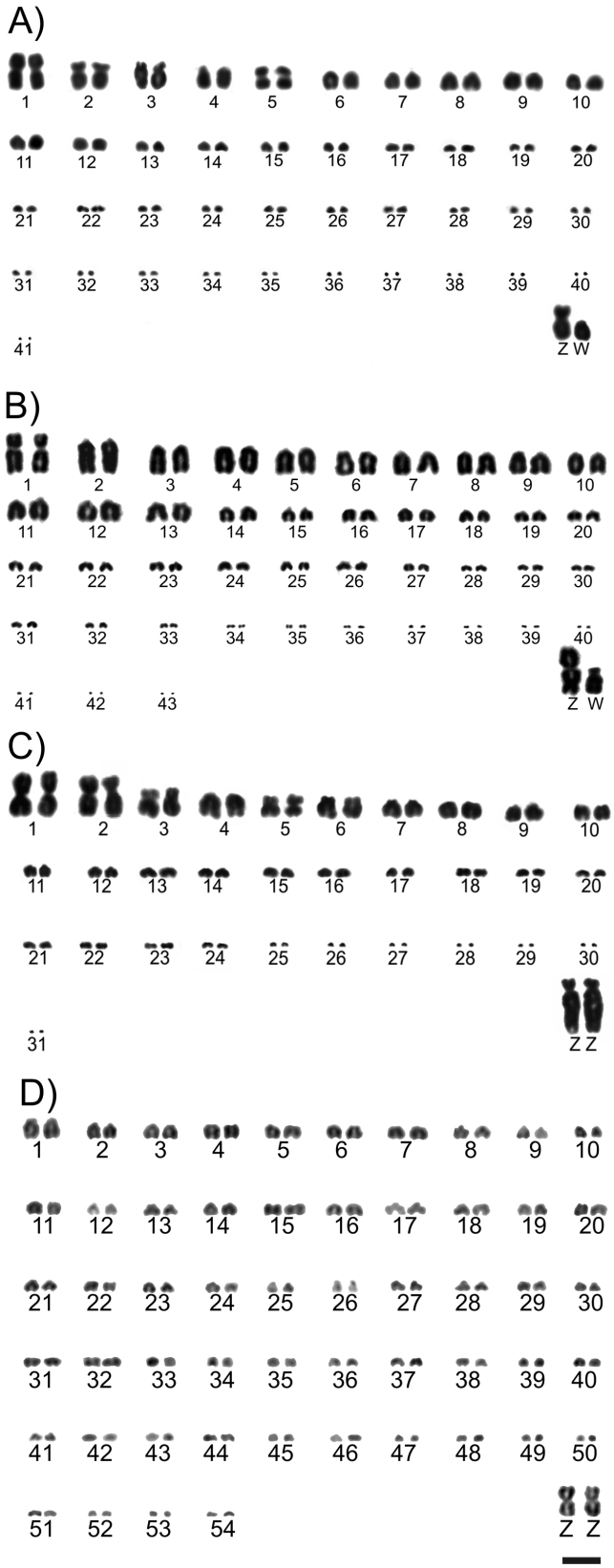
Figure 3 – BAC-FISH in *Veniliornis spilogaster* (VSP). Chromosomal rearrangment between chromosome 2 of VSP and the ancestral chromosome homolog to the chicken microchromosome 12 (GGA12 4M5 FITC and 60P3 Texas Red). FITC= Fluorescein isothiocyanate. GGA = *Gallus gallus*. Bar 5 μm.

Figure 4 – Hypothetical rearrangement occurred between chromosome 2 of *Veniliornis spilogaster* and homolog of microchromosome 12 of *Gallus gallus* (GGA12).

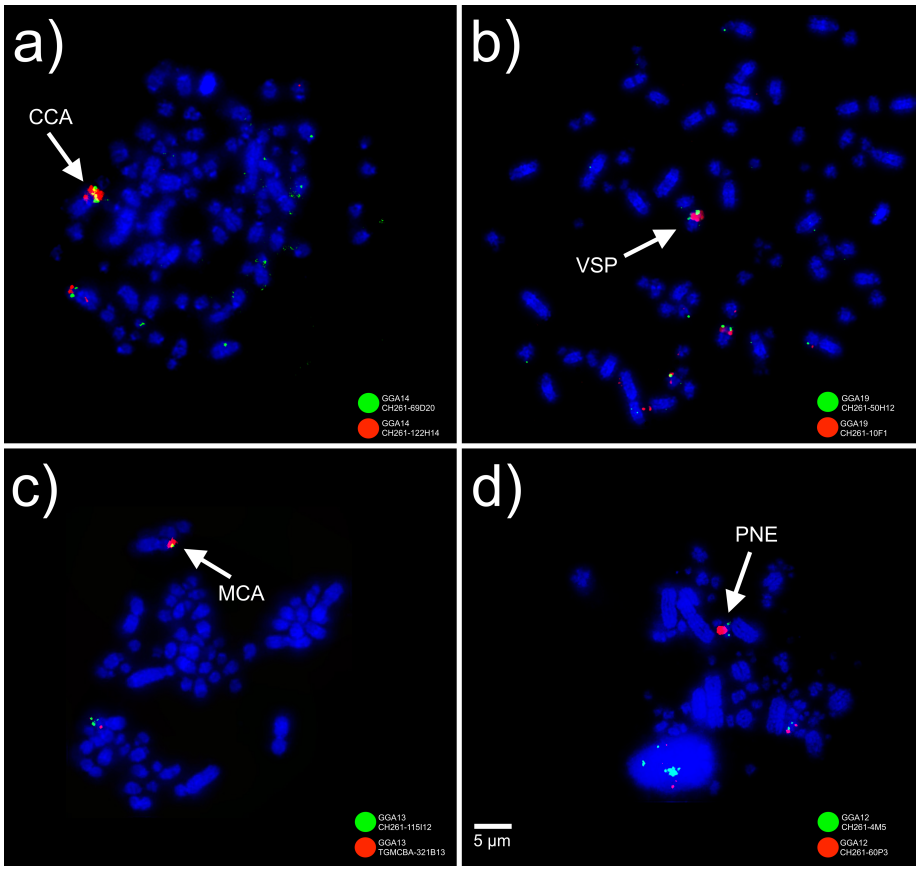
Figure 5 – Phylogeny showing diploid numbers in some species of Piciformes (Picidae and Ramphastidae). Chromosome numbers were obtained from Bird Chromosome Database and from the present paper (Degrandi et al. 2020). The phylogenetic tree was created by TimeTree using its databases (http://www.timetree.org, accessed on 11 Jun 2023).

Figures

Figure 1



608 Figure 2



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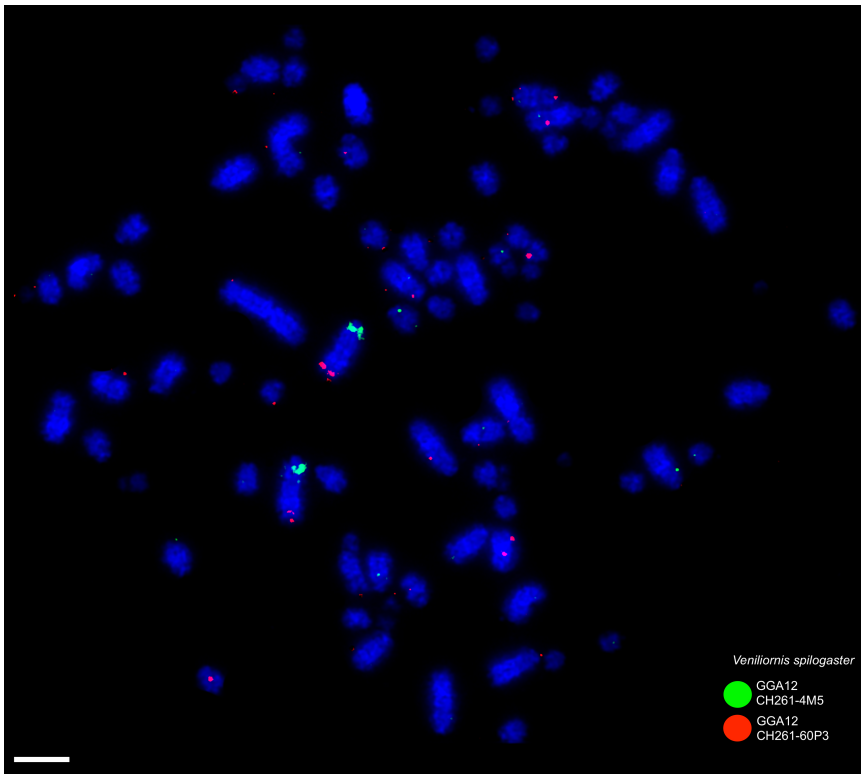
610 Figure 3



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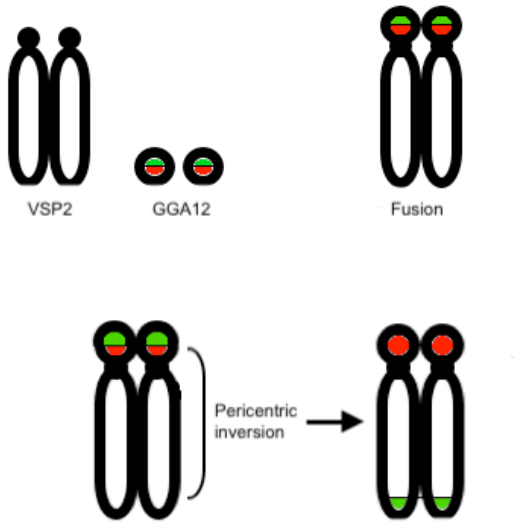
612 Figure 4



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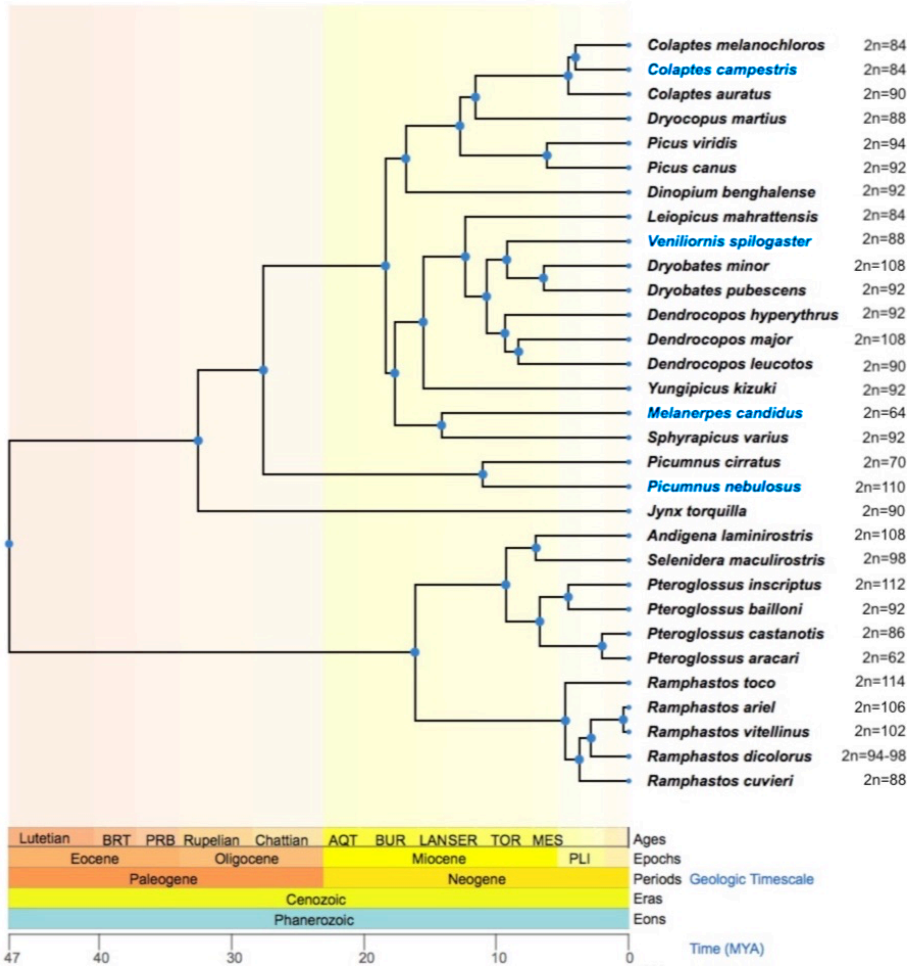
614 Figure 5



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