









Research Article
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The distribution of 45S rDNA sites in bird chromosomes suggests multiple evolutionary histories

Tiago Marafiga Degrandi¹ , Ricardo José Gunski² , Analía del Valle Garneró² , Edivaldo Herculano Correia de Oliveira^{3,4} , Rafael Kretschmer⁵ , Marcelo Santos de Souza² , Suziane Alves Barcellos²  and Iris Hass¹ 

¹Universidade Federal do Paraná (UFPR), Departamento de Genética, Curitiba, PR, Brazil.

²Universidade Federal do Pampa (UNIPAMPA), São Gabriel, RS, Brazil.

³Universidade Federal do Pará (UFPA), Belém, PA, Brazil.

⁴Instituto Evandro Chagas (IEC), Belém, PA, Brazil.

⁵Universidade Federal do Rio Grande do Sul (UFRGS), Instituto de Biociências, Porto Alegre, RS, Brazil.

Abstract

The distribution of 45S rDNA cluster in avian karyotypes varies in different aspects, such as position, number of bearer chromosomes, and bearers being macro- or microchromosomes. The present study investigated the patterns of variation in the 45S rDNA-bearer chromosomes of birds in order to understand the evolutionary dynamics of the cluster configuration and its contribution to the evolution of bird karyotypes. A total of 73 bird species were analyzed, including both published data and species for which rDNA-FISH was conducted for the first time. In most birds, the 45S rDNA clusters were located in a single pair of microchromosomes. Hence, the location of 45S rDNA in macrochromosomes, observed only in Neognathae species, seems to be a derived state, probably the result of chromosomal fusion between microchromosomes and distinct macrochromosomes. Additionally, the 45S rDNA was observed in multiple microchromosomes in different branches of the bird phylogeny, suggesting recurrence of dispersion processes, such as duplications and translocations. Overall, this study indicated that the redistribution of the 45S rDNA sites in bird chromosomes followed different evolutionary trajectories with respect to each lineage of the class Aves.

Keywords: FISH, chromosome, chromosome evolution, cytogenetics, Aves.

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Introduction

The rDNA genes are extremely important for cell function, given that they encode the rRNA involved in ribosome biogenesis (Hadjiolov, 1985; Shaw and Brown, 2012). In this process, two rDNA clusters are involved: the 45S rDNA composed by 18S, 5.8S, and 28S genes, and internal (ITS1 and ITS2) and external (5'ETS and 3'ETS) transcribed spacers; and the 5S rDNA, composed by a 5S gene separated by an intergenic spacer region (IGS) (Daniels and Delany, 2003; Dyomin *et al.*, 2016). In the eukaryotic genome, multiple copies of these clusters are organized in tandem in the DNA, forming the 5S and 45S rDNA sites in the chromosome (Daniels and Delany, 2003; Dyomin *et al.*, 2016).

Identification of chromosomes that bear 45S rDNA can be performed by the silver nitrate impregnation tech-

nique (Ag-NOR) (Howell and Black, 1980). However, this procedure only identifies the chromosomes with 45S rDNA sites in transitional activity, exhibiting intercellular, and interindividual variation (Zurita *et al.*, 1997). In this way, fluorescence *in situ* hybridization (FISH) experiments are more appropriate for this type of study, since they allow the precise identification of the bearing chromosomes when using probes for the genes that make up the rDNA cluster even when they are not active (O'Connor, 2008).

In recent years, FISH has been increasingly used to detect rDNA-bearer chromosomes in a range of vertebrate and invertebrate species (e.g., Roy *et al.*, 2005; Cazaux *et al.*, 2011; Mazzoleni *et al.*, 2018; Sochorová *et al.*, 2018). These studies have shown that 45S and 5S rDNA sites are most frequently found in a single chromosome pair per diploid genome, although considerable variation has been observed, with up to 74 chromosome copies for the 5S rDNA cluster sites and 54 for the 45S (Sochorová *et al.*, 2018). In addition, no significant correlation has been found between the number of 5S and 45S loci, which suggests that their

distribution and amplification within the karyotype follow independent evolutionary trajectories (Sochorová *et al.*, 2018).

The location of rDNA sites has been related to hotspots of chromosomal breakage (Cazaux *et al.*, 2011). This fragility is probably originated by the repetitive nature of clusters or their intense gene expression activity (Huang *et al.*, 2008). In the chromosome, these breakages may result in different types of rearrangements, such as translocation, fusions, duplications, and inversions, leading to rapid changes in the chromosomal distribution of the rDNA sites in closely related species (Datson and Murray, 2006; Degrandi *et al.*, 2014).

Birds are a highly diversified biological group with more than 10,000 species. On the other hand, less than 12% of the species have a known karyotype (Kretschmer *et al.*, 2018a). The diploid number ranges from $2n = 40$, as found in *Burhinus oedicephalus*, to $2n = 136-142$ in *Corythaixoides concolor* (Christidis, 1990; Nie *et al.*, 2009). However, the karyotype of birds is relatively conserved, and most species have $2n = 80$. Generally, their karyotypes are characterized by the presence of macrochromosomes, which are 2.5–6 μm in length, and microchromosomes, which are less than 2.5 μm long (Rodionov, 1996; Kretschmer *et al.*, 2018a). This basic karyotype structure can be seen in the species of both the Paleognathae and Neognathae clades (Kretschmer *et al.*, 2018a).

Studies that have mapped the chromosomal location of 45S rDNA sites have shown considerable divergence among birds (Nishida-Umehara *et al.*, 2007; Nishida *et al.*, 2008, 2013; Nie *et al.*, 2009; Tagliarini *et al.*, 2009; de Oliveira *et al.*, 2013; Kretschmer *et al.*, 2014; Degrandi *et al.*, 2017; de Oliveira *et al.*, 2017). In Paleognathae birds, the 45S rDNA is normally found in a single microchromosome pair (Nishida-Umehara *et al.*, 2007). However, in the Neognathae birds, a significant variation has been observed, including species with 45S rDNA clusters in multiple microchromosomes, in a single macrochromosome pair, or in both (Nishida *et al.*, 2008; de Oliveira *et al.*, 2013; Tagliarini, 2013; Degrandi *et al.*, 2017; de Oliveira *et al.*, 2017). However, the origin of this variation and its possible evolutionary implications are still poorly understood.

Thus, the aim of this study was to investigate this variation in 45S rDNA-bearing chromosomes of birds in order to understand the evolutionary dynamics of the cluster configuration and its contribution to the evolution of the bird karyotype.

Materials and Methods

Specimens

In this work, we analyzed the basic karyotype structure and distribution of the 45S rDNA sites in bird karyotypes. The following data were considered in each species: diploid number, number of 45S rDNA-bearing chromo-

somes, their type (macro- or microchromosome), and position of the clusters on the chromosome arm. First, the data were obtained from the literature, considering only the species in which the 45S rDNA clusters were identified by FISH-rDNA. Ag-NORs data were disregarded due to the intercellular and individual variations or possible false positive results, already reported in the literature.

Additionally, 29 species were selected from the sample bank of the Laboratory of Animal Genetic Diversity at Universidade Federal do Pampa for the first rDNA-FISH screening of each taxon: order Passeriformes/family Thraupidae: *Tachyphonus coronatus*, *Coryphospingus cucullatus*; Icteridae: *Agelaioides badius*, *Molothrus bonariensis*, Tyrannidae: *Pitangus sulphuratus*, *Myiarchus ferox*; Tityridae: *Schiffornis virescens*; Furnariidae: *Dendrocolaptes platyrostris*, *Anumbius annumbi*, *Synallaxis albescens*, *Furnarius rufus*, *Cranioleuca obsoleta*, *Syndactyla rufosuperciliata*; Coraciiformes/Alcedinidae: *Chloroceryle americana*; Piciformes/Ramphastidae: *Ramphastos tucanus*; Accipitriformes/Accipitridae: *Pseudastur albicollis*, *Buteogallus urubitinga*; Pelecaniformes/Ardeidae: *Syrigma sibilatrix*; Charadriiformes/Stercorariidae: *Stercorarius antarcticus*; Caprimulgiformes/Trochilidae: *Amazilia versicolor*, Nyctibiidae: *Nyctibius griseus*, Caprimulgidae: *Hydropsalis torquata*; Cuculiformes/Cuculidae: *Coccyzus melacoryphus*, *Piaya cayana*, *Guira guira*; Columbiformes/Columbidae: *Columbina talpacoti*; Tinamiformes/Tinamidae: *Nothura maculosa* and *Rhynchotus rufescens* (Table 1).

Chromosome preparation

Mitotic chromosomes were obtained following standard protocols, including direct preparation from bone marrow, fibroblast culture, and lymphocyte culture (Moorhead *et al.*, 1960; Sasaki *et al.*, 1968; Garner and Gunski, 2000).

FISH for 18S rDNA

FISH using probes specific for the 18S rDNA gene identified the 45S rDNA-bearing chromosomes. Primers were developed from sequences obtained from the fish *Hoplias malabaricus* (Cioffi *et al.*, 2009). This generated a fragment of approximately 1,400 base pairs, which was labeled by polymerase chain reaction (PCR), using the primers 18SF (5'CCGAGGACCTCACTAAACCA 3') and 18SR (5'CCGCTTTGGTGACTCTTGAT-3'), with fluorescein dUTP in the PCR mix.

The PCRs were run in a final volume of 25 μL containing 2 ng of genomic DNA from *H. malabaricus*, 0.2 μM of each primer (18SF and 18SR), 0.2 mM of dNTP, 10X buffer (1x), 50 mM of MgCl_2 (2 μM), 1 mM of Fluorescein-12-dUTP solution, 1 U/ μL of *Taq* polymerase, and sterile H_2O to complete to final volume. The thermal cycling parameters were 94 °C for 60 s, 30 cycles of 94 °C for 60 s, 60 °C for 60 s, 72 °C for 90 s, followed by an elongation step of 5 min at 72 °C (Cioffi *et al.*, 2009).

Table 1 - Distribution of 45S rDNA clusters in bird karyotypes.

| Infraclass/ order | Family | Species | 2n | N° | Type of chromosome | Position | Reference |
|-------------------|----------------|-------------------------------------|-------|----------|--|----------|-------------------------------------|
| Neognathae | | | | | | | |
| Passeriformes | Turdidae | <i>Turdus rufiventris</i> | 78 | 6 | Micro | NA | Kretschmer <i>et al.</i> , 2014 |
| Oscines | | <i>Turdus albicollis</i> | 78 | 4 | Micro | NA | Kretschmer <i>et al.</i> , 2014 |
| | Thraupidae | <i>Saltator similis</i> | 80 | 2 | Micro | NA | dos Santos <i>et al.</i> , 2015 |
| | | <i>Saltator aurantirostris</i> | 80 | 2 | Micro | NA | dos Santos <i>et al.</i> , 2015 |
| | | <i>Tachyphonus coronatus*</i> | 80 | 2 | Micro | NA | Present study |
| | | <i>Coryphospingus cucullatus</i> | 80 | 2 | Micro | NA | Present study |
| | Icteridae | <i>Agelaioides badius</i> | 80 | 4 | Micro | NA | Present study |
| | | <i>Molothrus bonariensis</i> | 80 | 2 | Micro | NA | Present study |
| | Fringillidae | <i>Serinus canaria</i> | 80 | 4 | Micro | NA | dos Santos <i>et al.</i> , 2017 |
| | Parulidae | <i>Basileuterus culicivorus</i> | 80 | 2 | Micro | NA | Present study |
| | Estrildidae | <i>Taeniopygia guttata</i> | 80 | 2 | Micro | NA | dos Santos <i>et al.</i> , 2017 |
| | | <i>Elaenia spectabilis</i> | 80 | 4 | Micro | NA | Kretschmer <i>et al.</i> , 2015 |
| Passeriformes | Tyrannidae | <i>Pitangus sulphuratus</i> | 78 | 2 | Micro | NA | Present study |
| Suboscines | | <i>Myiarchus ferox</i> | 76 | 2 | Micro | NA | Present study |
| | Tityridae | <i>Schiffornis virescens</i> | 82 | 2 | Micro | NA | Present study |
| | Furnariidae | <i>Dendrocolaptes platyrostris*</i> | 82 | 2 | Macro, 1 th | P | Present study |
| | | <i>Anumbius annumbi</i> | 82 | 2 | Micro | NA | Present study |
| | | <i>Synallaxis albescens</i> | 82 | 2 | Micro | NA | Present study |
| | | <i>Furnarius rufus*</i> | 82 | 2 | Micro | NA | Present study |
| | | <i>Cranioleuca obsoleta</i> | 82 | 2 | Micro | NA | Present study |
| | | <i>Syndactyla rufosuperciliata</i> | 82 | 2 | Micro | NA | Present study |
| Psittaciformes | | <i>Psittacus erithacus</i> | 62-64 | 8 | Micro | NA | Seibold-Torres <i>et al.</i> , 2015 |
| Falconiformes | Falconidae | <i>Falco tinnunculus</i> | 52 | 4 | Micro | NA | Nishida <i>et al.</i> , 2008 |
| | | <i>Falco peregrinus</i> | 50 | 12 or 14 | Micro | NA | Nishida <i>et al.</i> , 2008 |
| | | <i>Falco columbarius</i> | 40 | 9 | Micro | NA | Nishida <i>et al.</i> , 2008 |
| Coraciiformes | Alcedinidae | <i>Chloroceryle americana</i> | 94 | 2 | Micro | NA | Present study |
| Piciformes | Picidae | <i>Colaptes campestris</i> | 84 | 2 | Macro, 13 th | I | de Oliveira <i>et al.</i> , 2017 |
| | | <i>Colaptes melanochloros</i> | 84 | 2 | Macro, 13 th | I | de Oliveira <i>et al.</i> , 2017 |
| | | <i>Melanerpes candidus</i> | 64 | 2 | Micro | NA | de Oliveira <i>et al.</i> , 2017 |
| | Ramphastidae | <i>Ramphastos tucanus*</i> | 112 | 2 | Micro | NA | Present study |
| Trogoniformes | Trogonidae | <i>Trogon s. surrucura</i> | 82 | 6 | Micro | NA | Degrandi <i>et al.</i> , 2017 |
| Accipitriformes | Pandionidae | <i>Pandion haliaetus</i> | 74 | 2 | Macro, 2 th | P, q | Nishida <i>et al.</i> , 2014 |
| Eagles | Accipitridae | <i>Pseudastur albicollis</i> | 66 | 2 | Macro, 8 th | P, q | Present study |
| | | <i>Buteogallus urubitinga*</i> | 68 | 2 | Macro, 8 th | P, q | Present study |
| | | <i>Buteo nitidus</i> | 68 | 2 | Macro, 8 th | P, q | de Oliveira <i>et al.</i> , 2013) |
| | | <i>Rupornis magnirostris</i> | 68 | 2 | Macro, 8 th | P, q | de Oliveira <i>et al.</i> , 2013 |
| | | <i>Buteogallus meridionalis</i> | 68 | 2 | Macro, 8 th | P, q | de Oliveira <i>et al.</i> , 2013 |
| | | <i>Harpia harpyja</i> | 58 | 4 | Macro, 6 th and Micro, 25 th | S | Tagliarini, 2013 |
| | | <i>Morphnus guianensis</i> | 82 | 2 | Macro, 1 th | S | Tagliarini, 2013 |
| | | <i>Nisaetus n. orientalis</i> | 66 | 2 | Micro, 29 th | NA | Nishida <i>et al.</i> , 2013 |
| Accipitriformes | Cathartidae | <i>Sarcoramphus papa</i> | 80 | 2 | Micro | NA | Tagliarini <i>et al.</i> , 2009 |
| Vultures | | <i>Cathartes burrovianus</i> | 80 | 2 | Micro | NA | Tagliarini <i>et al.</i> , 2009 |
| | | <i>Cathartes aura</i> | 80 | 2 | Micro | NA | Tagliarini <i>et al.</i> , 2009 |
| | | <i>Gymnogyps californianus</i> | 80 | 2 | Micro | NA | Raudsepp <i>et al.</i> , 2002 |
| Pelecaniformes | Ardeidae | <i>Syrigma sibilatrix*</i> | 62 | 2 | Micro | NA | Present study |
| Charadriiformes | Stercorariidae | <i>Stercorarius antarcticus</i> | 84 | 2 | Micro | NA | Present study |
| | Burhinidae | <i>Burhinus oediconemus</i> | 42 | 2 | Macro, 13 th | I | Nie <i>et al.</i> , 2009 |
| Caprimulgiformes | Trochilidae | <i>Amazilia versicolor</i> | 82 | 2 | Micro | NA | Present study |
| Hummingbirds | | | | | | | |
| Caprimulgiformes | Nyctibiidae | <i>Nyctibius griseus</i> | 86 | 2 | Micro | NA | Present study |
| Nighthjars | Caprimulgidae | <i>Hydropsalis torquata</i> | 74 | 2 | Micro | NA | Present study |

Table 1 (cont.)

| Infraclass/ order | Family | Species | 2n | N° | Type of chromosome | Position | Reference |
|---------------------|---------------|---------------------------------|----|----|-------------------------|----------|---|
| Cuculiformes | Cuculidae | <i>Coccyzus melacoryphus</i> | - | 2 | Micro | NA | Present study |
| | | <i>Piaya cayana</i> | 88 | 2 | Macro 7 th | P, p | Present study |
| | | <i>Guira guira</i> | 76 | 2 | Macro, 6 th | P, q | Present study |
| Columbiformes | Columbidae | <i>Columbina talpacoti</i> | 76 | 2 | Micro | NA | Present study, Kretschmer <i>et al.</i> , 2018b |
| | | <i>Zenaida auriculata</i> | 76 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Geotrygon montana</i> | 86 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Geotrygon violacea</i> | 86 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Leptotila verreauxi</i> | 78 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Patagioenas cayennensis</i> | 76 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Columba livia</i> | 80 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Columbina passerina</i> | 76 | 2 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| | | <i>Columbina picui</i> | 76 | 6 | Micro | NA | Kretschmer <i>et al.</i> , 2018b |
| Galliformes | Phasianidae | <i>Coturnix japonica</i> | 78 | 6 | Micro | NA | McPherson <i>et al.</i> , 2014 |
| | | <i>Meleagris gallopavo</i> | 80 | 2 | Micro, 18 th | NA | McPherson <i>et al.</i> , 2014 |
| | | <i>Gallus gallus</i> | 78 | 2 | Micro, 16 th | NA | Dyomin <i>et al.</i> , 2016 |
| Paleognathae | | | | | | | |
| Tinamiformes | Tinamidae | <i>Nothura maculosa</i> | 78 | 4 | Micro | NA | Present study |
| | | <i>Eudromia elegans</i> | 80 | 4 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |
| | | <i>Rhynchotus rufescens</i> | 78 | 2 | Micro | NA | Present study |
| Casuariiformes | Dromaiidae | <i>Dromaius novaehollandiae</i> | 80 | 2 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |
| | Casuariidae | <i>Casuaris casuarius</i> | 92 | 2 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |
| Struthioniformes | Struthionidae | <i>Struthio camelus</i> | 80 | 2 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |
| Rheiformes | Rheidae | <i>Rhea pennata</i> | 80 | 2 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |
| | | <i>Rhea americana</i> | 80 | 2 | Micro | NA | Nishida-Umehara <i>et al.</i> , 2007 |

2n = diploid number; N° = Number of 45S rDNA-bearing chromosomes; Type of chromosome: Macro = Macrochromosome; Micro = Microchromosome; Nomenclature for the position in the chromosome: I= Interstitial; S = subtelomeric; P = Pericentromeric; Arm location: Short arm = p; Long arm = q; NA = Not applicable; *Shown in the fig. 1; Species names are in accordance with Gill and Donsker (2018).

For the FISH procedures, slides with metaphases were treated with RNase A (10 µg/mL) for 20 min and then denatured in 70% formamide at 70°C for 80 s. Subsequently, 300 ng of the 18S probe were added to each slide, which was then sealed with a cover slip and incubated overnight at 37 °C (Daniels and Delany, 2003). The slides were then washed in 50% formamide at 42 °C for 1 min (x2), 2xSSC at 40 °C for 2.5 min (x2), and once in 4xSSC Tween (1X) at room temperature. The chromosomes were counterstained with DAPI. Hybridization results were analyzed using a Zeiss Axioplan2 fluorescence microscope.

Chromosomal analyses

The diploid number of each specimen was determined from the analysis of approximately 30 mitotic cells stained with Giemsa observed under an optical microscope. Variation in the number of rDNA clusters was evaluated based on the number of chromosomes that presented a fluorescent sig-

nal. The rDNA cluster-bearing chromosomes were classified as either macrochromosomes or microchromosomes, according to their length (Rodionov, 1996). The position of the 45S rDNA cluster was classified as: (i) pericentromeric (adjacent to the centromere), (ii) subtelomeric (adjacent to the telomere), and (iii) interstitial (between the centromere and the telomere) (Cazaux *et al.*, 2011). Ideograms were created using these characteristics to represent the rDNA-bearing chromosomes in each species.

Phylogenetic comparison

The species were compared using the phylogenetic relationships proposed by Jarvis *et al.* (2014) and Prum *et al.* (2015). In this step, the chromosomal locations of the 45S rDNA clusters were plotted in a modified phylogenetic tree of Jarvis *et al.* (2014). In this tree, we used the Mesquite software to exclude groups of birds for which rDNA location data were not available. We considered the pres-

ence of 45S rDNA in a single pair of microchromosomes as an ancestral condition for birds, according to the hypothesis of Nishida-Umehara *et al.* (2007). Based on this hypothesis, we analyzed the evolutionary relationships and the probable chromosomal rearrangements that would explain the variations observed in chromosomes carrying 45S rDNA.

Results

The number of chromosomes ($2n$), number of 45S rDNA sites, and the characteristics of these bearing chromosomes from 29 selected species for rDNA-FISH screening in this work are shown in Table 1 (see species identified as 'present study' in Table 1). The rDNA-FISH results of some selected species are shown in Figure 1.

Overall, the analysis of the chromosomal distribution of the 45S rDNA included 73 bird species, representing 17 orders of the class Aves (Table 1). Eight of these species were Paleognaths, representing four orders, the Casuariiformes, Rheiformes, Struthioniformes, and Tinamiformes. The other 65 species were Neognaths, belonging to 13 orders, the Accipitriformes, Caprimulgiformes, Charadriiformes, Columbiformes, Coraciiformes, Cuculiformes, Falconiformes, Galliformes, Passeriformes, Pelecaniformes, Piciformes, Psittaciformes, and Trogoniformes.

Variation in the diploid number in birds

Considering only the bird species for which the location of 45S rDNA sites is available (73), diploid numbers ranged from $2n = 40$ to $2n = 112$ (Table 1). Despite this ample variation, most (38) of the species had diploid numbers between 78 and 82, and 21 were $2n = 80$ (Figure 2A). While

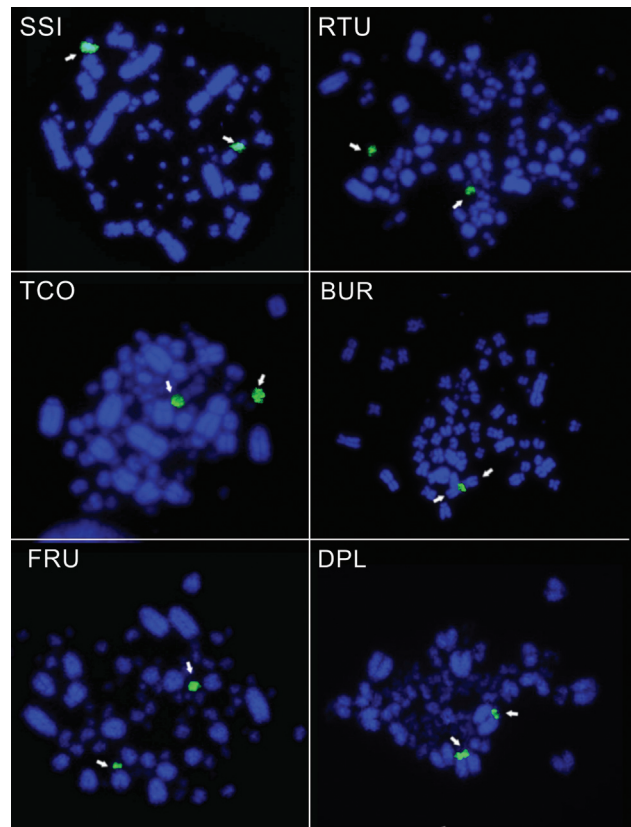


Figure 1 - Examples of the metaphases analyzed in the present study using the 18S rDNA probe (green) to identify the chromosomes (blue) carrying 45S rDNA sites (arrows). The acronym shown in the upper right corner of each metaphase indicates the species: *Syrrigma sibilatrix* (SSI), *Ramphastos tucanus* (RTU), *Tachyphonus coronatus* (TCO), *Buteogallus urubitinga* (BUR), *Furnarius rufus* (FRU), and *Dendrocolaptes platyrostris* (DPL).

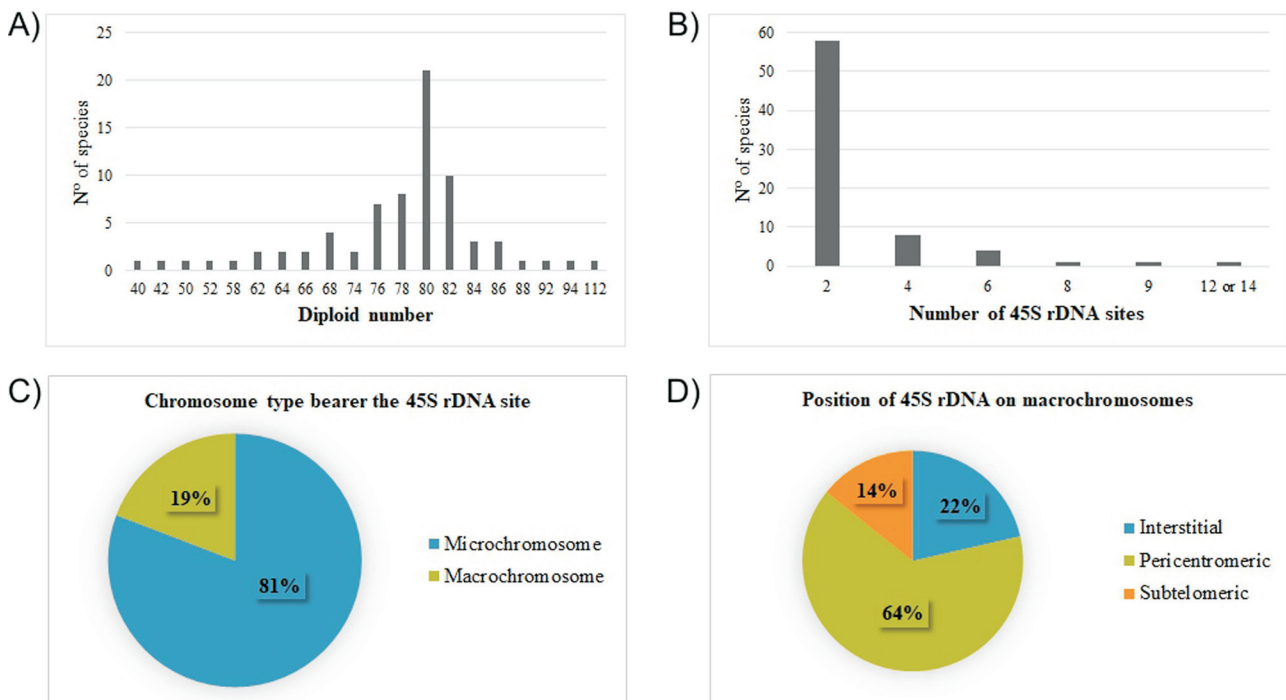


Figure 2 - Chromosomal location of the 45S rDNA sites in all 73 bird species analyzed in the present study. (A) variation in the diploid number; (B) variation in the number of 45S rDNA bearer chromosomes; (C) the proportion of the species with 45S rDNA located in macrochromosomes or microchromosome; (D) location of the 45S rDNA cluster in the chromosome arm.

the Paleognathae species were relatively conserved, with most species having around 80 chromosomes, higher variability in 2n was observed in Neognathae (Table 1).

Number of 45S rDNA sites

The analysis of the number of 45S rDNA-bearing chromosomes highlighted that most (58) species had a cluster in a single chromosome pair (Figure 2B). In the Paleognathae, *Nothura maculosa* and *Eudromia elegans* were exceptions, with two rDNA-bearing chromosome pairs. In the Neognathae, the 45S rDNA clusters were found in a single chromosome pair and in up to six or seven pairs (Table 1).

Types of rDNA-bearing chromosomes

In the bimodal analysis of macrochromosomes vs. microchromosomes, the 45S rDNA sites of most (59) species were observed on microchromosomes (Figure 2C). In the Paleognathae, the rDNA was located exclusively on microchromosomes. The Neognathae presented different configurations, by contrast, with some species having the cluster in the microchromosomes, others in the macrochromosomes, and some in both types of chromosome, as observed in the Accipitriformes, *Harpia harpyja* (Table 1).

The location of the rDNA in macrochromosomes was observed in 14 Neognathae species (Table 1), representing a number of different orders: *Pandion haliaetus*, *Pseudastur albicollis*, *Buteogallus urubitinga*, *Buteo nitidus*, *Rupornis magnirostris*, *B. meridionalis*, *H. harpyja* and *Morphnus guianensis* (Accipitriformes), *Burhinus oedincnemus* (Charadriiformes), *Piaya cayana* and *Guira guira* (Cuculiformes), *Dendrocolaptes platyrostris* (Passeriformes), *Colaptes campestris*, and *Colaptes melanochloros* (Piciformes). In some cases, it was possible to identify homologies between the macrochromosomes and those of *Gallus gallus* (Table 2).

Position of the 45S rDNA site in the chromosomes

As microchromosomes have a limited resolution, the species with rDNA sites in these tiny elements were excluded from the analysis of the rDNA topology in the chromosomes in order to avoid biases in data interpretation. Therefore, the position of the rDNA cluster was analyzed only in the 14 species in which the 45S rDNA is located in macrochromosomes.

The 45S rDNA was observed in a pericentromeric position in most (64%) cases, that is, in *P. haliaetus*, *P. albicollis*, *B. urubitinga*, *B. nitidus*, *R. magnirostris*, *B. meridionalis* (Accipitriformes), *G. guira*, *P. cayana* (Cuculiformes), and *D. platyrostris* (Passeriformes). The interstitial position was the second most frequent, being observed in 22% of the species, *B. oedincnemus* (Charadriiformes), *C. campestris*, and *C. melanochloros* (Piciformes). Finally, a subtelomeric position was recorded in two (14%) species, *M. guianensis* and *H. harpyja* (Accipitriformes) (Figure 2D, Table 1).

Phylogenetic comparisons

For the phylogenetic comparisons, the presence of the 45S rDNA cluster in a single pair of microchromosomes was considered to be the ancestral condition, based on the hypothesis of Nishida-Umehara *et al.* (2007). This analysis revealed that the variation in the number of 45S rDNA-bearer chromosomes was independent of the phylogenetic relationships among the species (Figure 3). The presence of rDNA in macrochromosomes was observed in species belonging to different orders from infra class Neognathae (Figure 3).

Discussion

Here we present for the first time a broad analysis of the distribution of 45S rDNA in avian karyotype. Although an impressive variation was observed in the chromosomes carrying the 45S rDNA cluster, we recorded that in most

Table 2 - Associations of 45S rDNA sites with macrochromosomes and their respective homologies with *Gallus gallus* (GGA) chromosomes.

| Order | Species | 45S rDNA chromosome location | Homologous GGA segment * | Reference |
|-----------------|---------------------------------|--------------------------------------|--------------------------|----------------------------------|
| Accipitriformes | <i>Pandion haliaetus</i> | 2 th | GGA1 | Nishida <i>et al.</i> , 2014 |
| | <i>Harpia harpyja</i> | 6 th and 25 th | GGA1 | Tagliarini, 2013 |
| | <i>Morphnus guianensis</i> | 1 th | GGA3 | Tagliarini, 2013 |
| | <i>Pseudastur albicollis</i> | 8 th | GGA7 | de Oliveira <i>et al.</i> , 2010 |
| | <i>Buteo nitidus</i> | 8 th | GGA7 | de Oliveira <i>et al.</i> , 2013 |
| | <i>Rupornis magnirostris</i> | 8 th | GGA7 | de Oliveira <i>et al.</i> , 2013 |
| | <i>Buteogallus meridionalis</i> | 8 th | GGA7 | de Oliveira <i>et al.</i> , 2013 |
| Charadriiformes | <i>Burhinus oedincnemus</i> | 13 th | 2 Micro | Nie <i>et al.</i> , 2009 |
| Cuculiformes | <i>Piaya cayana</i> | 7 th | GGA2 | Unpublished data |
| | <i>Guira guira</i> | 6 th | GGA2 | Unpublished data |

*Homologies established by chromosome painting; Micro: Microchromosome.

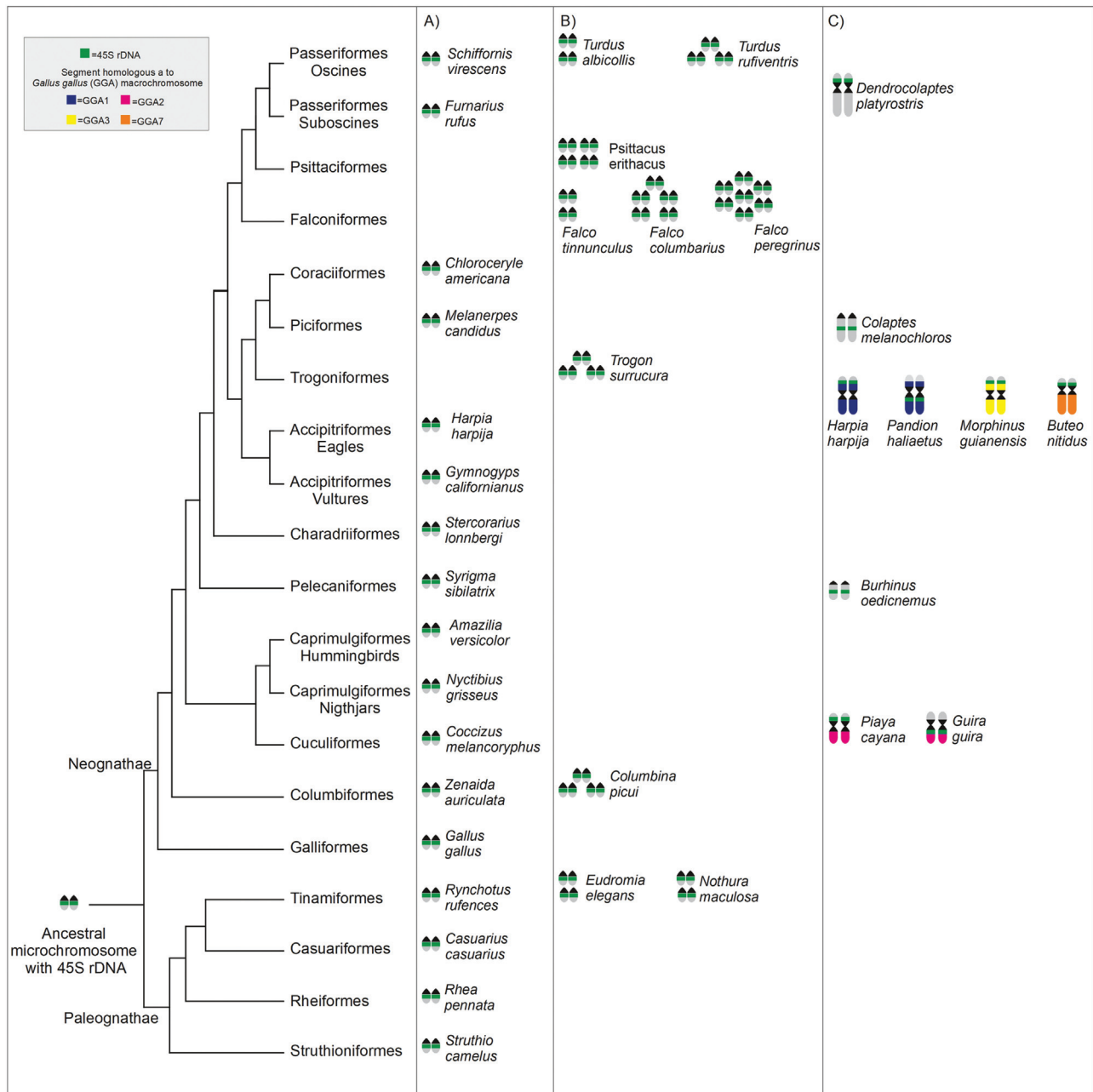


Figure 3 - Phylogenetic relationships among the birds modified from Jarvis *et al.*, 2014. The data of chromosomal location of the 45S rDNA from species analyzed in the present study were plotted in the tree. (A) Species with rDNA located only in a microchromosome pair; (B) species with rDNA in multiples microchromosomes; (C) species in which the rDNA is located in macrochromosomes. The complete data are shown in Table 1.

species it is located in a single pair of microchromosomes. Interestingly, most of these species have a karyotype with $2n = 80$ chromosomes (Figure 2A).

A study with rodents indicated that there is no relationship between the $2n$ and the number of 45S rDNA cluster bearing chromosomes (Cazaux *et al.*, 2011). Nevertheless, birds with $2n = 80$ chromosomes that carry only a single pair of 45S rDNA microchromosomes seem to reflect the karyotype conservation status of these species in relation to the ancestral karyotype of birds (PAK), as pro-

posed by Griffin *et al.* (2007). This karyotype uniformity of birds has also been observed in species from Paleognathae and Neognathae using the GGA whole chromosome paint (Kretschmer *et al.*, 2018a).

The presence of a single pair of microchromosomes with 45S rDNA conserved among the species of Paleognathae (*Dromaius novaehollandiae*, *Casuarus casuaris*, *Struthio camelus*, *Rhea pennata*, and *Rhea americana*) suggests that this would be an ancestral condition of rDNA (Nishida-Umehara *et al.*, 2007). Using the phylogenetic re-

relationships proposed by Jarvis *et al.* (2014) and Prum *et al.* (2015), we compared these data and identified that several species of the Neognaths infraclass preserve the 45S rDNA in a pair of microchromosome (Figure 3), a fact that reinforces the hypothesis of PAK ancestral condition (Griffin *et al.*, 2007).

The 45S rDNA-bearer chromosome is related to the presence or absence of the process of karyotypic diversification. For example, Accipitriformes, where species of the Cathartidae family have karyotypes with 80 chromosomes, the 45S rDNA was located in only a single pair of microchromosomes (Raudsepp *et al.*, 2002; Tagliarini *et al.*, 2009). In contrast, the Accipitridae family shows a diploid number quite derived ($2n = 58-82$), and chromosome painting evidenced an extensive karyotypic reorganization, originated by breaks and fusions of macrochromosomes (GGA) and microchromosomes. In this group, it was observed that 45S rDNA is associated with different macrochromosomes (Table 2) (de Oliveira *et al.*, 2013, Tagliarini, 2013; Nishida *et al.*, 2014).

45S rDNA in multiple microchromosomes

Multiple microchromosomes carrying 45S rDNA can be found in some species of the orders Tinamiformes, Columbiformes, Trogoniformes, and Falconiformes, and notably, even phylogenetically related species may differ in the number of rDNA bearing chromosomes. For instance, Paleognath birds from the order Tinamiformes show variation in the number of clusters. In *R. rufescens* a single microchromosome pair containing the 45S rDNA was observed, whereas in *N. maculosa* and *E. elegans*, the 45S rDNA is located in two pairs of microchromosomes (Figure 3). Similarly, such numerical variation is also seen in species of the same genus, as in the genus *Falco* (Falconiformes), where *F. tinnunculus* has 45S rDNA in four microchromosome pairs, *F. columbarius* in five pairs, and *F. peregrinus* shows this cluster in six or seven pairs (Nishida *et al.*, 2008) (Figure 3). Considering the phylogenetic relationships between these orders, the most plausible explanation for the origin of these variation are recurrent processes of 45S rDNA cluster duplications or translocations, resulting in the numerical variation observed in these species.

45S rDNA distribution in macrochromosomes

The 45S rDNA location in macrochromosomes can be considered a derived characteristic in birds (Kretschmer *et al.*, 2018a). The available data on chromosomal homologies with *G. gallus* (GGA) (Table 2), demonstrated that the rDNA sites are clearly associated with distinct macrochromosomes. This scenario might have been originated by multiple independent events of chromosomal fusion, which are supported by several different types of evidence.

In Accipitriformes, for example, multiple associations were recorded, including GGA1, GGA3, and GGA7.

In *B. nitidus*, *R. magnirostris*, and *B. meridionalis*, an association with the homologous GGA7 segment was found, although the short arm of the chromosome pair containing the rDNA of these species was not hybridized by any of the GGA probes used (de Oliveira *et al.*, 2013). This unhybridized region probably corresponds to the homologous of the ancestral microchromosome containing the rDNA, reinforcing the fusion hypothesis. Similarly, in *P. haliaetus*, the rDNA located on the q-arm of chromosome 2 was associated with the homologous GGA1 segment (Nishida *et al.*, 2014). In this species, the short arm did not hybridize by any GGA probe. However, *P. haliaetus* showed rDNA in the long arm, suggesting that a pericentric inversion should have occurred after fusion with the 45S rDNA microchromosome, shifting the cluster position to the long arm.

45S rDNA related to intrachromosomal rearrangements

Intrachromosomal rearrangements have been reported in bird karyotypes, and our data revealed that two cases involved the 45S rDNA-bearer chromosome (Degrandi *et al.*, 2017). For example, in Cuculiformes, *Piaya caiana* and *Guira guira* showed the association of 45S rDNA with a segment homologue to chromosome GGA2 (Table 2). In *P. caiana*, the cluster was in the pericentromeric region of the short arm of the submetacentric chromosome pair 7, whereas in *G. guira* the cluster was in the long arm pericentromeric region of the metacentric chromosome 6 (Figure 3). In Accipitriformes *Harpia harpyja* and *Pandion haliaetus*, the association was with a segment homologue to chromosome GGA1 (Table 2). However, in *H. harpyja*, the rDNA cluster was seen in the subtelomeric region of macrochromosome 6, and in *P. haliaetus*, the cluster occupied the pericentromeric region of the long arm on chromosome 2 (Figure 3) (Tagliarini, 2013; Nishida *et al.*, 2014). The translocation or a pericentric inversion may explain this position variation of the internal 45S rDNA cluster in the bearer chromosome, which corroborates the hypothesis that the 45S rDNA cluster is related to chromosomal break-points, according to Cazaux *et al.* (2011).

Conclusion

In birds, the 45S rDNA site is located predominantly in a single pair of microchromosomes, although a number of deviations from this basic pattern exist, with some species having rDNA located in more than one microchromosome pair or in macrochromosomes, or in both types of chromosome. The present study also demonstrated that the redistribution of rDNA sites within the chromosome complement has resulted from chromosomal rearrangements, which have resulted from the distinct evolutionary histories of each group of the class Aves.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

TMD outlined the study and conducted it fully at all stages. RK, MSS, SAB, RJG, ADVG, participated in field work, laboratory methods and data acquisition. TMD, RJG, EHCO, IH, ADVG data analysis. TMD wrote of the manuscript.

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