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CITOGENÉTICA EVOLUTIVA EM ESPÉCIES DA FAMÍLIA COLUMBIDAE (AVES, COLUMBIFORMES)

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SUMÁRIO

Lista de Abreviaturas

CAK ancestral karyotype of the Columbiformes

CLI *Columba livia*

CPA *Columbina passerina*

CPI *Columbina picui*

FISH Fluorescent *in situ* hybridization

GGA *Gallus gallus*

GMO *Geotrygon montana*

GVI *Geotrygon violacea*

JJA *Jacana jacana*

LAL *Leucopternis albicollis*

LVE *Leptotila verreauxi*

PAK Putative ancestral avian karyotype

PCA *Patagioenas cayennensis*

ZAU *Zenaida auriculata*

Resumo

Columbidae é uma família da Classe Aves, Ordem Columbiformes que inclui os pombos, pombas e rolas e compreende cerca de 300 espécies, distribuída em todos os continentes. Devido a diversidade deste grupo, espécies desta família foram alvos de vários estudos, incluindo citogenéticos. Apesar de que a maioria dos estudos citogenéticos em espécies da família Columbidae foram baseados apenas na citogenética clássica (coloração convencional e bandeamentos cromossômicos), resultados interessantes foram observados, tais como a variação do número diploide e a ocorrência de rearranjos intercromossômicos e intracromossômicos. Estes estudos influenciaram na escolha da família Columbidae para o desenvolvimento desta Tese. Nas últimas décadas houve um grande esforço para reconstruir a filogenia das aves atuais, mas a análise dos cariótipos através de técnicas de citogenética molecular, tais como a pintura cromossômica ainda limita-se a poucas ordens. Considerando que a última revisão dos dados citogenéticos é de 2007, no capítulo I realizamos uma revisão sobre o genoma das Aves, incluindo dados de citogenética clássica e molecular. No capítulo II nós realizamos a caracterização do cariótipo de nove espécies da família Columbidae, sendo que uma delas foi descrita pela primeira vez (*Geotrygon violacea*) e mapeamos a distribuição de sequências repetitivas (rDNA 18S e microssatélites). No capítulo III realizamos a pintura cromossômica comparative em quatro espécies da família Columbidae (*Zenaida auriculata, Columba livia*, *Columbina picui* e *Leptotila verreauxi*). A pintura cromossômica foi realizada utilizando sondas cromossomo-específica de *Gallus gallus* (GGA), *Leucopternis albicollis* (LAL) e de *Z. auriculata* (ZAU). As sondas de ZAU foram desenvolvidas durante o doutorado sanduíche relalizado na Universidade de Cambridge (2017). A pintura cromossômica com as sondas de GGA e ZAU demonstraram a conservação da maioria dos macrocromossomos, exceto a fusão entre os cromossomos ancestrais 6 e 7 em *L. verreauxi*. Entretanto, os sinais de hibridização das sondas de ZAU foram mais intensos do que GGA. As sondas de LAL confirmaram os resultados obtidos com as sondas de GGA e ZAU, mas revelaram também uma complexa reorganização do cromossomo homólogo ao GGA1 nas quatro espécies analisadas, involvendo inversões paracêntricas e pericêntricas. Além disso, inversões nos cromossomos homólogos ao GGA2 foram identificadas em *C. picui* e *L. verreauxi*. A ocorrência da reorganização dos cromossomos homólogos ao GGA1 nas quatro espécies analisadas neste capítulo e em

espécies da Ordem Passeriformes analisados previamente, corroboram com a recente proposta de divergência das Neoaves (Columbea e Passerea). No capítulo IV realizamos a pintura cromossômica com as sondas de ZAU e GGA na espécie *Jacana jacana* (Charadriiformes), com o objetivo de verificar a eficiência das sondas desenvolvidas durante o doutorado sanduíche. Observamos sinais de hibridização mais intensos para as sondas de ZAU do que GGA, o que diminui o viés na interpretação dos dados. Também identificamos uma extensa reorganização cromossômica na espécie *J. jacana*, que em comparação com dados da literatura, demonstra que espécies da Ordem Charadriiformes passaram por uma evolução cromossômica exclusiva. Os resultados desta Tese demonstram que distintos rearranjos ocorreram durante a evolução cromossômica das espécies da família Columbidae e também na espécie *J. jacana*. Além disso, as sondas de ZAU mostraram-se como uma importante ferramente para comparações cromossômicas em espécies de Aves, principalmente Neoaves.

Abstract

Columbidae is a family of Class Aves, Order Columbiformes that includes the pigeons, doves and rolas and comprises about 300 species, distributed in all the continents. Due to the diversity of this group, species of this family were the targets of several studies, including cytogenetics. Although most cytogenetic studies on species of the Columbidae family were based only on classical cytogenetics (conventional staining and chromosomal banding), interesting results were observed, such as diploid number variation and the occurrence of interchromosomal and intrachromosomal rearrangements. These studies influenced the choice of the Columbidae family for the development of this thesis. In recent decades there has been a great effort to reconstruct the phylogeny of current birds, but the analysis of karyotypes through molecular cytogenetic techniques such as chromosome painting is still limited to a few orders. Considering that the last revision of the cytogenetic data is from 2007, in chapter I we conducted a review on the genome of Birds, including classical and molecular cytogenetic data. In chapter II we performed the karyotype characterization of nine species of the Columbidae family, one of which was described for the first time (*Geotrygon violacea*) and mapped the distribution of repetitive sequences (18S rDNA and microsatellites). In Chapter III we performed comparative chromosome painting on four species of the family Columbidae (*Zenaida auriculata*, *Columba livia*, *Columbina picui* and *Leptotila verreauxi*). Chromosome painting was performed using chromosome-specific probes from *Gallus gallus* (GGA), *Leucopternis albicollis* (LAL) and *Z. auriculata* (ZAU). The ZAU probes were developed during the "Doutorado sanduiche" at the University of Cambridge (2017). The chromosome painting with GGA and ZAU probes demonstrated the conservation of most of the macrochromosomes except the fusion between the ancestral chromosomes 6 and 7 in *L. verreauxi*. However, hybridization signals from the ZAU probes were more intense than GGA. LAL probes confirmed the results obtained with the GGA and ZAU probes, but also revealed a complex rearrangement of the chromosome homologous to GGA1 in the four species analyzed, involving paracentric and pericentric inversions. In addition, inversions in chromosomes homologous to GGA2 were identified in *C. picui* and *L. verreauxi*. The occurrence of the reorganization of homologous GGA1 chromosomes in the four species analyzed in this chapter and in species of the Passeriformes Order analyzed previously, corroborate with the recent proposal of divergence of the Neoaves

(Columbea and Passerea). In chapter IV we performed the chromosome painting with the ZAU and GGA probes in the *Jacana jacana* (Charadriiformes) species, with the objective of verifying the efficiency of the probes developed during the "Doutorado sanduiche". We observed more intense hybridization signals for the ZAU probes than GGA, which reduces the bias in the interpretation of the data. We also identified an extensive chromosome reorganization in the *J. jacana* species, which, in comparison with literature data, shows that species of the Order Charadriiformes underwent a unique chromosomal evolution. The results of this thesis demonstrate that distinct rearrangements occurred during the chromosome evolution of the species of the family Columbidae and also in the species *J. jacana*. In addition, the ZAU probes proved to be an important tool for chromosome comparisons in species of Birds, especially Neoaves.

1. INTRODUÇÃO - Considerações gerais sobre as Aves e Columbiformes

As Aves representam o grupo de vertebrados terrestre mais diversificado, somando aproximadamente 10.000 espécies, todos descendentes de uma radiação ancestral que pode ser traçada até o famoso *Archaeopteryx lithographica*, há 150 milhões de anos. Estudos moleculares e morfológicos contemporâneos dividem as Aves modernas (Neornithes) em três grupos monofiléticos: Palaeognathae (Tinamiformes e Struthioniformes), Galloanseres (Galliformes e Anseriformes) e Neoaves (todas demais ordens) (LIVEZEY & ZUSI, 2007; JARVIS *et al*., 2014; PRUM *et al*., 2015).

Columbiformes é uma das ordens inseridas no grupo Neoaves e representa uma das ordens mais facilmente reconhecidas em todo o mundo, com mais de 300 espécies, sendo tradicionalmente dividida em duas famílias: Columbidae (pombos e rolas) e Raphidae (extintos Dodô e Solitário de Rodrigues) (PEREIRA *et al*., 2007). Três grandes clados são apoiados em Columbiformes e foram referidos como A, B, e C por PEREIRA *et al*. (2007), com base em dados de sequências de DNA mitocondrial e nuclear. O clado A é subdividido em dois subclados bem apoiados: um subclado refere-se a gêneros exclusivos das Américas e o outro inclui pombos e rolas do Velho e do Novo Mundo. O clado B agrupa somente espécies de pombos do Novo mundo e o clado C inclui muitos gêneros encontrados na África, Ásia, Austrália, Índia Oriental e Nova Zelândia.

A radiação adaptativa dos gêneros modernos de Columbiformes iniciou-se no início do Eoceno, supostamente facilitada por sua alta capacidade de dispersão (PEREIRA *et al*., 2007), o que lhes permitiu diverenciar-se em um grande número de espécies e colonizar uma gama extremamente diversificada de habitats em todos os continentes, exceto na Antártida (GIBBS *et al*., 2001). Em vista dessa diversidade, os Columbiformes têm sido alvo de vários estudos, tais como mudanças comportamentais e fenotípicas, seleção natural e citogenética (DE LUCCA, 1984; SOL, 2008; LAPIEDRA *et al*., 2013; SHAPIRO *et al*., 2013).

Os estudos citogenéticos demonstraram resultados interessantes em espécies de Columbiformes, assim como a variação do número diploide, o qual varia entre 68 (*Uropelia campestris*) e 86 cromossomos (*Geotrygon montana*) (DE LUCCA & DE AGUIAR, 1976; DE LUCCA, 1984; GUTTENBACH *et al*., 2003; DERJUSHEVA *et al*., 2004). Além disso, rearranjos cromossômicos foram propostos para alguns gêneros através

da comparação do padrão de Bandeamento G em 14 espécies Neotropicais de Columbiformes: inversões pericêntricas em *Patagioenas*; fusões e translocações em *Uropelia*; fissões cêntricas em *Geotrygon*; fusões, translocações, inversões paracêntricas e pericêntricas em *Columbina*, *Leptotila* e *Zenaida* (DE LUCCA, 1984). Resultados similares foram encontrados em duas espécies de pombos domesticados, *Streptopelia risoria* e *Columba livia* (STOCK & MENGDEN, 1975). Estes autores relataram duas inversões paracêntricas, uma para cada braço do cromossomo 1 em ambas as espécies e a presença de dois pares a mais de macrocromossomos bibraquiais médios em *S. risoria* do que em *C. livia*, provavelmente devido a ocorrência de fusões cromossômicas.

Em relação à citogenética molecular, apenas duas espécies desta ordem foram estudadas até o momento, e somente com sondas derivadas de *Gallus gallus*: *Streptopelia roseogrisea* (2n=78) e *Columba livia* (2n=80) (GUTTENBACH *et al*., 2003; DERJUSHEVA *et al*., 2004). *Columba livia* possui o mesmo padrão de hibridização proposto para o suposto cariótipo ancestral das aves (PAK) (GUTTENBACH *et al*., 2003; GRIFFIN *et al*., 2007). Em *Streptopelia roseogrisea* os supostos cromossomos ancestrais 1-3 e 5 mostraram-se conservados, o cromossomo 4 mostrou a mesma característica derivada observada em *Gallus gallus* (fusão do PAK 4 com o PAK 10) e as sondas GGA 6-9 hibridizaram cada uma em um dos braços longos ou curtos dos cromossomos 4-7 (o cromossomo exato não pôde ser identificado devido as similaridades morfológicas destes cromossomos) (DERJUSHEVA *et al*., 2004).

Como mencionado, os rearranjos intracromossômicos parecem ter desempenhado importante papel durante a evolução cariotípica de espécies da ordem Columbiformes. De fato, rearranjos intracromossômicos têm sido relatado com frequência em espécies de Aves, tanto por dados *in silico*, quanto por dados de hibridização *in situ* (WARREN *et al*., 2010; KRETSCHMER *et al*., 2014; 2015). Neste contexto, espécies da Ordem Columbiformes tonam-se interessantes do ponto de vista citogenético.

2. OBJETIVOS

2.1 OBJETIVO GERAL

O estudo tem por finalidade avaliar a variabilidade cromossômica de espécies da família Columbidae (Aves, Columbiformes) buscando a relação entre rearranjos

cromossômicos e proximidade filogenética. Além disso, pretende-se produzir sondas cromossomico-específicas da espécie *Zenaida auriculata* para posterior comparação com outras espécies desta família e da Classe Aves.

2.2 OBJETIVOS ESPECÍFICOS

- Obter cultura celular e preparações cromossômicas de diferentes espécies da família Columbidae;
- Realizar o mapeamento de sequências repetitivas em metáfases das espécies amostradas;
- Realizar a citometria de fluxo, amplificação e marcação das sondas cromossomo-específicas da espécie *Zenaida auriculata*;
- Identificar os segmentos homólogos entre *Gallus gallus* e *Zenaida auriculata*;
- Identificar os segmentos homólogos entre *Leucopternis albicollis* e as espécies da família Columbidae amostradas;
- Construir um mapa de homologia entre *Gallus gallus* e as espécies da família Columbidae amostradas;
- Compreender a evolução cariotípica nos representantes da família Columbidae;
- Avaliar a eficiência das sondas cromossomo-específicas da espécie *Zenaida auriculata* na espécie *Jacana jacana*.

3. Capítulo I

Karyotype Organization in Birds: from Conventional Staining to Chromosome Painting

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Abstract: In the last few decades, there have been great efforts to reconstruct the phylogeny of Neoaves based mainly on DNA sequencing. Despite the importance of karyotype data in phylogenetic studies, especially with the advent of FISH techniques using different types of probes, the use of chromosomal data to clarify phylogenetic proposals is still minimal. Additionally, comparative chromosome painting in birds is restricted to a few orders, while in mammals, for example, virtually all orders have already been analyzed by this method. Most reports are based on comparisons using *Gallus gallus* probes, and only a small number of species have been analyzed with more informative sets of probes, such as those from *Leucopternis albicollis* and *Gyps fulvus*, which show ancestral macrochromosomes rearranged in alternative patterns. Despite this, it is appropriate to review the available cytogenetic information and possible phylogenetic conclusions. In this report the authors gather both classical and molecular cytogenetic data and describe some interesting and unique characteristics of karyotype evolution in birds.

Keywords: Avian genome; classical and molecular cytogenetics; sex chromosomes; Avian cytotaxonomy.

Avian phylogenomics and their impact

With approximately 10,600 species, birds represent the class of Tetrapoda with the highest number of species [1]. Modern birds (Neornithes) are divided traditionally in Palaeognathae (tinamous and flightless ratites), Galloanseres [Galliformes (landfowl) and Anseriformes (waterfowl)], and Neoaves (all other extant birds) [2]. In the last few decades, there have been great efforts to reconstruct the phylogeny of birds using morphologic [3], nuclear DNA sequencing [2], and whole genome sequence [4,5] data. Nevertheless, this task has proved to be a hard challenge, due to the rapid adaptive radiation of birds, which resulted in short internal nodes [2].

Birds are used as model organisms in many fields of biology, such as the evolution of brain, cognition, behavior, phylogenetic relationships, vocal learning and sex determination [4,6-8]. In addition, some birds such as the Psittaciformes provided multiple services acting as genetic linkers, seed facilitators for secondary dispersers, and plant protectors, through their feeding activities and therefore can be considered key mutualists with a pervasive impact on plant assemblages [9].

Avian Genome: An overview

Although birds represent the second most specious group of Vertebrates, and the most specious group of Tetrapoda. Until recently, genome size was known in only 2% of avian species (the lowest proportion among Vertebrates). Data show that the avian genome is extremely constant, with an average size of 1.4 pg of DNA [10]. So far, the lowest and the highest content of DNA vary by only two fold: 1 pg in *Amadina fasciata* and 2.2 pg in *Struthio camelus*, while in Mammals it ranges from 1.7 to 8.4 pg, for example [10]. *Gallus gallus* has 1.2 pg equivalent to the 1.12 Gb calculated from the sum of chromosome measurements in the GGA flow karyotype [11]. The small size of avian genome results mainly from loss of repetitive sequences [12], deletion of large segments and gene loss [13]. It is known that the intron size in chicken (*Gallus gallus*) is smaller than in humans [14].

Chicken microchromosomes constitute 23% of the female genome [15], are GC-rich [16] and have a higher CpG content than the macrochromosomes [17]. Some authors suggest that the small amounts of repetitive sequences in these tiny elements facilitate the pairing process and chiasma formation during meiosis [18,19]. However, the reduction of repetitive sequences is also observed in macrochromosomes, indicating that other selective factors are in action.

Other authors claim that as the smallest genomes are found in excellent flyers, while the largest ones are found in birds that do not fly, this genome reduction may be an adaptative characteristic, subject to the action of natural selection [20,21]. According to these authors, when analyzed from a phylogenetic context, the high metabolic needs related to some aspects of avian physiology, including flight, led to the diminution of introns and the genome as a whole [20]. However, this view is criticized because the evidence is insufficient to determine which came first, the ability to fly or the decrease in genome size [14,22]. Also, taxa other than birds have small genomes, including turtles and crocodiles that have genome sizes and GC content similar to chicken [11].

Despite the need for better knowledge of the avian genome because of their economical and biological importance, and their successful evolution, until recently only a few species have had their genomes sequenced - chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and the zebrafinch (*Taeniopygia guttata*) [23-25], together with a

few others more recently [26,27]. However, sequencing of 48 different species reported important information concerning avian genome organization, as well as aspects concerning their origin, evolution and phylogeny [4,13,28].

Consistent with previous reports on zebra finch and chicken, almost all avian species possess a small amount of repetitive sequences (4-10% of the total genome). The only exception is a species of woodpecker (*Picoides pubescens*), with transposons derived from a species-specific LINE type CR1 amounting to 22% of their genomes [13]. Apparently, this is a consequence of the accumulation of repetitive sequences in sex chromosomes, as this species has a large Z chromosome with more blocks of repetitive sequences than other birds. Indeed, the application of microsatellite probes in three species of woodpeckers has shown that the Z chromosome is the largest element in the karyotype due mostly to the accumulation of microsatellite sequences [29].

Karyotype organization: insights from classical cytogenetics

Despite these important alterations in repetitive sequences, avian genomes are highly conserved in chromosome number and gene order [13,28]. Most species have high diploid numbers close to 80, and chromosomes divided into two types – macro and microchromosomes. Macrochromosomes are the first 5 to 10 largest pairs, and are easily classified by their morphology. On the other hand, microchromosomes are punctiform elements, virtually impossible to distinguish from each other.

Although this uniqueness is assumed for most birds, it is important to highlight that only a little more than 12% of bird species have been characterized cytogenetically at least using conventional staining. The most comprehensive overview to date is the classic work of Christidis [30] with 800 species, and there have been no more than a few hundred additions since then. Most of these studies, especially the older ones, are incomplete, describing only the macrochromosomes and identifying the sex chromosomes [31]. Birds have a conserved ZW system of sex determination, in most cases of which the W chromosome is much smaller than the Z. There are some exceptions, such as the Palaeognathes, which have homomorphic sex chromosomes [32]. In addition, in two species, the crimson finch, *Neochmia phaeton* (Passeriformes), and the paddy bird, *Ardeola grayii* (Pelecaniformes), the W is larger than the Z chromosome [33,34].

The karyotypes of only a small percentage of birds have been studied by banding techniques. However, G-banding is of poor quality in birds, and it is difficult to evaluate and understand chromosomal rearrangements using this technique. Because of their small size, no G-banding patterns are seen in the smallest macrochromosomes or in microchromosomes. Hence, other chromosomal markers, based on the distribution of constitutive heterochomatin or on the sites of nucleolar organization regions (NORs), have been important in studying evolutional relationships [35].

C-banding indicates that heterochromatic blocks are usually confined to centromeric regions and are also found conspicuously in the W chromosome [36,37]. This scarcity of constitutive heterochromatin may be related to the small amount of repetitive sequences, as discussed earlier.

Finally, the studies based on Ag NOR-banding, which reveals transcriptionally active nucleolar organization regions, have shown that many species have only one NOR-bearering pair, usually a microchromosome [32,38]. However, a number of species show more than one pair with NORs, such as some birds of prey and Passerines [36,39,40]. As species of different groups, including basal ones such as Ratites and Galloanserae (except *Coturnix japonica*, with three pairs) [41], show only one pair of NOR-bearing microchromosomes, the occurrence of more than one pair must indicate a derived characteristic, probably due to the duplication and transposition of ribosomal gene clusters.

Chromosomal variation: classical cytogenetic contributions

Most bird species have diploid numbers ranging from 74 to 86 chromosomes, most of which are microchromosomes (Figure 1). However, there are some groups with interesting chromosomal variations, not only in number, but also in chromosome morphology based on the centromere position and due to pericentric inversions or centromere repositioning/neocentromere formation [42]. Extremes in diploid numbers are found in species such as *Ceratogymna bucinator*, with 2n=40, and *Corythaixoides concolor*, with 2n=136-142 [30].

For instance, Palaeognathes have diploid numbers close to 80. Groups such as Tinamiformes [43], Strutioniformes [32] have similar karyotypes, some with small variations in chromosomal morphology. An important feature to highlight in this group is the morphology of the sex chromosomes, which are homomorphic in most species of

Strutioniformes, except *Rhea sp*., which shows a slight difference between the Z and W, the sixth largest pair in the species [44].

Conversely, birds of prey, currently including Falconiformes and Accipitriformes, have a variety of rearranged karyotypes with species with diploid numbers close to 80, such as in Cathartidae, but also species with fewer chromosomes, or with only a few pairs of microchromosomes, as in some hawks and eagles, and low diploid numbers, as in some falcons with 2n=40-42 [40,45-47]. Because of this, birds of prey have been the subject of many cytogenetic studies. Based on conventional staining, the most usual explanation for the reduced number of microchromosomes, was the occurrence of fusions involving these elements [45], an idea that would be corrected only after the advent of chromosome painting [40,48,49].

Between these two extremes, there are groups of birds which show that 2n=80 may not be the rule. Among Charadriiformes, with most species ranging from 2n=78-82, genus *Burrhinus* includes species with some of the lowest diploid numbers among birds: 2n=42 [50] or, in Piciformes, with some species of genus *Ramphastus* with diploid numbers of more than 100 [51].

Psittaciformes are an interesting order because of their variable karyotypes, which, although not very different from 2n=80, exhibit important differences in chromosomal morphology, which have been used as criteria for phylogenetic proposals [52]. Recently, this group, which includes parrots, macaws, parakeets and alleys, has been shown to be of special interest. For example, the karyotype of *Myiopsitta monachus*, a South American species with 2n=48, has the lowest diploid number among Psittaciformes, and an exceptionally large W chromosome, due to the accumulation of microsatellite sequences [53].

In summary, despite their usually conserved karyotypes, birds do show some interesting chromosomal variability, both in diploid number and chromosomal morphology, although most data are based only on macrochromosomes. Additionally, as we will discuss in the next section, with the advent of molecular cytogenetics and DNA sequence data, the observed variation is an underestimate of avian chromosomal reorganization, which is based mainly on intrachromosomal rearrangements, such as pericentric and paracentric inversions [36,54,55].

Molecular Cytogenetics: Colorful insights on Avian Cytogenetics

Comparative chromosome painting in Aves has helped to overcome the limitations of karyotype analysis because of the poor quality of G-banding. So far, 77 species of birds have been analyzed by chromosome painting, in studies exploring evolutionary approaches such as chromosome diversification mechanisms, differentiation of sex chromosomes, and chromosome homology. In addition, different types of probes based on repetitive sequences have contributed to our understanding of avian genome organisation.

However, it is important to emphasize that, despite the development of DNA markers that help identify chicken microchromosomes [55,56,57], avian cytogenetics has not reached its full potential, and most comparative data refer only to macrochromosomes.

Probes for Cross-Species Comparative Chromosome Painting

So far, chromosome painting sets of four different species have been used in Avian comparative cytogenetics: *Gallus gallus* (GGA) (2n=78), *Burhinus oedicnemus* (BOE) (2n=40), *Leucopternis albicollis* (LAL) (2n=66) and *Gyps fulvus* (GFU) (2n=66). Of these, most studies have used *Gallus gallus* probes, not only for its economic importance and well known genome, but also because this species has a chromosomal organization similar to the putative avian ancestral karyotype, except for one rearrangement [56,58,59].

GGA probes have shown strong homology between macrochromosomes of many different species, even in species phylogenetically distant. For each analyzed species, an average of two different rearrangements was found, except for species with more derived karyotypes, such as birds of prey [60-62]. For the latter, characterized by the small number of microchromosomes, at least 19 to 22 interchromosomal rearrangements per species have been described [40,62].

B. oedicnemus (Charadriiformes, Burrinidae, BOE) probes were described by Nie et al [50] and applied to eight species of six different orders [63,64]. Although BOE probes do not add much information on GGA macrochromosomes, because they are conserved in both species, the use of BOE paints indicates the involvement of some microchromosome pairs in evolutionary rearrangements. The results confirm that some ancestral pairs of microchromosomes fuse to form metacentric chromosomes in BOE, while remaining as individual microchromosomes in most Neognathes [63].

Leucopternis albicollis (Accipitriformes, Accipitridae) (LAL) was the first bird of prey for which whole-chromosome probes were produced, and these were described first in reciprocal cross-species painting with GGA by de Oliveira et al. [49]. The most striking results show that although many fusions involving microchromosomes contributed to the reduction of the diploid number to 2n=66, the largest ancestral macrochromosome pairs have undergone multiple fissions leading to 2 to 5 separate pairs. This finding has made the set of LAL probes especially useful for the detection of intrachromosomal rearrangements, such as paracentric inversions, which cannot be identified by GGA or BOE probes. In fact, a series of intrachromosomal rearrangements were identified in all species of Passeriformes analyzed with LAL probes [36,37,65,66].

The most recent set of probes were developed from *Gyps fulvus* (Acciptriformes, Accipitridae, GFU) [64]. GFU probes were used in *Buteo buteo* (2n=68), *Gallus gallus*, *Gyps himalayensis* (3n=66) and *B. oedcinemis*, and the results, together with data from other reports have been used in a cladistics analysis of birds of prey.

Chromosome painting and Avian Phylogeny

A sufficient number of species have been analyzed by chromosome painting in only a few orders to allow firm phylogenetic proposals based on chromosomal events. It is noted that most species studied showed similar chromosomal findings, with the exception of Accipitriformes and Falconiformes. Thus, chromosomal rearrangements that were available for cladistic purposes are rare and mostly based on fissions. Similar karyotypes based on homologies with GGA macrochromosomes were described in species of Ratites, Galliformes, Anseriformes and New World Vultures (Cathartidae) [47,48,58,67,68]. In Passeriformes, it was shown that all species studied shared a fission of GGA1 [36,37,60,61,65]. Because of this, a putative avian ancestral karyotype (PAK) was proposed, in which the first eleven macrochromosome pairs corresponded to GGA1-GGA3, GGA4q, GGA5-GGA10 and GGA4p [59].

In 2005, the results of a comparative chromosome painting using GGA probes in the harpy eagle were reported, showing that fission of some GGA macrochromosomes produced two to five separate pairs [40]. Then, in 2010, a set of probes derived from an Accipitridae, the white hawk (*Leucopternis albicollis*, LAL) was described [49] which revealed similar multiple fusions of LAL in the GGA macrochromosomes. This showed

that LAL probes could be used as region-specific probes to identify intrachromosomal rearrangements in the macrochromosomes of many other avian species. Firstly, they were applied to different species of South American buteoninae, and this confirmed that the rearrangements observed by LAL probes constituted a cytogenetic signature for this group [69]. In Passeriformes, the probes allowed the detection of a series of complex intrachromosomal rearrangements, both in Oscines and Suboscines, confirming that these inversions had occurred early in the history of this group, before the split of these two suborders [36,37,65,66,70]. Finally, different species of macaws (Psittaciformes) have been analyzed by FISH experiments using both GGA and LAL probes, and the results allowed the authors to propose phylogenetic relationships and cytogenetic signatures for this group [71].

Distribution of telomeric sequences

As the most distal structures of eukaryotic chromosomes, telomeres play a critical role in maintaining their stability and function [72]. The use of telomeric sequence probes has revealed that, sometimes, these sequences may be found in interstitial positions (ITS, Interstitial Telomere Sequences), and are usually interpreted as the remnants of previous chromosomal fusions [72,73].

In birds, the use of telomeric sequences as probes produces terminal signals, with the interesting finding that much brighter signals are observed in microchromosomes compared to macrochromosomes [37,70]. Additionally, ITS have been seen in different groups of birds, especially in more basal groups. For instance, many ITS are observed in Palaeognathae, due to ancestral fusions, and their gradual disappearance has been noted during the divergence of Palaeognathae and Neognathae [72].

Another example of ITS on the long arm of chromosome 3 in *Falco columbarius*, was critical for the identification of an ancestral fusion [73]. However, many cases of tandem chromosome fusions or centric fusions do not have the expected ITS, probably due to loss of telomeric DNA during these rearrangements [74-76].

On the other hand, in Passeriformes, while studies in species of four different families in both Suborders, Suboscines (Tyrannidae) and Oscines (Thraupidae, Estrildidae and Fringillidae) did not detect any ITS [37,66,70], other studies in Turdidae and Fringillidae

(*F. coelebs*) have detected numerous ITS [61,72], which have not yet been explained phylogenetically.

Ribosomal DNA clusters

As in most aspects of avian cytogenetics, information about the distribution of 18/28S and 5SrDNA are restricted to a few species, especially with the use of FISH probes. However, the data collected from Ag-NOR staining reveals that most species including Ratites [32], and Galloanserae [77] have only one pair of microchromosomes bearing these clusters. However, some species showed a higher number of rDNA bearing chromosome pairs [36,65], and some birds of prey have ribosomal gene clusters in macrochromosomes [69] (Figure 2).

Because Ratites and Galloanserae (except *Coturnix japonica*, with three pairs) [41] have only one pair of microchromosomes bearing 18/28rDNA, this is accepted as ancestral. More than one pair of microchromosomes bearing these clusters is regarded as the derived state, possibly due to translocation following amplification of ribosomal genes [78].

Information on 5SrDNA is even more restricted. In six of only seven species of two different orders, Galliformes and Passeriformes, 5SrDNA clusters are located in a pair of microchromosomes. However, in the zebra finch (*Taeniopygia gutata*), these clusters are found in the long arm of pair 1, in an interstitial position [37,41,70,79]. As GGA painting did not detected any interchromosomal rearrangement involving this segment (corresponding to GGA2) in *T. gutata*, transposition is a possible explanation [80]. Studies of these repetitive sequences should be extended to additional avian orders.

Detailed Putative Avian Ancestral karyotype (PAK)

The presence of species with karyotypes similar to the putative avian ancestral karyotype in virtually every group of birds has reinforced its authenticity. Additionally, current information using different sets of FISH probes, especially those from *L. albicollis*, allows us to propose a more detailed version of the PAK.

In many species of different orders LAL probes are found in the same arrangement as in *Gallus gallus* [49]. This is the case in species of Cathartidae [47], Charadriiformes [81],

Strigiformes, Anseriformes and Strutioniformes (unpublished data, figure 3). These observations suggest to us that the arrangement of LAL probes detected in GGA macrochromosomes also reflects their organization in the putative ancestral karyotype (Figure 3).

This assumption has been made by different authors who have characterized the sequence of intrachromosomal rearrangements observed in groups such as Passeriformes and Psittaciformes [36,37,65,66,70,71,82]. Furthermore, the data enabled these authors to define certain rearrangements as cytogenetic signatures of groups within these orders which corroborate phylogenetic proposals [66,69,71,81,82].

Karyotypical Evolution based on Chromosome painting

As indicated above, and even in the absence of chromosomal signatures, some of the events revealed by chromosome painting can act as important characters in phylogenetic analyses. We review here the main findings that have been made in the following different groups of birds (**Supplementary Materials)**.

Palaeognathae

Six different species of Struthioniformes and Tinamiformes have shown 2n=80, except for the cassowary (*Casuarius casuarius*), which has 92 chromosomes. Despite this, the results of GGA probes show the conservation of all syntenic groups corresponding to the macrochromosomes of PAK [32,60]. It can be inferred that fissions involving the microchromosomes must have been involved in the origin of the highest diploid number found in the cassowary, as already postulated for the coscoroba swan (*C. coscoroba*), with 98 chromosomes and conserved macrochromosomes [68]. Although there are no reports of LAL probes applied to Paleognathae birds, it has been observed that at least *Rhea americana* shows that pairs 1, 2 and 3 have the same sequence observed in PAK/GGA (Figure 4, unpublished data).

Galloanseres (Galliformes and Anseriformes)

Thirteen species of Galliformes have been analyzed by FISH [60,67]. Fusions and fissions seem to be the most common rearrangements in this order. *Coturnix c. japonica*

has the same fusion observed in GGA4 (PAK4/PAK10). Fission of ancestral chromosome 2 (PAK2) occurs in seven species (*Phasianus colchicus*, *Chrysolophus pictus*, *Lophura nycthemera*, *Chrysolophus amherstiae*, *Meleagris gallopavo*, *Tetrao urogallus* and *Callipepla californica*). The rearrangement seems to have occurred at the centromere in all of them, although only GGA probes were used. Associations PAK6/PAK7, PAK6/PAK8 and PAK8/PAK9 are observed in *Numida meleagris*, *Tetrao urogallus* and *Pavo cristatus*, respectively. Finally, *Bambusicola thoracica*, *Ortalis vetula* and *Coturnix chinensis* have karyotypes similar to PAK.

In Anseriformes, even though some species are common, only three have been hybridized with GGA probes: *Anser anser*, 2n=80 [60], *Aix sponsa*, 2n=80 [83], and *Coscoroba coscoroba*, 2n=98 [68]. Interestingly, all show conserved macrochromosomes corresponding to PAK1-PAK10, except *Anser anser* that has the same fusion found in GGA4 (PAK4/PAK10), and *C. coscoroba* whose high diploid number, as already mentioned, is probably due to rearrangements involving microchromosomes.

Neoaves

Neoaves includes almost 95% (30 orders) of all bird species, comprising all contemporary avian lineages except Palaeognathae (ratites and tinamous) and the Galloanserae (chicken and ducks). Despite this great diversity, species of only ten orders have been studied by chromosome painting: Columbiformes, Gruiformes, Eurypygiformes, Charadriiformes, Strigiformes, Trogoniformes, Falconiformes, Accipitriformes, Psitaciformes and Passeriformes. Of them, the most striking chromosomal rearrangements are found in birds of prey (Falconiformes and Accipitriformes), Psittaciformes and Passeriformes, although other taxa such as *Burrhinus oedicnemus* (Charadriiformes), with 2n=42 [50] have extremely rearranged karyotypes.

Two species of Columbiformes have been analyzed with GGA probes. *Columba livia* (2n=80) shows the same organization as PAK [59,61], while *Streptopelia roseogrisea* (2n=78) has a derived karyotype, with PAK4 and PAK10 fused as in GGA4, and paints GGA6-9 hybridizing to the long arms of biarmed pairs 4-7 [60].

In Gruiformes, two species were analyzed with GGA probes - *Fulica atra* and *Gallinula chloropus* [83] *F. atra* and *G. chloropus* share associations PAK 4/5 and PAK

6/7, as well as fissions of PAK 4 and 5. The fission of PAK 5 may be a synapomorphy for this order.

Although formely a member of Gruiformes, E*urypyga*. *helias* is now included in the order Eurypygiformes [4]. This species has been analyzed by both GGA and LAL probes, and showed the association PAK 2/5, followed by an inversion, and fissions in PAK 1, 2 and 5. Additionally, LAL were arranged in the same order as observed in *G. gallus* in chromosomes of *E. helias* corresponding to PAK1 (EHE 2 and 5) and PAK 3 (EHE 3). It also presented the fission of PAK 5, which could reinforce its close relationship with Gruiformes.

Charadriiformes, have very heterogeneous karyotypes. *Burhinus oedicnemus* has been analyzed with both GGA and *Gyps fulvus* probes [50,64], *Vanellus chilensis* with GGA and LAL probes [81], and *Larus argentatus* with *Burhinus oedicnemus* probes [63]. The low diploid number observed in *B. oedicnemus* (2n=42) was shown to be a result of multiple fusions involving microchromosomes [50]. In *L. argentatus*, chromosomes corresponding to PAK 5-9 are fused with other undefined elements [63], while in *V. chilensis* the association PAK8/PAK9 was detected. Additionally, LAL probes revealed that their arrangement was identical to that observed in GGA macrochromosomes.

Three species of owl (Strigiformes) have already given a glimpse of the interesting chromosomal variation in this order. *Bubo bubo* has the association PAK4/2, while *Strix nebulosa* shows the association PAK4/5 [60,63]. *Pulsatrix perspicillata* reveals the most impressive karyotype with the associations PAK1/2, PAK5/4, PAK6/7; PAK9/4 and PAK5/8 [75]. As possible synapomorphies, these three species share the fission of PAK5, while the centromeric fission of PAK1 is shared by *B. bubo* and *S. perspicillata*. Despite these rearrangements, *P. perpicillata* shows a similar arrangement of LAL probes as *G. gallus* (Figure 5), reinforcing this sequence as ancestral for birds.

In Trogoniformes only *Trogon surrucura surrucura* has been studied by comparative chromosome painting, and this reveals the association PAK 6/7, and fission of PAK2 and PAK5 [38].

Birds of prey which have been subject to numerous cytogenetic analyses since the advent of conventional staining, fall into two different orders: Falconiformes, which embraces the former Falconidae family, and Accipitriformes, which includes the Accipitridae and Cathartidae families [2,4,5]. Within Falconiformes, diploid numbers

range from 2n=40, in *Falco columbaris* (the lowest diploid number found in birds), to 2n=92, in *Polyborus plancus* [73,84]. However, only three species of genus *Falco* have been analyzed with GGA probes: *F. columbaris* (2n=40), *F. peregrinus* (2n=50) and *F. tinnunculus* (2n=52) [73]. The latter two species share the associations PAK2/m; PAK4/m; PAK5/m; PAK6/m; PAK7/m (where m corresponds to microchromosome). *F. columbarius* has a lower diploid number due to additional rearrangements involving associations: PAK2/5/m; PAK3/2/m; PAK3/4/m; PAK4/m; PAK7/m/5/m; PAK8/6/m. Fissions of PAK 2, 3 and 5, which, together with the associations observed in *F. peregrinus* and *F. tinnunculus*, must have had been present in the ancestral karyotype of these three species.

Fourteen species of Accipitriformes have been analyzed by comparative chromosome painting, ranging from species with karyotypes resembling the putative ancestral karyotype, to hawks and eagles with many rearrangements. Only one of the families of Accipitriformes (Sagitariidae) has not been analyzed. For Cathatidae, two species have been studied: *Gymnogyps californianus* and *Cathartes aura*, both with 2n=80, and similar to *G. gallus*. Additionally, the latter has been analyzed by LAL probes, showing that the segments are found in the same order as *G. gallus*, indicating no additional intrachromosomal rearrangements [47,48]. *Pandion haliaetus*, the only species of the family Pandionidae, was analyzed by Nishida et al. [85], and this shows the fission of PAK1 into different segments, (PAK1seg/9, PAK1seg/m, PAK1seg/4 and PAK1seg/6). Fission of PAK5 was also observed.

Eleven species of Accipitridae were analyzed by chromosome painting: *Harpia harpyja*, *Rupornis magnirostris*, *Asturina nitida*, *Buteogallus meridionallis*, *Leucopternis albicollis*, *Buteo buteo*, *Gyps himalayensis*, *Nisaetus nipalensis orientalis*, *Gyps rueppelli*, *Gyps fulvus* and *Gypaetus barbatus* [40,49,62,64,69,76]. All of them are characterized by the fission of ancestral chromosomes PAK1-3 and 5, and fusions involving macrochromosomes (or segments of macrochronosomes) and microchromosomes, which have led to lower diploid numbers (despite the numerous macrochromosome fissions), a low number of microchromosome pairs and a high number of biarmed chromosomes. Some chromosomal signatures have been described, such as fusion PAK1seg/6 in South American Buteoninae [69]. However, due to this high chromosomal variability, more species must be analyzed to detect possible synapomorphies that could help in understanding the phylogeny of this group.

Although only seven species of Psittaciformes have been analyzed by comparative chromosome painting, the results have been more promising and have helped to trace aspects of the chromosomal evolution of this order: *Agapornis roseicollis*, *Nymphicus hollandicus* and *Melopsittacus undulatus* [74], *Ara macao* [86], *Ara chloropterus* and *Anodorhynchus hyacinthinus* [71], *Psittacus erithacus* [87]. Firstly, all the species had a fission of PAK1 into two separate pairs (except for *Ara macao*, which had two fissions leading to three distinct segments). Associations PAK1/4q, PAK6/7, PAK8/9 or others derived from them are present in most species, and probably in their common ancestor. For instance, *Ara macao*, *Ara chloropterus*, *Anodorhynchus hyacinthinus* and *Psittacus erithacus* share the associations PAK1/4q, PAK6/7, PAK8/9, as well as the fission of PAK1. Fission in PAK1 and fusion of PAK6/7 were found in *Nymphicus hollandicus*, while PAK 8/9 had a further fusion, becoming PAK4/8/9. In a similar manner, *Melopsittacus undulatus* has the associations PAK5/6/7 and PAK4/8/9, as well as fission in PAK1 and 6. *Agapornis roseicollis*, with 2n=48, is a species with many associations (PAK6q/7, PAK1/4, PAK8/9 and PAK2/9) and fissions (PAK1, 2 and 9). Although centric fissions tend to produce homoplasic characters, it is interesting to note that the fission found in PAK1 in all species of Psittaciformes so far has also been detected in all Passeriformes studied by FISH, corroborating a recent proposal that Passeriformes and Psittaciformes are sister-groups [2,4,5].

Fifteen species of Passeriformes, most belonging to suborder Oscines, are the subject of different reports [36,37,60,61,70,83,88]. Although most of them share the same organization of PAK, plus the fission of PAK1, the results of LAL probes reveal a complex set of paracentric and pericentric inversions in PAK1q. These rearrangements must have occurred before the split of Oscines and Suboscines, as both suborders share some of the same inversions [65,66].

Structure and evolution of the avian sex chromosomes

The largely homomorphic and euchromatic Z and W chromosomes of paleognathous birds are regarded as the ancestral state of avian sex chromosomes, characterized by a large pseudoautosomal region of the W chromosome [32]. In contrast, the Z and W chromosomes of the Neognathes generally show significant differences in size and

morphology [89,90] although the Z chromosome initially was considered to be highly conserved in all birds.

Based on the uniform size and morphology of the Z chromosome in various avian species, Ohno [91] first proposed that the Z was highly conserved throughout avian lineages, and this seemed to be confirmed by comparative FISH mapping [32,92]. More recently, the mapping of microsatellites by FISH in different species of birds has shown that the Z chromosome of birds exhibits some variability in the accumulation of repetitive sequences. While in *Myiopsitta monachus* (Psittaciformes) the microsatellite probes revealed the accumulation of CAG sequences, the use of 11 different microsatellite probes did not produce any signals in the Z chromosome of nine species of Columbidae [53,93]. In addition, in three species of woodpeckers (Piciformes), a large accumulation of microsatellite sequences is present in the Z chromosome which, in consequence, is the largest element in the karyotype [29].

Recent molecular analysis reveals that degeneration of the W chromosome occurs at different rates among neognathous birds, and that each species may lose different amounts of the differential/non-recombining region [7]. While in *Gallus gallus* the W chromosome is punctiform, in some species of Accipitriformes the W is a larger, sometimes biarmed chromosome [40,69]. However, independent of its size, the W chromosome tends to be largely heterochromatic and may be identified by C-banding. The homomorphic pair of sex chromosomes in *Myiopsitta monachus* (Psittaciformes) is of special interest as, due to the accumulation of three different microsatellite sequences in the W chromosome, whereas the Z chromosome of this species accumulated only one of these sequences [53].

The first case of a multiple sex chromosome system in birds was described recently in the penguin *Pygoscelis adeliae* (Sphenisciformes), in which males have $Z_1Z_1Z_2Z_2$ and females Z_1Z_2W [94]. This finding indicates that sex chromosomes in birds can follow different paths of evolution, and that these differences represent distinct stages of differentiation in each of their lineages.

Avian Cytotaxonomy

Despite the strong conservation of karyotypes in birds, compared to mammals and fish, chromosomal data have been used in many cytotaxonomic and phylogenetic studies. With the introduction of FISH technology, cross-species homology and changes in

chromosome size and morphology have been characterized more precisely, and this has contributed to a better understanding of avian phylogenetic relationships (Fig. 6).

As an example, Rodrigues et al. [68] were able to support the close phylogenetic relationship of two species of Anseriformes, *Coscoroba coscoroba* and *Cereopsis novaehollandiae*, first suggested by molecular phylogenetic analysis [95]. It was observed that the *C. coscoroba* had 2n=98, the highest among Anseriformes, so far, and close to *C. novaehollandiae* (2n=92). Additionally, ancestral macrochromosomes PAK1-PAK10 were conserved, and similar in size and shape to other Anseriformes, including *C. novaehollandiae*. Hence, the authors suggested that fissions in microchromosomes are responsible for the high diploid number in these two species.

As in Anseriformes, FISH studies in Gruiformes species suggest that PAK5q fission might be a synapomorphy for Gruiformes and that fissions in PAK1 and PAK2 that are found only in Eurypygyformes (in only one species, *Eurypyga helias*), might also occur in Rynochetidae (only one species, *Rhynochetos jubatus*), because of the similar chromosomal morphology of *E. helias* and *R. jubatus* [82]. A close phylogenetic relationship between Eurypigidae and Rynochetidae is suggested, indicating their separation from a common ancestor by the Gondwana vicariancy in South America and New Caledonia.

Birds of prey still have a confusing phylogeny, and from the traditional proposals in which they were included in one order, Falconiformes, they have been reassigned to a group within Ciconiiformes [96] and more recently separated into two different orders – Falconiformes and Accipitriformes [2,4,5]. In order to search for cytogenetic signatures in different lineages within Accipitriformes, Nie et al., [64] performed a cladistic analysis using chromosomal characters. Their chromosomal phylogeny suggests that Falconiformes have unique chromosomal rearrangements, differing from those of Accipitriformes species. In addition, they suggest that *Pandion haliaetus* (Pandionidae) may well be a member of Accipitridae and that *Buteo buteo*, a supposed buteoninae species, is much closer to other accipitrids than to the Neotropical buteoninae species. In addition, species in Cathartidae (the New World vultures) have typical avian karyotypes and show a high degree of conservation in chromosomal synteny with *Gallus gallus*, thus differing from other species in Accipitriformes and Falconiformes.

Despite having the highest number of avian species analyzed by FISH, comparative chromosome painting has revealed a low degree of chromosomal variation within Passeriformes, although these species share a complex pattern of paracentric and pericentric inversions. Additionally, as this pattern has been observed both in Oscines and Suboscines, the rearrangements must have occurred before the separation of these two groups [36,65,66].

Chromosome painting in Passeriformes supports the proposal that Psittaciformes is their sister-group (Psittacopasserae) [97], with which they share the PAK1 centric fission in all their species [2,4,5]. Similarly, previous studies have suggested that Piciformes may be closely related to Passeriformes [98,99]. However, Piciformes are characterized by high diploid numbers, probably due to multiple fissions involving macrochromosomes, leading to a karyotype quite distinct from Passeriformes. Indeed, our preliminary studies show that fission of PAK1-PK5 generates 2-6 different pairs in *Ramphastos tucannus* (2n=112) (unpublished data).

Studies in Psittaciformes using conventional staining have been used in a citotaxonomic analysis of Neotropical parrots [52]. However, a number of species have been analyzed by comparative chromosome painting which provides important information, not only concerning their phylogeny, but also their biogeography and karyotypical evolution [53,71,74,82]. Recent studies in two different genera of macaws show that fusions and fissions also have an important role in the karyotypical diversification of Neotropical Psittacidae [71,86]. A fusion of PAK6/PAK7 was observed in all the Psittaciformes analyzed so far, and in most of them, the newly formed chromosome has undergone a paracentric inversion. This is the situation in Neotropical parrots and macaws and in the African *Psittacus erithacus* and *A. roseicollis* [71,74,86,87], indicating that PAK6/PAK7 could represent a synapomorphy for this group. This fusion was also reported in Australian species, however without any apparent inversion, as in *Melopsyttacus undulates,* or showing a different pattern of inversion, as in *Agapornis roseicollis* [74]. Based on this, it was suggested the PAK6/PAK7 fusion must represent a synapomorphy for Psittaciformes, but the different patterns of inversion and fusion still need to be clarified.

Conclusions: Current state of Avian Cytogenomics

The examples discussed here show that the increasing chromosomal data provide important information on phylogenetic relationships in many different groups of birds, despite the apparent conservation of karyotypes. Additionally, the progress of avian cytogenomics has been rapid. Until recently, whole genome sequence assessment was limited to three species, the chicken (*Gallus gallus*), domestic turkey (*Meleagris gallopavo*), and zebra finch (*Taeniopygia guttata*). These studies have inspired plans for sequencing projects of thousands of species [8, 100]. For example, the Genome 10K Project biospecimen list includes specimens from approximately 50% of the 10,500 species of birds [100]. However, even the best-assembled genomes (using contemporary technologies) consist of subchromosomal-sized scaffolds [57]. The biggest challenge is to assemble scaffolds into chromosomes. The difficulties are due mostly due to gaps associated with heterochromatin and the presence of numerous microchromosomes [8]. Recently, Damas et al., [57] combined computational algorithms for ordering scaffolds into predicted chromosome fragments (PCFs), retaining local structures of the target genome after verification of a limited number of scaffolds, and physical mapping of PCFs directly to chromosomes using a universal set of avian bacterial artificial chromosome (BAC) probes. In this study, they developed an approach to upgrade fragmented genome assemblies (pigeon and falcon) to the chromosome level, allowing them to be used to address novel biological questions related to avian genome evolution. Hence, the assembly of scaffolds into chromosomes of more bird species, and the merging of chromosomal and sequencing data will expand our knowledge of avian genome evolution, helping to identify intrachromosomal rearrangements and leading to improved understanding of the phylogenies discussed in this review.

Supplementary Materials: Table 1 - The diploid number, associations and fissions in chicken homologous segments (GGA1-10) in avian genomes using chicken and White Hawk probes. Seg= segment; M= microchromosome

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Figure legends

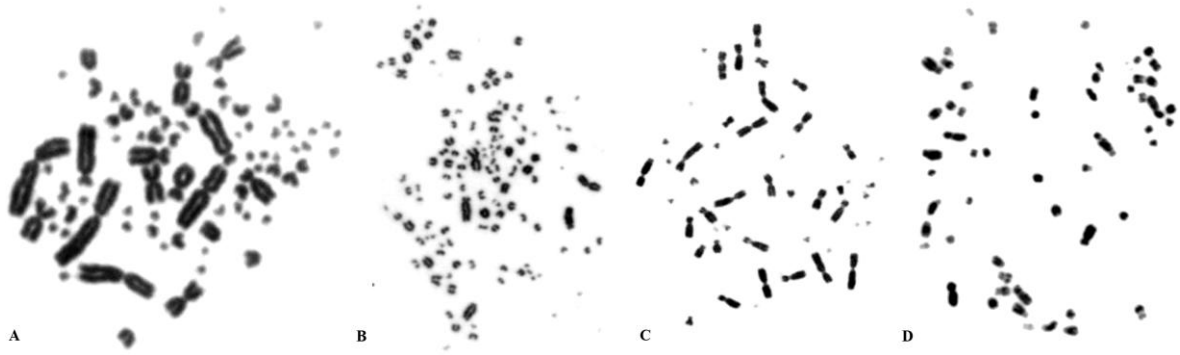

Figure 1. Chromosomal diversity in birds: (A) the most typical formulae, with 2n close to 80, such as in *Vanellus chilensis* (2n=78); (B) an extreme high diploid number, such as *Ramphastos tucanus* (2n=112), an atypical low diploid numbers: (C) *Myiopsitta monachus* (2n=48); and an example of bird of prey (D) *Spizaetus tyrannus* (2n=68).

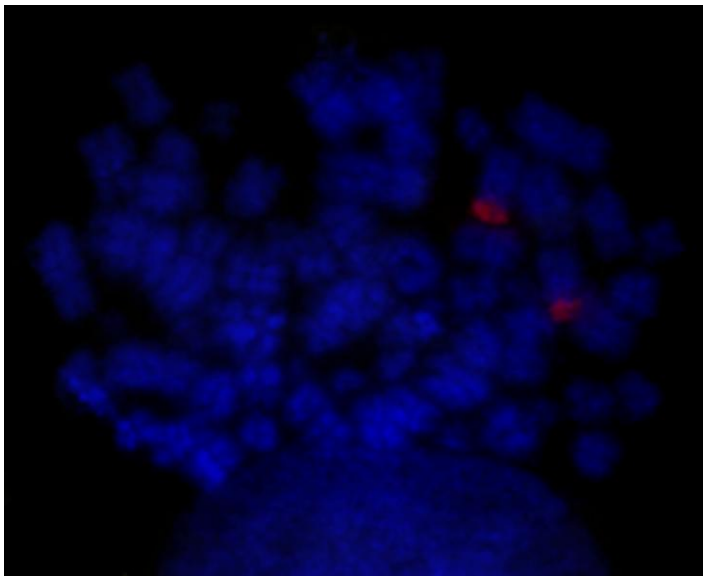

Figure 2. Distribution of 18/28SrDNA in *Buteogallus meridionallis* (Accipitriformes), in the short arm of a medium pair of macrochromosomes.

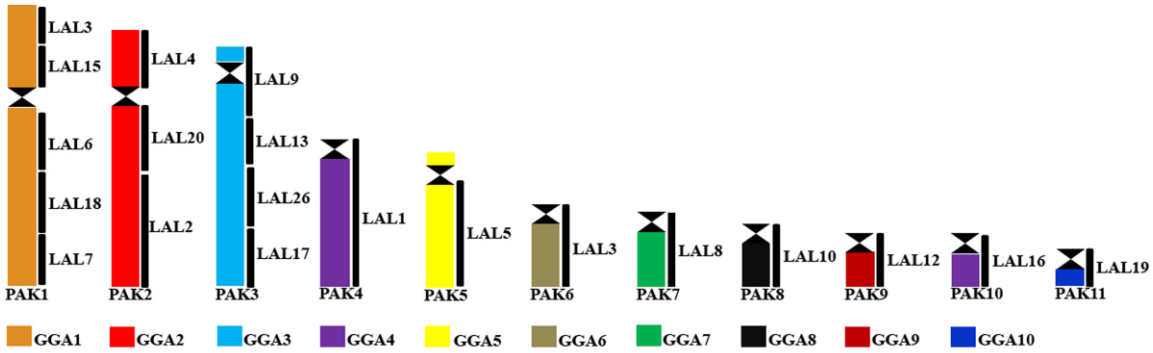

Figure 3. Refined putative avian ancestral karyotype, based on the homology with *L. albicollis*.

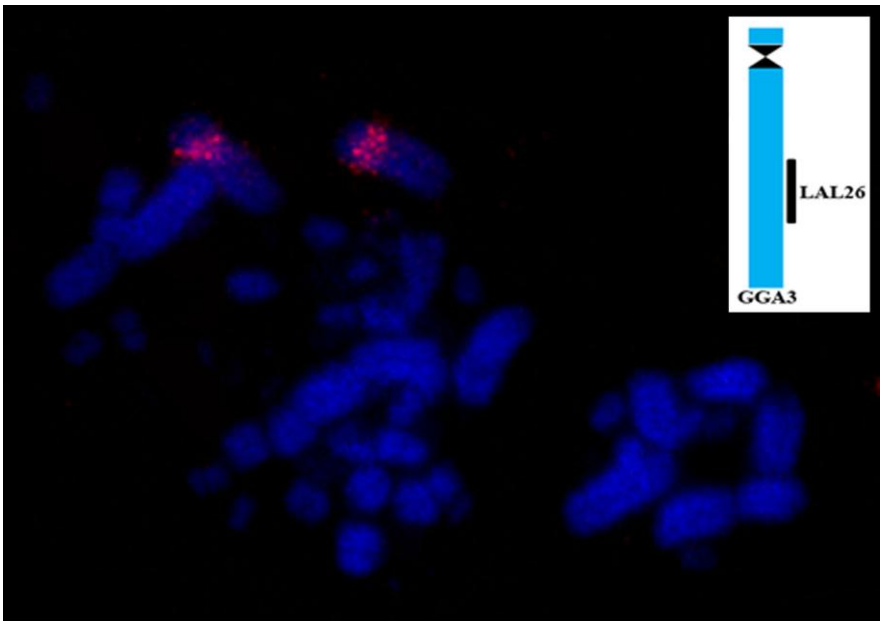

Figure 4. Result of comparative chromosome painting using LAL probes on metaphases of *Rhea americana*. These probes hybridize on the same position as in *Gallus gallus*, confirming that the organization of Ratitas and *G. gallus* are similar and might correspond to the ancestral organization found in PAK.

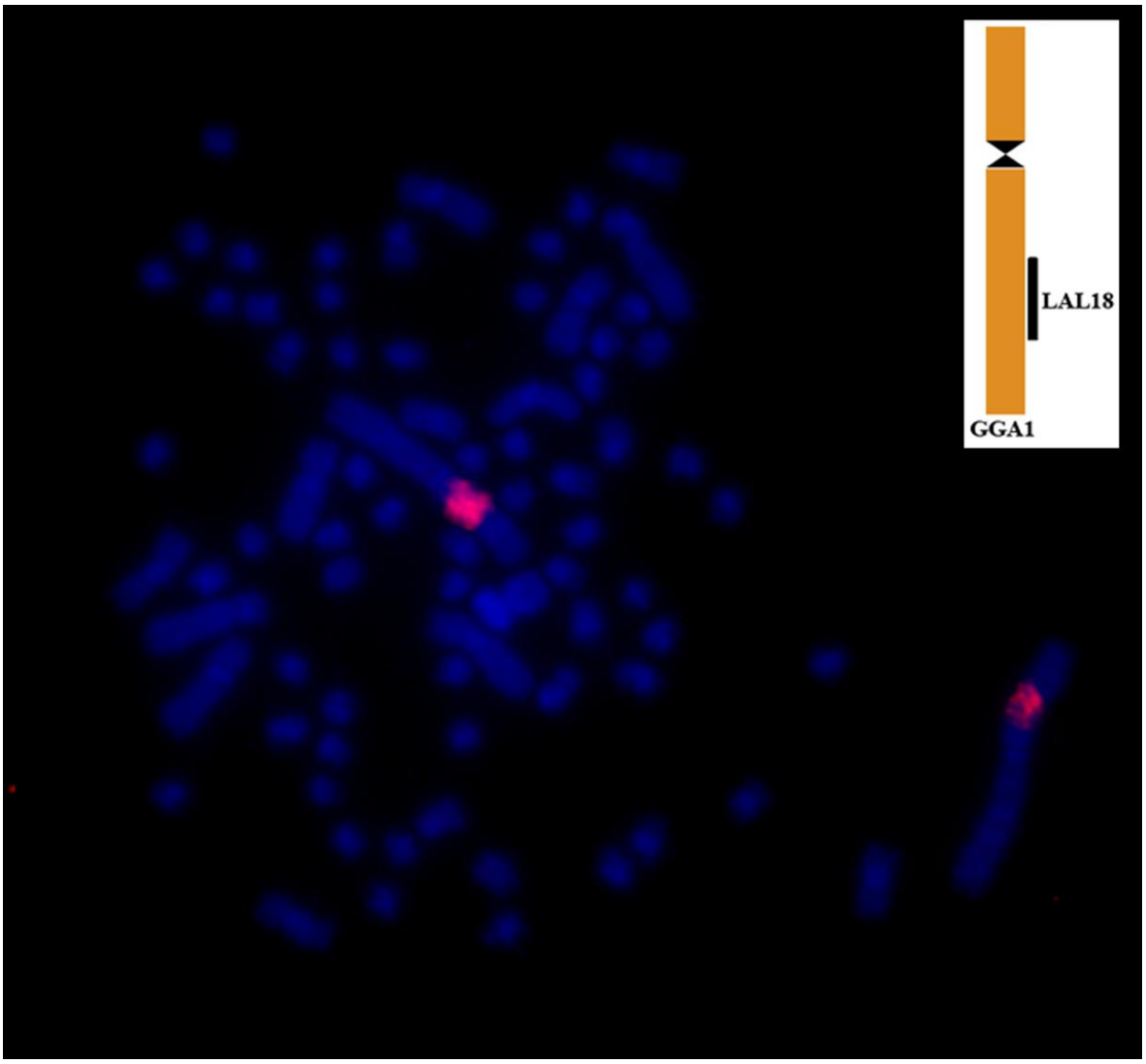

Figure 5. Result of comparative chromosome painting using LAL probes on metaphases of *Pulsatrix perspicillata*. These probes hybridize on the same position as in *G. gallus*, confirming that despite the reorganization of owl's chromosomes, they retained the ancestral organization found in PAK.

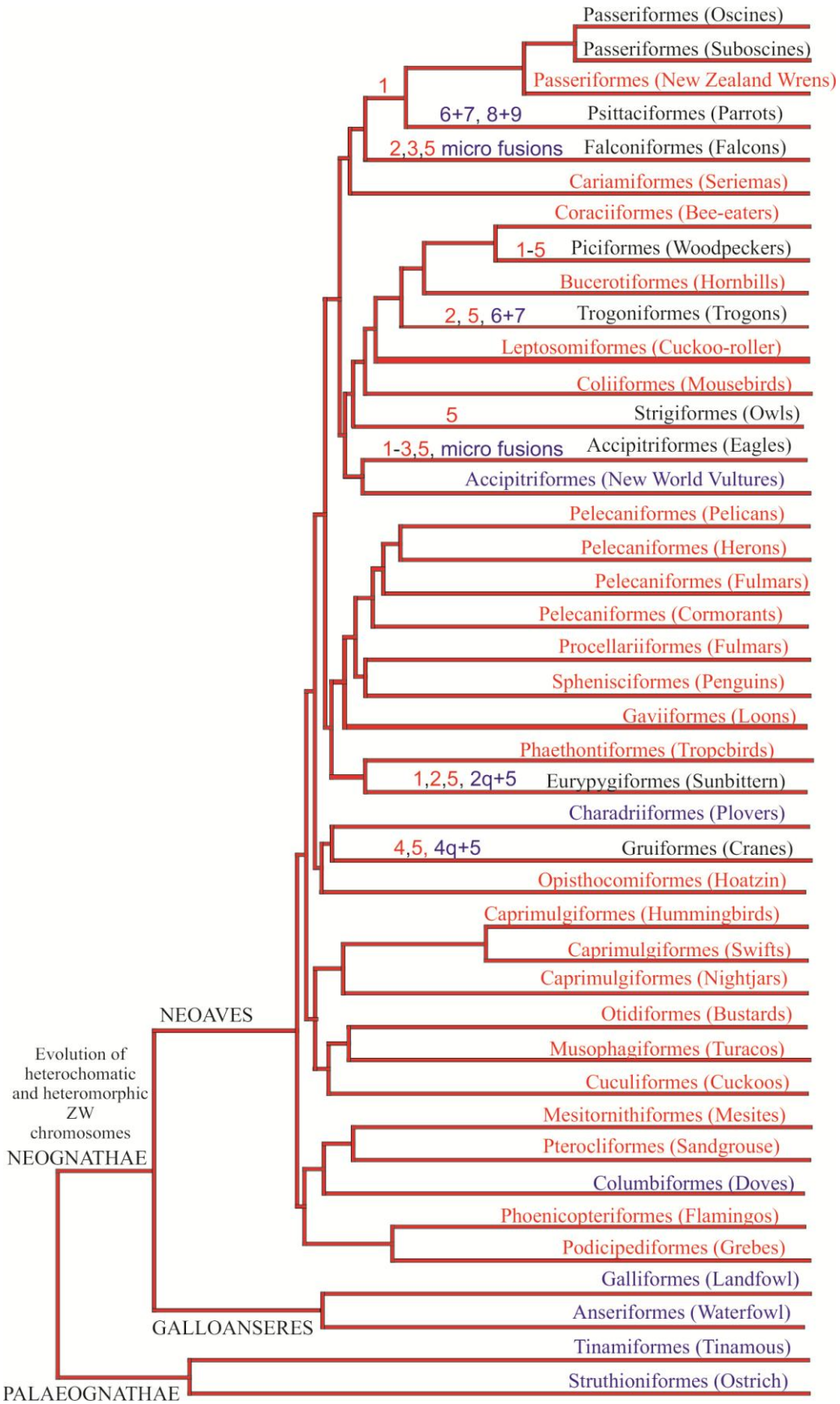

Figure 6: Chromosomal rearrangements based on PAK plotted in a current avian phylogeny (Jarvis et al., 2014) [4]. Rearrangements are represented by fissions (red) and

fusions (blue). Orders in red represent those without chromosomal data up to now, while the blue ones represent groups currently without chromosomal synapomorphies.

4. Capítulo II

Repetitive DNAs and shrink genomes: A chromosomal analysis in nine Columbidae species (Aves, Columbiformes)

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Abstract

An extensive karyotype variation is found among species belonging to the Columbidae family of birds (Columbiformes), both in diploid number and chromosomal morphology. Although clusters of repetitive DNA sequences play an important role in chromosomal instability, and therefore in chromosomal rearrangements, little is known about their distribution and amount in avian genomes. The aim of this study was to analyze the distribution of 11 distinct microsatellite sequences, as well as clusters of 18S rDNA, in nine different Columbidae species, correlating their distribution with the occurrence of chromosomal rearrangements. We found 2n values ranging from 76 to 86 and nine out of 11 microsatellite sequences showed distinct hybridization signals among the analyzed species. The accumulation of microsatellite repeats was found preferentially in the centromeric region of macro and microchromosomes, and in the W chromosome. Additionally, pair 2 showed the accumulation of several microsatellites in different combinations and locations in the distinct species, suggesting the occurrence of intrachromosomal rearrangements, as well as a possible fission of this pair in *Geotrygon* species. Therefore, although birds have a smaller amount of repetitive sequences when compared to other Tetrapoda, these seem to play an important role in the karyotype evolution of these species.

Keywords: Birds, FISH, microsatellites, sex chromosomes, chromosomal rearrangements.

Introduction

Columbiformes is one of the most easily recognized bird orders in the world, with more than 300 species and traditionally divided into two families: Columbidae (pigeons and doves) and Raphidae (Pereira *et al.*, 2007). Three large clades are supported on Columbiformes, referred to as A, B, and C by Pereira *et al*. (2007), based on mitochondrial and nuclear DNA data. Clade A is subdivided into two well-supported subclasses: one referring exclusively to America genera and the other includes pigeons and turtle doves from the Old and New Worlds. Clade B groups only New World pigeon species and Clade C includes many genera found in Africa, Asia, Australia, the East Indies, and New Zealand.

Cytogenetic studies based mainly on conventional staining have shown an interesting variation in diploid number, which ranges from 76 to 86 (Takagi and Sasaki,

1974; de Lucca and de Aguiar, 1976; de Lucca, 1984). Other aspects of their karyotypical organization remain unknown, although the observed variation in chromosome morphology suggests the occurrence of intra- and interchromosomal rearrangements (de Lucca, 1984).

There is evidence supporting that some groups of vertebrates with a high metabolic demand have smaller cells, and as consequence, smaller genomes (Szarski, 1983). In accordance with this hypothesis, the relationship between flying and the reduced genome size of birds, bats and possibly pterosaurs, has been interpreted as an evidence that the high energetic demand of flying exerted selective pressures for small cells and small genomes (Hughes and Hughes, 1995; Organ and Shedlock, 2009; Zhang and Edwards, 2012). Conformingly, birds have the lowest average genome sizes among Tetrapoda (Andrews *et al*., 2009) while bats show the smallest genomes when compared to most Mammalian species (Smith and Gregory, 2009). In addition, humming birds have the smallest genomes among birds, probably associated with their intense necessity of energy to hover during flight (Gregory *et al*., 2009).

Repetitive DNAs represent an important proportion of the genome in eukaryotes, being composed by sequences *in tandem* (satellites, minisatellites and microsatellites) and transposable elements (transposons and retrotransposons) (Charlesworth *et al*., 1994; López-Flores and Garrido-Ramos, 2012). These repetitive sequences play an important role in genome evolution in eukaryotes (Biémont and Vieira, 2006). For example, it was proposed that the genome evolution in mammals has been driven by chromosomal rearrangements in fragile sites, composed by in tandem repetitive sequences (Ruiz-Herrera *et al*., 2006). In addition, transposable elements can also influence the occurrence of chromosomal rearrangements by inducing chromosomal breakage (Biémont and Vieira, 2006).

An important class of repetitive sequences is formed by the microsatellites, small sequences (1–6 base pairs) repeated in tandem and dispersed through the genome. Mono-, di-, tri-, and tetranucleotide repetitions are the most common types of microsatellites (Ellegren, 2004). Mutation rates in these sequences are 10-100,000 folds higher than the mean of other genome regions, making them important markers for genetic variability studies of natural and captive populations (Gemayel *et al*., 2010). Cytogenetic mapping of these sequences has also contributed to a better comprehension of sex chromosome evolution and chromosomal differentiation, and have been extensively analyzed in fishes

(Cioffi and Bertollo, 2012). In general, repetitive sequences accumulate preferentially in centromeric and heterochromatic regions, as observed in many fishes (Cioffi *et al*., 2012), lizards (Pokorná *et al*., 2011) and plant species (Kejnovsky *et al*., 2013). However, little is known about the dynamic of repetitive sequences in birds. In sauropsids (reptiles and birds), many microsatellites have been intensely amplified in sex chromosomes Y/W in seven species (six reptiles and *Gallus gallus*), associated to the differentiation and heterochromatinization of these chromosomes (Matsubara *et al*., 2015).

Recently, distinct hybridization patterns of microsatellite sequences have been demonstrated in species of two different orders of birds (de Oliveira *et al*., 2017; Furo *et al*., 2017). In Piciformes, a large accumulation of 10 sequences was observed on autosomes and especially on the Z sex chromosome in three woodpecker species (Picidae). The Z chromosome corresponds to the larger element of their karyotype due to the accumulation of such sequences, which increased its size (de Oliveira *et al*., 2017). On the other hand, in *Myiopsitta monachus* (Psittaciformes, Psittacidae) these sequences accumulated preferentially in the W sex chromosome, which has the same size of the Z chromosome, unlike most Neognathae bird species (Furo *et al*., 2017). These two examples show that the analysis and mapping of repetitive sequences in the genome of avian species may contribute for a better understanding of the processes underlying sex chromosomes differentiation and karyotype evolution.

Thus, the analysis of microsatellite sequences in groups of birds showing chromosomal variation both in diploid number and chromosomal morphology, such as Columbiformes, may bring important information concerning their karyotypical evolution. In this study, we report the chromosomal mapping of different repetitive sequences, including 18S rDNA clusters and 11 different microsatellite sequences in Columbidae species in order to verify the role of these sequences in their karyotypical diversity. The results suggest that, despite their lower amount in the genome, repetitive DNAs seem to play an important role in the karyotype evolution of these species.

Material and Methods

Specimens and chromosome preparations

Nine species of Columbidae family were analyzed in this study. Individuals were collected in their natural habitat, except for *G. montana* and *G. violacea*, which were

collected from captivity (Table 1). Experiments followed protocols approved by the Ethics Committee on the Use of Animals (CEUA - Universidade Federal do Pampa, 026/2012, and permission number SISBIO 33860-1 and 44173-1).

Chromosomes were obtained from fibroblast cultures, according to Sasaki *et al*. (1968) or from bone marrow, following Garnero and Gunski (2000). Both techniques included exposition to colcemid (1 h, 37 ºC), hypotonic treatment (0.075MKCl, 15 min, 37 ºC) and fixation with methanol/acetic acid (3:1).

Chromosome probes and FISH experiments

18S rDNA fragments were amplified by PCR using primers NS1 5'-GTA GTC ATA TGC TTG TCT C-3' and NS8 5'-TCC GCA GGT TCA CCT ACG GA-3' and nuclear DNA of *Ocyurus chrysurus* (Perciformes: Lutjanidae) (White *et al*., 1990). Subsequently, fragments were labeled with digoxigenin by nick translation (Roche) and detected with anti-digoxigenin-rhodamine, following the manufacturer's instructions. Preparation of slides, hybridization and washes were performed according to Daniels and Delany (2003).

FISH experiments using microsatellite probes were done according to Kubat et al. (2008). Oligonucleotides $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CG)_{15}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(TA)_{15}$, directly labeled with Cy3 at the 5terminal were obtained from SIGMA. After denaturation, probes were applied on the slides and incubated for 16 h at 37 ºC in a humid chamber. Next, slides were washed twice in 2xSSC, twice in 1xSSC, and in PBS (phosphate buffered saline), and then dehydrated in an ascending ethanol series (70, 90 and 100%).

At least 30 metaphase spreads were analyzed to confirm the 2n, karyotype structure and FISH results. Images were captured using a Zeiss Imager Z2, coupled with the software Axiovison 4.8 (Zeiss, Germany). The chromosomes were classified as metacentric (m), submetacentric (sm), telocentric (t) or acrocentric (a) according to their arm ratios (Guerra, 1986).

Results

Diploid number and chromosomal morphology of the species analyzed are described in Table 2. Figures 1 and 2 show the karyotypes in conventional staining. We found a morphological variation in the Z chromosome of *L. verreauxi*, which corresponded to a submetacentric or acrocentric element (Figure 1). Additionally, pair 3 also showed morphological variation in *G. montana* as telocentric and acrocentric (Figure 2b).

18S rDNA probes hybridized onto microchromosomes in the nine species analyzed here. In *Z. auriculata, G. montana, G. violacea, L. verreauxi, P. cayennensis, C. livia, C. talpacoti* and *C. passerina* this sequences were detected in only one microchromosome pair, however, in *C. picui* these probes revealed the presence of clusters in three pairs of microchromosomes. Examples of 18S rDNA hybridization in the Columbidae are shown in Figure 3.

Chromosome mapping of microsatellite sequences

Of the nine species analyzed, only *C. picui* showed no hybridization signals for the microsatellite sequences used. In this species, we performed the hybridizations with chromosomal preparations obtained from two distinct protocols, fibroblasts and direct culture of bone marrow and obtained the same negative result. The other species showed an exclusive pattern of distribution for at least some of the microsatellite sequences used (Table 3). In general, these sequences were preferentially accumulated in the centromeric region of some macrochromosome pairs, in microchromosomes and in the W chromosome. There was no evident signal in the Z chromosome of any species. In addition, pair 2 showed an interesting accumulation of some sequences, of which the position varied in some species – a single band in the short arms in *Z. auriculata*, *C. passerina* and *C. talpacoti*, a single band in the long arms in *L. verreauxi*, *G. montana* and *P. cayennensis*, and two bands (GA_{15}) in the short arms in *P. cayennensis*. The highest number of sequences was found in *L. verreauxi* (Figure 4). Representative experiments of other species are shown in Figure 5.

Discussion

Corroborating previous studies (Takagi and Sasaki, 1974; de Lucca and de Aguiar, 1976; de Lucca, 1984) we observed a variation in the 2n number of the Columbidae species analyzed, ranging from 76 (*Z. auriculata, C. picui*, C. *passerina, P. cayennensis*

and *C. talpacoti*) to 86 (*G. violacea* and *G. montana*) *L. verreauxi* and *C. livia* showed an intermediate 2n (78 and 80, respectively). Among the species, the karyotype of *G. violacea* was described for the first time, showing that this species has a karyotype very similar to another species of this genus, *G. montana*, both in terms of chromosome morphology and in the diploid number.

In birds, it is accepted that the presence of one pair of microchromosomes bearing 18S rDNA clusters is the ancestral state, considering that this is the condition observed in basal groups, such as Ratites and Galloanserae (Ladjali-Mohammedi *et al*., 1999; Nishida-Umehara *et al*., 2007), and also in many species belonging to more derived groups, such as some Passeriformes and Accipitriformes (Tagliarini *et al*., 2011; dos Santos *et al*., 2015). This characteristic seems to be conserved also in Columbiformes, since, with the exception of *Columbina picui*, which showed three pairs of microchromosomes bearing 18S rDNA clusters, the other eight species analyzed presented only one microchromosome pair bearing these clusters, including two other *Columbina* species. One of the most accepted causes of this variation, even among phylogenetically related species, is the transposition or translocation of these sequences (Nishida *et al*., 2008; Kretschmer *et al*., 2014).

Considering the microsatellite sequences, we applied eleven different oligonucleotide probes, which gave different results for each species, demonstrating that the analysis of these repetitive sequences may represent an important chromosome marker in evolutionary and phylogenetic studies in birds. Only one species, *C. picui*, did not show a signal for any of the sequences used. A possible explanation is that microsatellites have a characteristic mutational behavior, with rates that are 10 to 100,000 times higher than the average mutation rates in other parts of the genome (Gemayel *et al*., 2010). Therefore, a microsatellite sequence can expand (addition of repeat units) or contract (deletion of repeat units) (López-Flores and Garrido-Ramos, 2012). It is possible that contraction of the microsatellites sequences occurred in C. picui, so the probes used were not complementary to the new sequence, considering the limitations inherent to FISH techniques, which needs at least 2–5 kb to be visible.

Accumulation of microsatellites in pair 2 was observed in practically all species, (the exceptions were *C. livia* and *C. picui*), although in different positions (Figure 6), probably due to intrachromosomal rearrangements, such as inversions, which are very frequent among birds (Warren *et al*., 2010; Kretschmer *et al*., 2014, 2015; dos Santos *et al*., 2015, 2017). Interestingly, while $(GGA)_{10}$ produced signals in pair 2 of *Zenaida*

auriculata, this sequence did not produce any signal in the two species of the genus *Geotrygon*. Instead, the sequence $(GA)_{15}$ hybridized in pair 2 of *G. montana* and G. *violacea*. In the remaining species, a higher number of sequences accumulated in pair 2: *L. verreauxi* [$(CA)_{15}$, $(GA)_{15}$, $(GAA)_{10}$, $(CAC)_{10}$, $(CGG)_{10}$ and $(GAG)_{10}$]; *P. cayennensis* [$(CA)_{15}$, $(GA)_{15}$, $(GAA)_{10}$ and $(GAG)_{10}$]; *C. talpacoti* [$(CA)_{15}$, $(GA)_{15}$, $(GAA)_{10}$ and $(CAC)_{10}$], and; *C. passerina* [$(CA)_{15}$, $(GA)_{15}$, $(GAA)_{10}$ and $(CAC)_{10}$].

From a phylogenetic point of view, the occurrence of the same sequences found in the same position in pair 2 of different species could be a reflection of a common origin, as for example the sequences $(CA)_{15}$, (GA)15, $(GAA)_{10}$ and $(CAC)_{10}$ in the species *L. verreauxi*, *C. talpacoti*, and *C. passerina*, and the three first ones in P. *cayennensis*. Furthermore, a more detailed analysis of these sequences in pair 2 of Columbidae species revealed that this pair is very informative about the karyotypical evolution in this group.

For instance, the presence of $(GA)_{15}$ in pair 2 of *Geotrygon* species, which is telocentric in this species but submetacentric in most of the other ones, suggests the occurrence of a chromosomal rearrangement, such as an inversion or fission in this pair. However, if we consider that the 2n of *Geotrygon* is higher than that for the other species (2n=86), with pair 2 being slightly smaller (Figure 1), it seems that fission is the most probable rearrangement to have occurred in this genus. Moreover, the sequence $(GA)_{15}$ hybridized in two different bands in the long arms of pair 2 in *P. cayennensis*, probably due to an inversion, which fragmented the block of repetitive sequences in two distinct ones. Similarly, the variation in the position of these repetitive sequences blocks in chromosome 2 – 2p in *C. passerina* and *C. talpacoti*, while 2q in *L. verreauxi*, *G. montana*, *G. violacea*, *P. cayennensis* – adds evidence for the occurrence of intrachromosomal rearrangements. A possible approach to test this hypothesis is the use of whole-chromosome probes of a species in which the syntenic group corresponding to GGA1 is found fragmented, such as *Leucopternis albicollis* (Falconiformes, Accipitridae), in which GGA2 corresponds to three different pairs (de Oliveira *et al*., 2010).

The importance of repetitive sequences in chromosomal instability has been proposed by some authors (e.g. Ruiz-Herrera *et al*., 2006). For example, the molecular characterization of evolutionary breakpoints in the genome of humans, primates and mouse has demonstrated that the genomic reorganizations mainly occur in regions with duplications or with some type of repetitive sequences, such as the dinucleotide $(TA)_n$, or close to these regions (Kehrer-Sawatzki *et al*., 2005; Fan et al., 2002; Kehrer-Sawatzki *et*

al., 2002; Locke *et al*., 2003). Although there is no single sequence responsible for the chromosomal instability, it is known that common fragile sites are enriched with A/T sequences and have the potential to form secondary structures (Schwartz *et al*., 2006; Glover, 2006). These features may affect the DNA replication and lead to chromosomal instability (Ruiz-Herrera *et al*., 2006). Interestingly, the dinucleotide $(TA)_{15}$ did not produce any positive signals in our studies, revealing a possible characteristic intrinsic to the genome of birds. Although the absence of signals may reflect not only the inexistence of clusters of this sequence, it may instead represent a lower number of repetitions, considering the limitations inherent to FISH techniques, which needs at least 2–5 kb to be visible. This lower number of repetitions may be related to the small size of the genome of birds, at the expense of loss of repetitive sequences (Hughes and Hughes, 1995; Organ and Shedlock, 2009; Zhang and Edwards, 2012).

Concerning sex chromosomes, it is widely accepted that the accumulation of repetitive sequences plays an important role in the differentiation of the element found exclusively in the heterogametic sex – W or Y (Matsubara *et al*., 2015). For instance, none of the sequences produced any signals in the Z chromosome, while different sequences were found accumulated in the W chromosome of the three species of which we analyzed female individuals: *C. livia* [$(CAA)_{10}$, $(CGG)_{10}$, $(GA)_{15}$ and $(GAG)_{10}$]; *G. violacea* [$(GA)_{15}$ and $(GAG)_{10}$], and *L. verreauxi* [$(CA)_{15}$, $(CAA)_{10}$, $(CGG)_{10}$, $(CAC)_{10}$, $(GAG)_{10}$, $(GAA)_{10}$ and $(GA)_{15}$]. Of these, two were also found in the W chromosome in *Gallus gallus*: sequences $(GA)_{15}$ and $(GAG)_{10}$ (Matsubara *et al*., 2015). Interestingly, these two sequences were shared by the three Columbidae species, possibly denoting some type of ancestral state. In fact, microsatellites are considered early colonizers of sex chromosomes and the differential accumulation of the same class of repeats on the W chromosome of distinct species reflects the inherent dynamism of these sequences (Charlesworth *et al*., 2005).

In summary, this study demonstrated the ubiquitous presence of repetitive elements in the genome of several Columbidae species, highlighting their possible role in the chromosomal diversification within this group. In addition, our data reinforced the view that the existence of one pair of microchromosomes bearing 18S rDNA clusters is apparently an ancestral character retained in Columbidae, and that repetitive sequences did preferentially accumulate in the centromeric regions of macro and microchromosomes, as well as in the W chromosomes. Additionally, despite the fact that studies with repetitive sequences in birds are still incipient, the comparison of our data with the ones for

Psittaciformes, Piciformes and Galliformes (Matsubara *et al*., 2015; de Oliveira *et al*., 2017; Furo *et al*., 2017) shows interesting variation in accumulation sites for some of them, reinforcing microsatellites as important markers for studies on karyotype evolution.

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Tables

Table 1. Information concerning the individuals sampled for this study.

Species	**Number of individuals/Sex**	**City/State***
Zenaida auriculata	2 ♂	São Gabriel/RS
Leptotila verreauxi	1 ♂ and 2 ♀	Santa Maria/RS
Columba livia	1 ♀	São Gabriel/RS
Columbina picui	2 ♂	Santa Maria and Porto Vera Cruz/RS
Columbina passerina	1 ♂	Belém/PA
Columbina talpacoti	3 ♂ and 1 ♀	Porto Vera Cruz/RS
Patagioenas cayennensis	2 ♂	Porto Vera Cruz /RS
Geotrygon violacea	1 ♀	Belém/PA
Geotrygon montana	1 ♂	Belém/PA

*RS= Rio Grande do Sul and PA= Pará Brazilian States.

Table 2 – Diploid number and chromosomal morphology of the nine Columbidae species included in this study.

		Chromosomes											
Species	2n	1	2	3	4	5	6	7	8	9	10	Z	W
Z. auriculata	76	SM	SM	A	SM	SM	T	T	T	T	T	M	-
G. montana	86	T	T	*	T	T	T	T	T	T	T	M	-
G. violacea	86	T	T	T	T	T	T	T	T	T	T	M	SM
L. verreauxi	78	SM	SM	A	M	A	A	A	M	T	T	*	SM
C. livia	80	SM	SM	A	SM	SM	T	T	T	T	T	M	M
P. cayennensis	76	SM	SM	A	M	A	A	A	T	T	T	M	-
C. talpacoti	76	SM	SM	A	M	M	T	T	T	T	T	M	-
C. passerina	76	SM	SM	A	M	M	T	T	T	T	T	M	-
C. picui	76	SM	T	T	T	T	M	A	T	M	T	T	-

2n = diploid number, M = metacentric, SM = submetacentric, A = acrocentric, T = telocentric, * = variable morphology.

Table 3 - Hibridization of 11 microsatellite sequences in nine Columbidae species. (-) no hybridization signals.

Repeat motif	Species								
	ZAU	LVE	PCA	GVI	GMO	CLI	CPI	CTA	CPA
$(CA)_{15}$	Centromere of machrocromosomes	Pericentromeric region of 2p; W centromere	Pericentromeric region of pairs 5 and 6; 2q	-	-	Centromere pairs 6-10	-	Pericentromeric region of 2p; telomere of 2p and 1p; centromere of pair 5	Pericentromeric region of 2p; telomere of 1p; centromere of pair 4; one pair of microchromosome
$(TA)_{15}$	-	-	-	-	-	-	-	-	-
$(GA)_{15}$	Most microchromosomes	Pericentromeric region of 2p; W q and p; centromere of pair 5	Two blocks in 2q	Chromosome W p and q; 2p	2q; 4q	Two pairs microchromosomes; all chromosome W	-	Pericentromeric region of 2p	2p
$(CAA)_{10}$	-	W centromere	-	Some microchromosomes	Two pairs of microchromosomes	Centromere pairs 6-10; all chromosome W	-	-	-
$(GAA)_{10}$	Pericentromeric region of 2p; centromere of most microchromosomes	Pericentromeric region of 2p; Wq; one pair of microchromosomes	2q	-	-	-	-	Pericentromeric region of 2p; centromere of pair 4; telomere of 1q	2p
$(CAT)_{10}$	-	-	-	-	-	-	-	-	-

$(GC)_{15}$	-	Some microchromosomes	-	-	-	-	-	-	-
$(CGG)_{10}$	One pair of microchromosomes	Pericentromeric region of 2p; terminal region of W	One pair of microchromosomes	-	Two pairs of microchromosomes	Two pairs of microchromosomes; all chromosome W	-	One pair of microchromosomes	-
$(CAG)_{10}$	-	Some microchromosomes	Three pairs of microchromosomes	-	-	-	-	One pair of microchromosomes; centromere of 6 pair	Some microchromosome; centromere of pair 6
$(CAC)_{10}$	-	Pericentromeric region of 2p; W centromere and q	Pericentromeric region of pair 5	-	-	-	-	Pericentromeric region of 2p; telomere of 1p	2p
$(GAG)_{10}$	Most microchromosomes	Pericentromeric region of 2p; Wq	Some microchromosomes; 2q	Some microchromosomes	Some microchromosomes	Some microchromosomes; all chromosome W	-	Telomere and centromere of pair 6; Some microchromosomes	Some microchromosome; centromere of pair 6

Figures

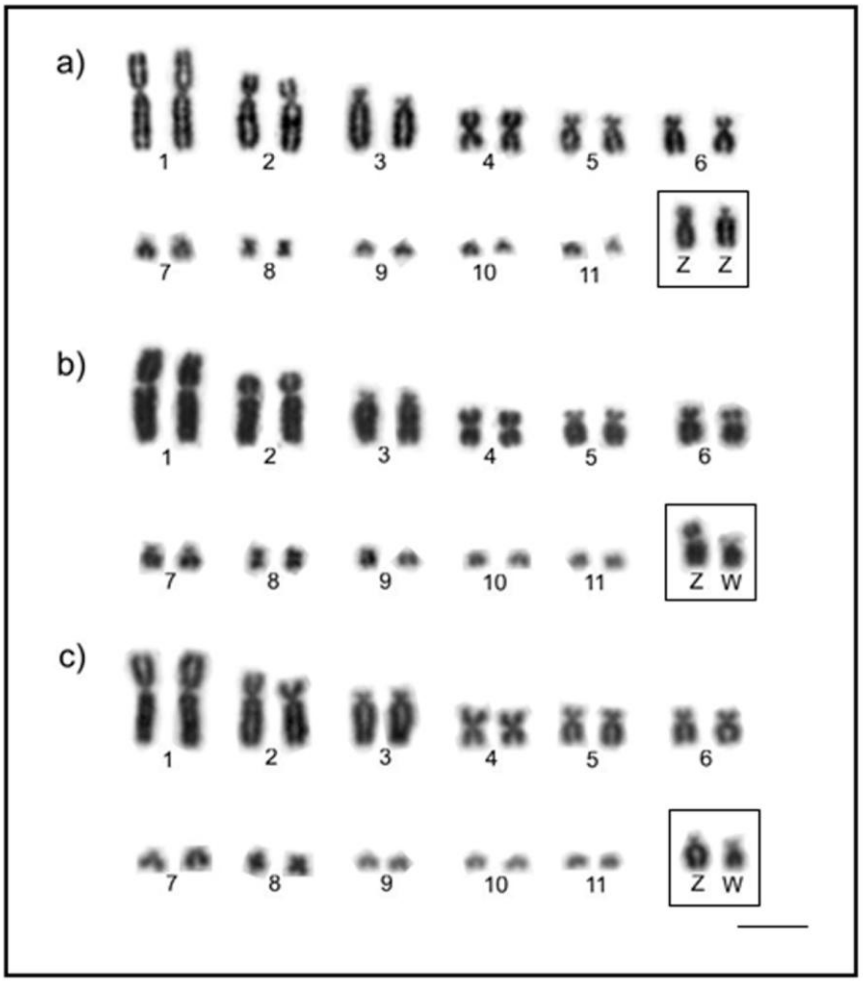

Figure 1 - Partial karyotype showing the largest autosomal pairs and ZW sex chromosomes of three *Leptotila verreauxi* individuals analyzed by conventional Giemsa-staining: (a) male with a submetacentric and acrocentric Z chromosomes; (b) female with submetacentric Z and W chromosomes, (c) female with an acrocentric Z and a submetacentric W chromosome. Sex chromosomes are boxed. Bar = 5µ.

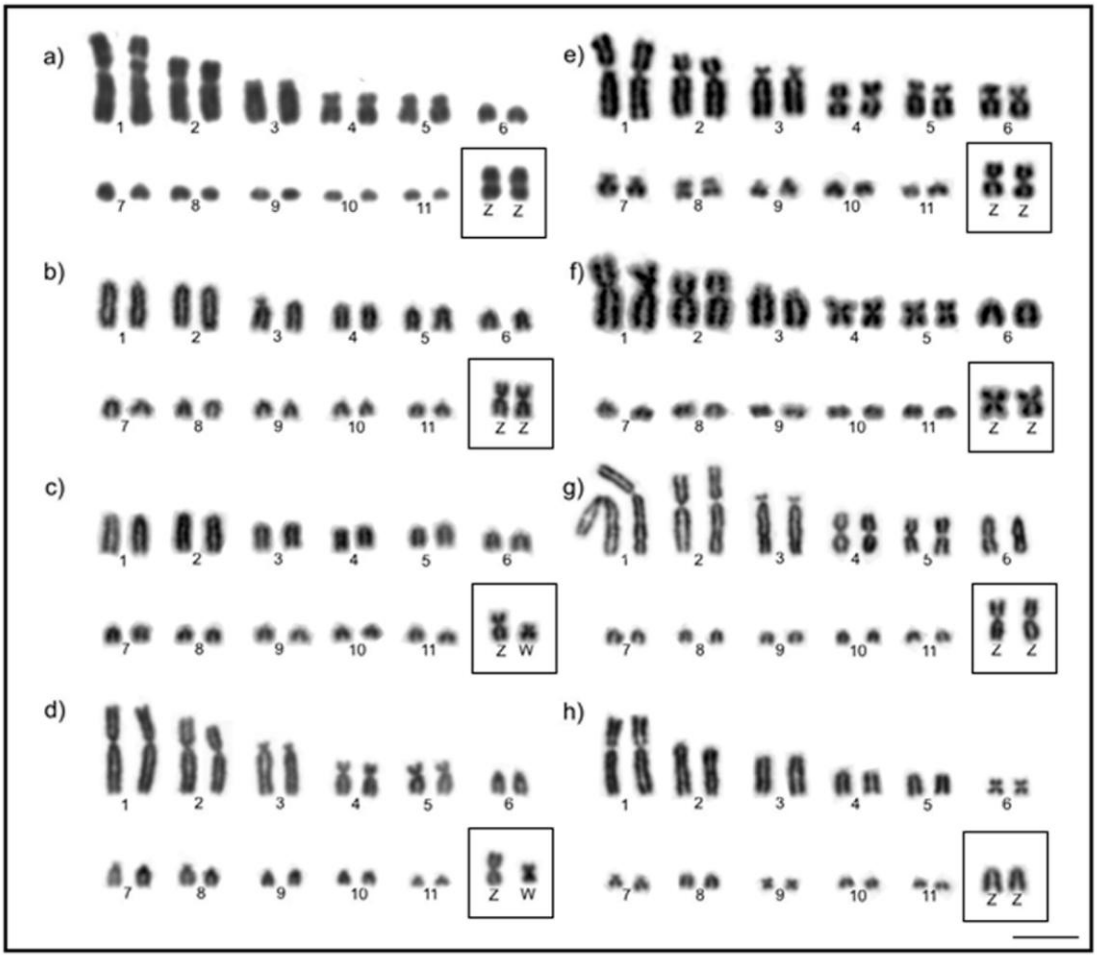

Figure 2 - Partial karyotype showing the largest autosomal pairs and ZW sex chromosomes of eight Columbidae analyzed by conventional Giemsa-staining: (a) *Zenaida auriculata*, male; (b) *Geotrygon montana*, male; (c) *Geotrygon violacea*, female; (d) *Columba livia*, female; (e) *Patagioenas cayennensis*, male; (f) *Columbina talpacoti*, female; (g) *Columbina passerina*, male; (h) *Columbina picui*, male. Sex chromosomes are boxed. Bar = 5 µ.

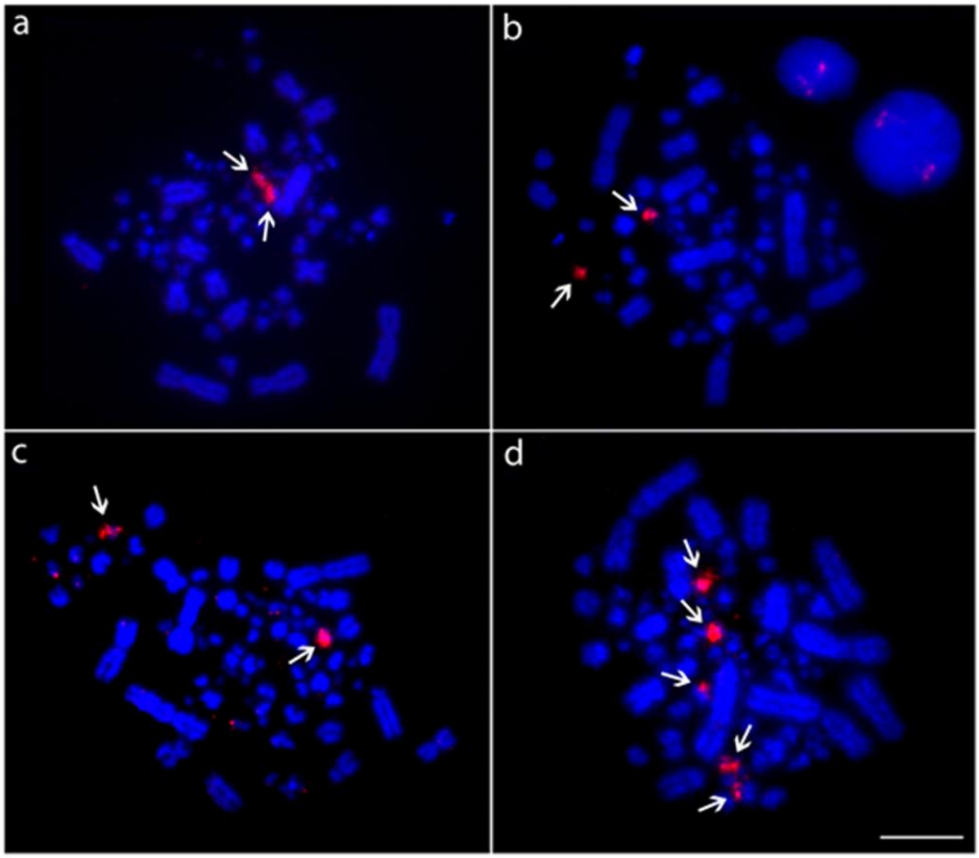

Figure 3 - Representative examples of FISH experiments using 18S rDNA probes in Columbidae species. (a) *L. verreauxi*; (b) *Z. auriculata*; (c) *C. livia*; (d) *C. picui*. The arrows point to the hybridization signals. Bar = 5 µ.

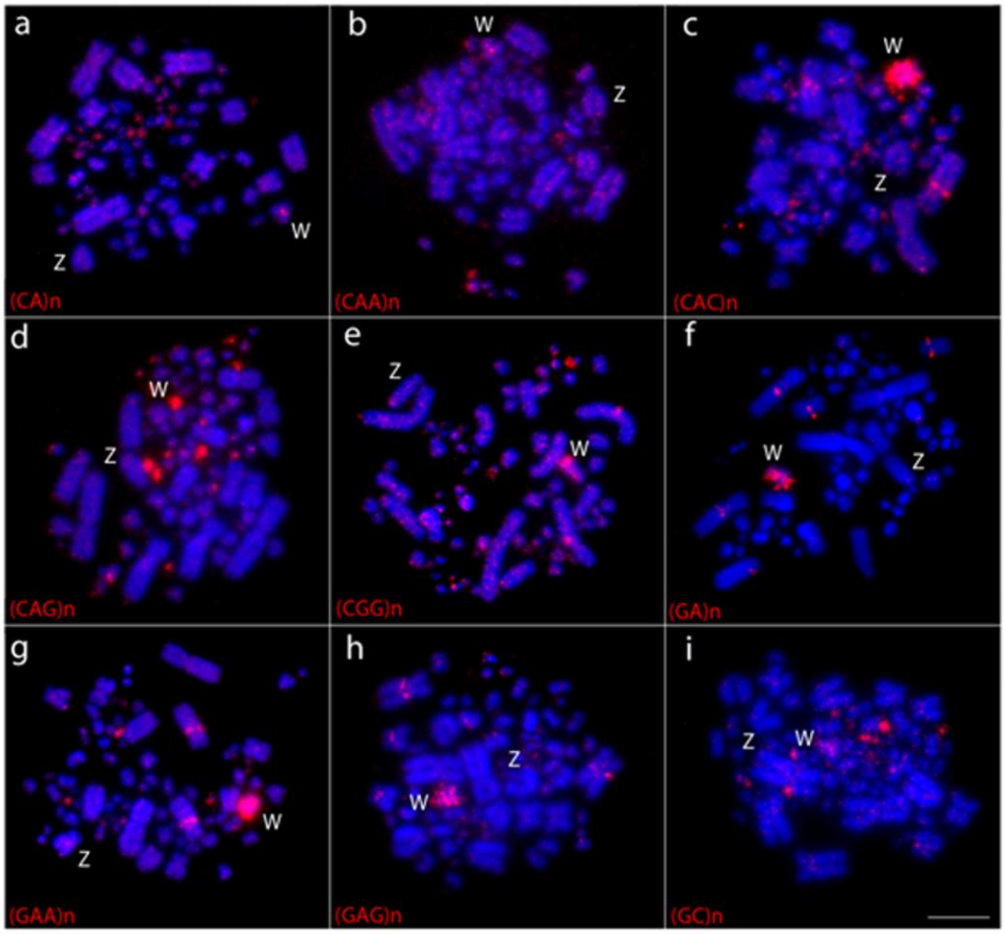

Figure 4 - Metaphases of a female *Leptotila verreauxi* in experiments of FISH using nine different microsatellite sequences (a-i). Chromosomes were counterstained with DAPI (blue) and probes were directly Cy3 (red) labeled. Microsatellite sequences are indicated on the bottom left of each figure. Sex chromosomes are indicated in each metaphase. Bar = 5 µ.

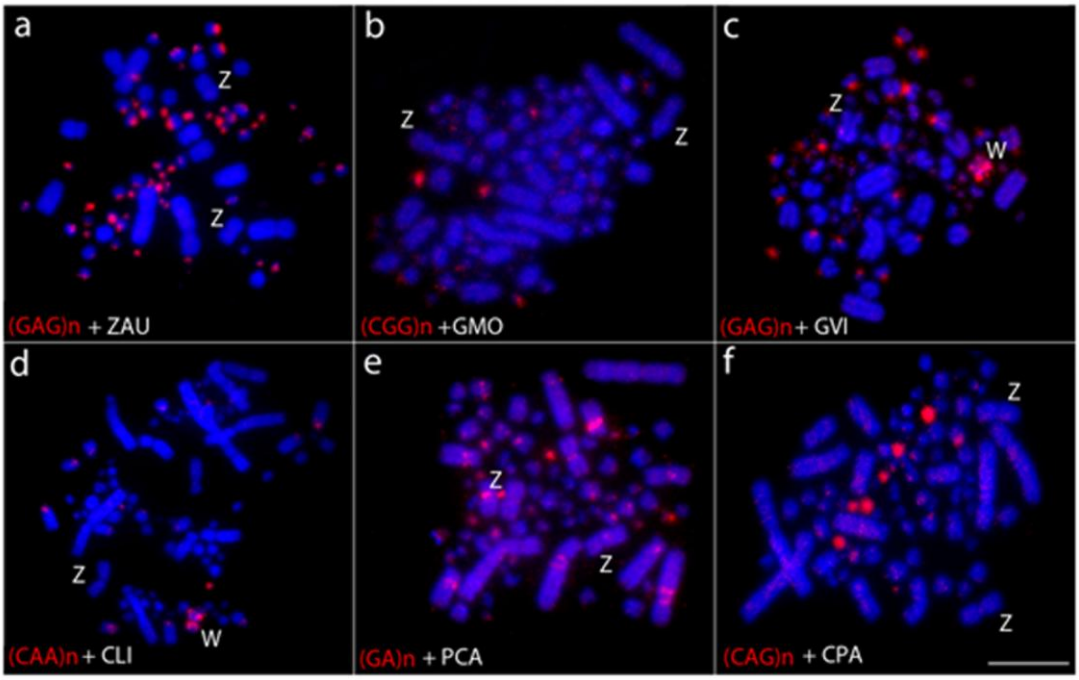

Figure 5 - Representative examples of FISH experiments using microsatellite sequences in six Columbidae species (a-f). Probes were directly labeled with Cy3 (red), while chromosomes were counterstained with DAPI (blue). Microsatellite sequences are indicated on the bottom left of each figure. Sex chromosomes are indicated in each metaphase. ZAU: *Zenaida auriculata* (a); GMO: *Geotrygon montana* (b); GVI: *Geotrygon violacea* (c); CLI: *Columba livia* (d); PCA: *Patagioenas cayennensis* (e); CPA: *Columbina passerina* (f). Sex chromosomes are indicated in each metaphase. Bar = 5 µ.

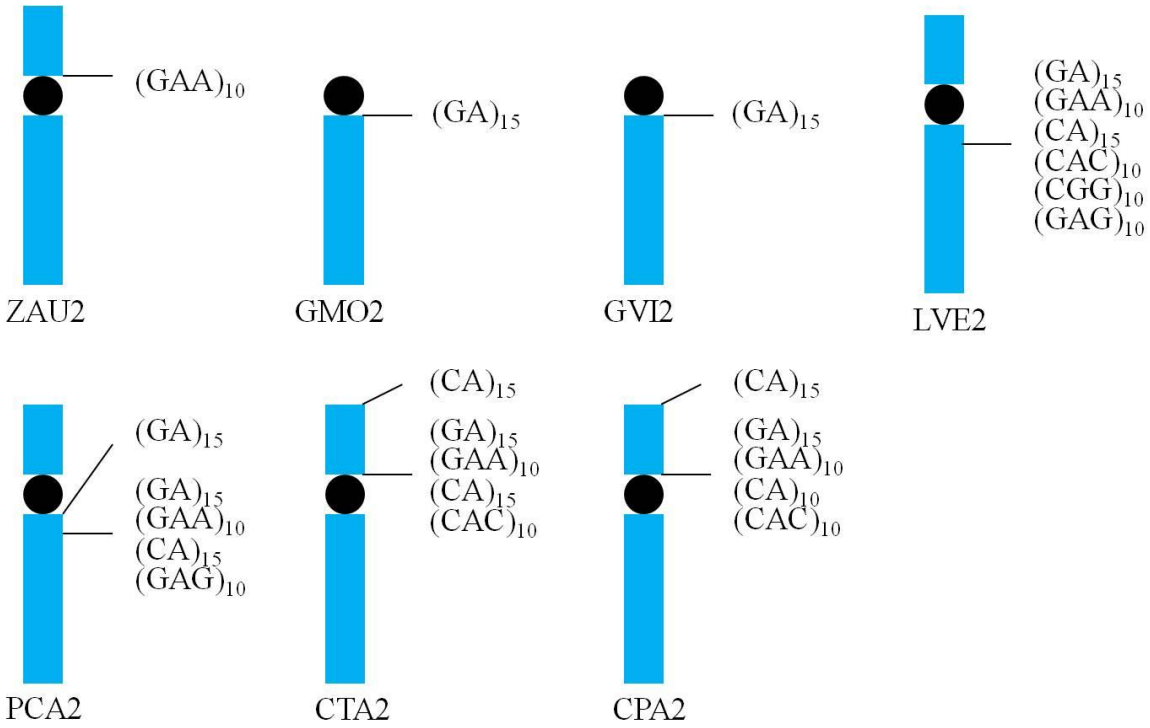

Figure 6 - Distribution and localization of microsatellite sequences in chromosome 2 of seven Columbidae species: ZAU (*Zenaida auriculata*), LVE (*Leptotila verreauxi*), PCA (*Patagioenas cayennensis*), GVI (*Geotrygon violacea*), GMO (*Geotrygon montana*), CTA (*Columbina talpacoti*) and CPA (*Columbina passerina*).

5. Capítulo III

Comparative chromosome painting in Columbidae (Columbiformes) reinforces divergence in Passerea and Columbea

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6. Capítulo IV

Extensive genomic reshuffling in wattled jacana indicates a exclusive karyotype evolution in Charadriiformes

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Artigo a ser submetido no periódico *Plos one*.

7. Considerações finais

O trabalho apresentado no capítulo I reúne informações sobre a citogenética de Aves e o grande esforço em reconstruir a história evolutiva das Neoaves principalmente através do sequenciamento do DNA. Entretanto, apesar da importância do conhecimento sobre o cariótipo em estudos filogenéticos, especialmente através das comparações cromossômicas com os resultados obtidos com a hibridização *in situ* fluorescente, a maioria das Ordens da Classe Aves ainda não possui nenhuma espécie analisada pela citogenética molecular. Além disso, a maioria dos artigos com pintura cromossômica são baseados apenas nas hibridizações de sondas de *Gallus gallus*, sendo que poucas espécies foram analisadas com diferentes conjuntos de sondas, tais como *Leucopternis albicollis* e *Gyps fulvus*, que são mais informativas, pois permitem identificar rearranjos intracromossômicos.

No capítulo II é demonstradas que ambas citogenética clássica e molecular foram úteis para elucidar a variabilidade cariotípica da família Columbidae. A distribuição das sequências de rDNA 18S demonstrou a conservação do cluster em todas as espécies analisadas, exceto em *Columbina picui*. A distribuição das sequências microssatélites revelou o acúmulo preferencial nas regiões centroméricas de alguns macrocromossomos, alguns microcromossomos, no cromossomo sexual W e no segundo par da maioria das espécies. Neste capítulo é levantada a hipótese da ocorrência de fusões, fissões e inversões durante a evolução cariotípica deste grupo. Além disso, com base na localização de blocos de sequências microssatélites é levantada a ideia de que talvez sequências repetitivas estejam associadas a esses rearranjos.

Os resultados apresentados e discutidos no capítulo III indicam que vários rearranjos cromossômicos ocorreram durante a evolução cromossômica em espécies da família Columbidae. Das quatro espécies analisadas pela pintura cromossômica com sondas de *G. gallus* e *L. albicollis*, apenas para *Leptotila verreauxi* identificamos um rearranjo intercromossômico (associação entre os cromossomos homólogos à GGA6 e GGA7 em LVE4). Entretanto, apesar da conservação sintênica, alguns macrocromossomos estão reorganizados através de rearranjos intracromossômicos. Os resultados também indicam que entre as espécies analisadas, *Z. auriculata* apresenta o cariótipo mais ancestral. A identificação dos rearranjos intracromossômicos no cromossomo correspondente ao

GGA1q em quatro espécies de diferentes gêneros, juntamente com comparação dos dados da literatura, corroboram com a recente divergência encontrada nas Neoaves (Passerea e Columbea). Interessantemente, o bloco de sequências microssatélites no cromossomo 2 da espécie *Leptotila verreauxi* (CAP II) está localizado na região em que foi proposto a ocorrência de rearranjos intracromossômicos (CAP III), indicando uma possível relação entre as sequências microssatélites e a ocorrência de rearranjos cromossômicos. Além disso, as sondas cromosomo-específicas desenvolvidas para *Z. auriculata* mostraram-se mais eficientes, em relação as sondas de *G. gallus*, para a comparação cromossômica em espécies da família Columbidae.

No capítulo IV foi realizada a hibridização das sondas cromossômicas de *Z. auriculata*, desenvolvidas neste trabalho (Cap III), bem como sondas de *G. gallus*, com o objetivo de verificar a eficiência das sondas de *Z. auriculata* em espécies não relacionadas. Para tanto, escolhemos a espécie *Jacana jacana*, a qual pertence à ordem Charadriiformes, caracterizada por uma extensa variação cariotípica. As sondas de *Z. auriculata* mostraram sinais de hibridização mais intensos do que as sondas de *G. gallus*. Nossas análises mostraram que o cariótipo da *Jacana jacana* sofreu uma extensa reorganização cromossômica envolvendo fissões de macrocromossomos e fusões entre os segmentos resultantes. Os resultados obtidos, em comparação com as demais espécies da ordem Charadriiformes analisadas pela pintura cromossômica (*Burhinus oedicnemus*, *Larus argentatus* e *Vanellus chilensis*) indicam que cada uma destas espécies possui uma organização cromossômica exclusiva.

Por fim, os resultados obtidos na presente Tese contribuíram para compreendermos a evolução cromossômica em representantes da família Columbidae e da Classe Aves. Além disso, as sondas cromossomo-específicas desenvolvidas para *Z. auriculata* mostraram-se como uma importante ferramenta na citogenética de Aves.

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