**UNIVERSIDADE FEDERAL DO PAMPA**

**THAYS DUARTE DE OLIVEIRA**

# ORGANIZAÇÃO GENÔMICA DE SEQUÊNCIAS REPETITIVAS EM PICA-PAUS (AVES PICIFORMES)

**São Gabriel**
**2017**

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (PPGCB) da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Ciências Biológicas.

Orientador: Dr. Ricardo José Gunski

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“Todos esses que aí estão
Atravancando o meu caminho,
Eles passarão…
Eu passarinho!”
(Poeminho do Contra - Mario Quintana)

## RESUMO

A caracterização da quantidade e distribuição da fração de DNA repetitivo em genomas auxilia no entendimento de sua organização cromossômica. As Aves são conhecidas por apresentar uma baixa proporção de DNA repetitivo quando comparada a outras classes de Vertebrados. Entretanto, a ordem Piciformes se destaca por apresentar uma quantidade percentual superior dessas sequências comparado com as outras aves. Com isso, o objetivo deste estudo foi determinar a distribuição de diferentes tipos de sequências repetitivas no genoma de três espécies da família Picidae, *Colaptes melanochloros* (2n=84), *Colaptes campestris* (2n=84) e *Melanerpes candidus* (2n=64), por meio de hibridização *in situ* fluorescente (FISH) com sondas de rDNA 18S, teloméricas $(TTAGGG)_n$ e microssatélites. Os resultados mostraram, nessas três espécies, o cromossomo sexual Z como o maior do complemento, esse fato deve-se ao acúmulo de diferentes sequências de microssatélites. Entretanto o cromossomo W de *C. melanochloros*, que é totalmente heterocromático, não apresentou acúmulo destas sequências. Os sítios ribossomais estão organizados em um par de cromossomos com uma constrição secundária e este teve o acúmulo da sequência $(CGG)_{10}$ nas três espécies. As sondas teloméricas apresentaram marcações nas regiões terminais dos cromossomos e marcações intersticiais em alguns macrocromossomos. As marcações intersticiais indicam fusões entre cromossomos ou acúmulo de sequências repetitivas similares as teloméricas. Com as sondas de microssatélites identificou-se o mesmo padrão de hibridização nas espécies de *Colaptes* e padrão distinto entre *Colaptes* e *M. candidus*. As nossas análises de FISH mostraram várias sequências de microssatélites amplificadas no cromossomo Z nas três espécies analisadas, o que pode explicar o fato deste ser o maior elemento do cariótipo e desta família conter maior quantidade de sequências repetitivas comparadas com outros grupos de aves. Curiosamente, nenhuma das sequências foi encontrada acumulada no cromossomo W, apesar de desempenharem um papel importante na diferenciação de cromossomos sexuais. Estes resultados evidenciam que, apesar da origem comum proposta para o sistema sexual ZW em aves, esses cromossomos seguiram diferentes trajetórias evolutivas em cada espécie, indicando uma alta plasticidade para a diferenciação cromossômica sexual neste grupo. Este trabalho é

o primeiro passo para esclarecer o papel das sequências satélites e microssatélites na diferenciação de cromossomos sexuais.

Palavras-Chave: Aves; Cromossomos sexuais; Microssatélites; Telomérica, rDNA.

## ABSTRACT

The characterization of the amount and distribution of the repetitive DNA fractions in genomes assists in the understanding of their chromosomal organization. The Birds are characterized by presenting a low proportion of repetitive DNA when compared to other classes of Vertebrates. However, the order Piciformes stands out for having a higher percentage of these sequences compared to other birds. The objective of this study was to determine the distribution of different types of repetitive sequences in the genome of three species of the family Picidae, *Colaptes melanochloros* (2n = 84), *Colaptes campestris* (2n = 84) and *Melanerpes candidus* (2n = 64) by fluorescence in situ hybridization (FISH) with 18S, telomeric (TTAGGG) and microsatellite rDNA probes. The results showed, in these three species, the sexual chromosome Z as the largest of the complement, this fact is due to the accumulation of different sequences of microsatellites. However, the W chromosome of *C. melanochloros*, which is totally heterochromatic, did not show accumulation of these sequences. The ribosomal sites are organized on a pair of chromosomes with a secondary constriction and this had the accumulation of the sequence $(CGG)_{10}$ in the three species. The telomeric probes showed markings in the terminal regions of the chromosomes and interstitial markings on some macrochromosomes. Interstitial markings indicate fusions between chromosomes or the accumulation of repetitive sequences similar to the telomeric ones. With the microsatellite probes the same pattern of hybridization was identified in the *Colaptes* species, distinct pattern between *Colaptes* and *M. candidus*. Our FISH analyzes showed several amplified microsatellite sequences on the Z chromosome in the three species analyzed, which may explain the fact that this is the largest element of the karyotype and that its genome contains the largest number of repetitive sequences compared to other groups of Birds. Interestingly, none of the sequences were found to be accumulated on the W chromosome, although they play an important role in the differentiation of sex chromosomes. These results show that, despite the common origin proposed for the ZW sexual system in birds, these chromosomes followed different evolutionary trajectories in each species, indicating a high plasticity for the sexual chromosome differentiation in this group. This work is the first step to clarify the role of satellites and microsatellite sequences in the differentiation of sex chromosomes.

Keywords: Birds; Sex chromosomes; Microsatellites; Telomerase, rDNA.

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### INTRODUÇÃO



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### MATERIAL E MÉTODOS



### CAPÍTULO 1

## LISTA DE ABREVIATURAS E SIGLAS

A - Acrocêntrico

CEUA - Comissão de Ética no Uso de Animais

CI - Índice Centromérico

COL - *Colaptes*

CR1 - Chicken Repeat 1

Cy3 - Cianina 3

DAPI - 4',6-diamidino-2-fenilindol

DMEM - Dulbecco's Modified Eagle Medium

DNA - Ácido Desoxirribonucleico

EDTA - Ethylene Diamine Tetraacetic Acid

FISH - Hibridização *in situ* fluorescente

IBAMA - Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis

ITS - Sequência Telomérica Intersticial

KCl - Cloreto de potássio

M - Metacêntrico

MCA - *Melanerpes candidus*

PBS - Tampão fosfato-salino

pb - Pares de base

PCR - Reação em Cadeia da Polimerase

rDNA - Ácido Desoxirribonucleico Ribosomal

RPMI - Roswell Park Memorial Institute

rRNA - Ácido Ribonucleico Ribosomal

SISBIO – Sistema de Autorização e Informação em Biodiversidade

SM - Submetacêntrico

SSC - Solução Salina de Citrato de Sódio

SSRs - Repetições de Sequências Simples

T - Telocêntrico

**SUMÁRIO**

# 1 INTRODUÇÃO

## 1.1 Genoma das Aves

As Aves possuem um genoma altamente compactado, os menores genomas entre os amniotas, cerca de três vezes menor quando comparado com mamíferos (SCHMID; BURT; NAZIONALE, 2000). Acredita-se que essa compactação do genoma e a diminuição do tamanho das células estejam relacionadas com exigências metabólicas para o vôo e podem estar associados ao mecanismo de reparo de DNA (GREGORY et al., 2009; SCHUBERT; VU, 2016; WRIGHT; GREGORY; WITT, 2014; ZHANG; EDWARDS, 2012).

Um modelo de evolução de genomas pressupõe que ocorrem menos mudanças evolutivas em genomas menores, o que corroborava com o conceito de que os genomas das aves possuem uma conservação incomum de cromossomos (ELLEGREN, 2010; OLIVER et al., 2007). Porém com o aumento dos estudos citogenéticos nos últimos anos observa-se uma alta variação de número cromossômico, variando de 40 cromossomos para *Falco columbarius* à 136-142 para *Corythaixoides concolor* (CHRISTIDIS, 1990; NISHIDA et al., 2008). O alto número de cromossomos em aves é devido à presença de microcromossomos (cromossomos menores que 20 MB), essa característica surgiu no ancestral das Aves devido à fissão do macrocromossomos ancestrais e cromossomos médios em microcromossomos ainda menores (BURT, 2002; HILLIER et al., 2004).

O sequenciamento recente de 48 espécies abrangendo toda a filogenia das aves ampliou o conhecimento sobre esses genomas, demonstrando que a quantidade de sequências repetitivas nesse táxon também é menor quando comparada com outros amniotas, sendo de 4% a 10% em aves enquanto em mamíferos pode chegar a 52% do genoma (KOEPFLI et al., 2015). Uma exceção encontrada foi em *Picoides pubenses* (Picidae) onde a fração repetitiva do genoma ultrapassa 22% (ZHANG et al., 2014).

## 1.2 Sequências Repetitivas

O DNA repetitivo consiste de sequências idênticas ou semelhantes, dispostas em *tandem* (lado a lado) ou dispersas por todo o genoma (MARTINS et al., 2010).

Com o avanço nos sequenciamentos e anotações dos genomas observou-se que essa fração do genoma é maior do que se suponha (KIDWELL, 2002; KORDIS, 2010; ZHANG et al., 2014). Os DNAs repetitivos são compostos tanto por sequências funcionais, quanto não codificantes. Entre as sequências repetitivas encontram-se elementos transponíveis, famílias multigênicas, satélites, minissatélites e microssatélites (JURKA et al., 2005).

As sequências repetitivas são responsáveis por causar variações genéticas entre espécies e até mesmo em indivíduos de uma mesma população, além de contribuir na replicação do DNA (LI et al., 2002), recombinação (BIET; SUN; DUTREIX, 1999), expressão gênica (LIU et al., 2001), na diferenciação de cromossomos sexuais em plantas (KUBAT et al., 2008), repteis (POKORNÁ; KRATOCHVÍL; KEJNOVSKÝ, 2011) e peixes (CIOFFI et al., 2010; YANO et al., 2014), ou seja, na organização estrutural e funcional do genoma em geral. Essas seqüências repetidas podem também estar envolvidas em rearranjos cromossômicos, tais como deleções, duplicações, inversões e translocações recíprocas, sendo responsáveis por proporções significativas das variações cariotípicas observadas em muitos grupos (KIDWELL, 2002).

Os elementos transponíveis diferenciam dos outros tipos de sequências repetitivas devido sua capacidade intrínseca de se movimentar no genoma hospedeiro (WICKER et al., 2007). O elemento mais abundante conhecido no genoma de aves é um retroelemento identificado em *Gallus gallus,* chamado *chicken repeat 1* (CR1), possuindo mais de 100000 cópias em seu genoma (VANDERGON; REITMANT, 1993; WICKER et al., 2005).

As famílias multigênicas são genes com alta similaridade estrutural e funcional, podem ocorrer em múltiplas cópias no genoma dispostos em *tandem* ou dispersos pelo genoma. As famílias gênicas responsáveis pelos genes de RNA ribossômico (rRNA) estão entre as mais estudadas, pois formam a estrutura básica das subunidades menor e maior dos ribossomos, ou seja, possuem importância estrutural e catalítica nas células (MARTINS, 2007; MARTINS et al., 2010). Os genes de rRNA em vertebrados superiores são organizados em *tandem* e constituídos por duas famílias gênicas, denominadas 45S DNA ribossômico (45S rDNA) e 5S rDNA. A família 45S rDNA contém genes que codificam 18S, 5,8S, e 28S rRNA, enquanto a segunda família codifica apenas 5S rRNA (MARTINS et al., 2010). As três moléculas de rRNA, que compõe a 45S rDNA, são transcritas a partir

de um único promotor pela RNA polimerase I, são separadas por espaçadores intergênicos e encontram-se na mesma localização cromossômica (EICKBUSH; EICKBUSH, 2007). Porém as duas famílias de genes ribossomais geralmente não estão na mesma localização cromossômica, embora haja exceções (LEITCH; HESLOP-HARRISON, 1993). As unidades de repetição de rDNA são evolutivamente dinâmicas encontram-se espalhadas pelo genoma criando novos *loci* de rDNA e também são altamente conservadas nas espécies (IBORRA; COOK, 2002). Utilizando sondas de rDNA 18S/28S em diferentes ordens de aves observa-se uma variação de números de sítios encontrados, variando de um par, como para *Rhea americana* a seis à sete pares para *Falco peregrinus* (NISHIDA-UMEHARA et al., 2007; NISHIDA et al., 2008).

Os DNAs satélites são sequências nucleotídicas com cerca de 100 a 300 pb diversas e altamente repetidas, organizadas em *tandem* e variam em seu número de cópias nos genomas eucariotos, estão organizadas principalmente nas regiões dos telômeros e dos centrômeros e são o principal componente da heterocromatina (MARTINS, 2007). Os minissatélites são repetições com cerca de 10 – 100 pb agrupadas geralmente próximos aos telômeros, comum a todos os eucariotos. Os microssatélites ou SSRs (repetições de sequências simples) são pequenas repetições de 1-6pb dispostas em *tandem* intercalas em todo o genoma dos eucariotos, podem estar associadas à heterocromatina ou em regiões eucromáticas (MARTINS, 2007). Um exemplo destas sequências são os telômeros, pois são constituídos por uma sequência simples de DNA repetida em *tandem*. Através da utilização de sondas marcadas, foram identificados telômeros contendo a sequência (TTAGGG)n em diversos grupos de aves, das paleognatas às neognatas. Em algumas aves, principalmente em ratitas, esta sequência não foi encontrada apenas nos telômeros. Como em *Struthio camelus* (avestruz) que apresentou marcações intersticiais em macrocromossomos, outros padrões de marcação foram encontrados em microcromossomos, como: marcações nas regiões centroméricas, em ambas as extremidades dos microcromossomos e em todo o cromossomo (NANDA et al., 2002).

### 1.3 Cromossomos sexuais em Aves

Em Aves o sistema de determinação sexual é do tipo ZZ/ZW, onde o par de cromossomos homomórficos ZZ são os machos e o par heteromórfico ZW são as fêmeas. O cromossomo Z contém de 7% a 10% do genoma, é conservado em tamanho, em geral apresenta o tamanho do quarto ou quinto maior par cromossômico, mas com a morfologia variável, devido a rearranjos intracromossomais que são comuns para este elemento do genoma (NANDA; SCHMID, 2002). O cromossomo W é heterocromático, possui um número substancial de genes ativos (ZHOU et al., 2014) e alta variabilidade de tamanho em diferentes espécies, contudo geralmente é menor que o cromossomo Z (SCHMID et al., 2005; STIGLEC; EZAZ; GRAVES, 2007).

As aves, Paleornithes (ratitas) são consideradas basais para a Classe, possuem os cromossomos sexuais Z e W idênticos morfologicamente e apresentam uma porção próxima ao centrômero diferente geneticamente (STIGLEC; EZAZ; GRAVES, 2007). As Neornithes (carinatas) apresentam, geralmente, diferenças significativas de tamanho e morfologia entre os cromossomos sexuais (CORREIA et al., 2009; GUNSKI et al., 2000; STIGLEC; EZAZ; GRAVES, 2007). Provando assim que as espécies de aves estão em diferentes estados de evolução nos cromossomos sexuais (ZHOU et al., 2014).

A teoria da evolução dos cromossomos sexuais propõe que os mesmos teriam se diferenciado a partir de um par de autossomos homólogos, sendo que o W sofreu diferenciação em diferentes graus e linhagens (OHNO, 1967). Essa teoria foi apoiada com a hibridização com sondas do cromossomo Z de *Gallus gallus*, em metáfases de *Dromaius novaehollandiae* (Rheiformes), onde demonstraram que ambos cromossomos (ZW) produziram sinais positivos de hibridização, sendo apenas uma pequena região próximo ao centrômero W onde não houve hibridização, o que comprova a alta homologia entre eles (SHETTY; GRIFFIN; GRAVES, 1999).

### 1.4 Características da família Picidae

A família Picidae, possui cerca de 215 espécies (MIKUSIŃSKI, 2006) e é popularmente conhecida como pica-pau, faz parte da ordem Piciformes. São amplamente conhecidos pela sua extraordinária capacidade de perfurar vários tipos de madeira. Sua alimentação é baseada principalmente em larvas de insetos, mas

também se alimentam de frutos (WINKLER; CHRISTIE; NURNEY, 1995). Vivem em bosques onde fazem seus ninhos abrindo uma cavidade nos troncos das árvores. Os ninhos são escavados em troncos de árvores o mais alto possível para proteção contra predadores. Os ovos, de 4 a 5, são chocados pela fêmea e também pelo macho durante 20 dias.

*Colaptes campestris* (pica-pau do campo) medem, em torno de 32 cm, possuem os lados da cabeça e do pescoço amarelos, assim como o peito, o alto da cabeça e a nuca são negros (DEVELEY; ENDRIGO, 2004) (Figura 1a). Alimentam-se de insetos, principalmente formigas e cupins e habitam campos e cerrados, vivem em casais e, às vezes em pequenos grupos. Terrícola, costumam capturar insetos no solo, mas ao se sentir ameaçados procuram árvores ou grandes pedras para se protegerem (DEVELEY; ENDRIGO, 2004). A espécie ocorre desde o nordeste do Brasil ao Uruguai, podendo ser avistado também no Paraguai, na Bolívia, na Argentina e no baixo Amazonas, inclusive no Suriname. Invade a Amazônia vindo do sul, estendendo seu domínio no Brasil oriental, em função dos desmatamentos (SICK, 1997) (Figura 2a).

*Colaptes melanochloros* (pica-pau verde barrado) medem aproximadamente 28 cm. Possuem plumagem de tom esverdeado, na cabeça, possuem divisão entre vermelho e preto, única entre os pica-paus, destaca a grande área branca da região dos olhos (DEVELEY; ENDRIGO, 2004) (Figura 1b). Alimentam-se de formigas e larvas de outros insetos, principalmente de besouros e também comem frutos carnosos, geralmente no inverno, quando diminui a quantidade de insetos (SICK, 1997). Vivem em matas de galeria, cerrados, caatingas, campos com árvores e na borda de florestas (AZPIROZ, 2012). Ocorrem desde a foz do rio Amazonas (ilha de Marajó) até o Rio Grande do Sul, e para oeste até o Mato Grosso. São encontrados também no Paraguai, Argentina e Uruguai (SICK, 1997) (Figura 2b).

*Melanerpes candidus* (pica-pau branco) medem entre 24 a 29 centímetros. Cabeça, pescoço, partes inferiores e dorso inferior são brancos, com a barriga amarela, as costas, asas e cauda são negras e uma linha negra que desce dos olhos até as costas. A região perioftálmica é amarelo-alaranjada e os machos diferenciam-se das fêmeas por possuírem a nuca amarela. (AZPIROZ, 2012) (Figura 1c). Alimentam-se de insetos e suas larvas, sementes, frutos e mel. Vivem em áreas campestres, pastos, eucaliptais, capoeiras ralas, buritizais, plantações e áreas rurais (SICK, 1997). Vivem também em cidades, parques, jardins, pomares, bordas de

brejos arborizados e no Pantanal de Mato Grosso. São encontrados em grupos de 6 a 10 indivíduos. Presentes em campos da foz do rio Amazonas e na região de Óbidos, estendendo-se para as regiões campestres de todo o Brasil. Encontrados também no Suriname, Bolívia, Argentina, Paraguai e Uruguai (SICK, 1997) (Figura 2c).

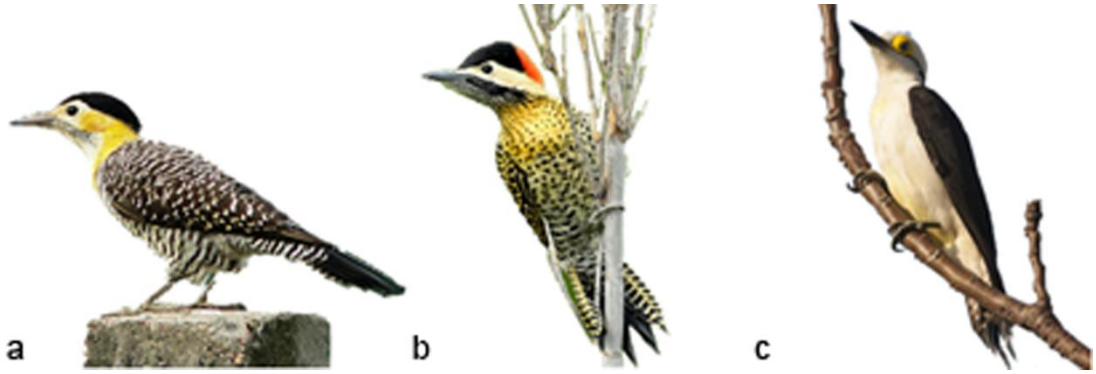


**Figura 1** - Imagem de um exemplar de *Colaptes campestris* (a), *Colaptes melanochloros* (b) e *Melanerpes candidus* (c). Adaptado de Wikiaves


**Figura 2** - Distribuição geográfica de *Colaptes campestris* (a), *Colaptes melanochloros* (b) e *Melanerpes candidus* (c). Adaptado de Wikiaves

Os pica-paus possuem inúmeras sinapomorfias e morfologias definidas que o torna um clado de fácil identificação, porém sua classificação esta baseada em características de anatomia geral e técnicas de caça semelhantes, pelas características de plumagem e distribuição geográfica. Notadamente, no entanto, as relações filogenéticas entre as espécies e gêneros de pica-paus ainda não foram claramente estabelecidas (WEBB; MOORE, 2005).

Os dados citogenéticos em Picidae são escassos, até o ano de 2016 apenas 15 espécies possuiam descrição cariotípica. Em comum estas espécies possuem um alto número diplóide, variando de 84 a 108 cromossomos para *Picoides*

*mahrattensis* e *Dendrocopos minor*, respectivamente (KAUL; ANSARI, 1978; SHIELDS; JARRELL; REDRUPP, 1982), e o cromossomo sexual Z é o maior do complemento cromossômico (BIAN; LI, 1989; HAMMAR, 1970; KAUL; ANSARI, 1978; SHIELDS; JARRELL; REDRUPP, 1982). Até o momento apenas *Picoides mahrattensis* e *Dendrocopos minor* possuem dados de bandamento C, apresentando heterocromatina constitutiva nas regiões centroméricas de todos os macrocromossomos e o cromossomo W totalmente heterocromático (KAUL; ANSARI, 1978; SHIELDS; JARRELL; REDRUPP, 1982), nas demais espécies são descritos apenas o número diploide e morfologias cromossômicas. A ausência de tais dados dificulta a compreensão da evolução cariotípica e as relações filogenéticas entre as espécies de pica-paus.

## 2 JUSTIFICATIVA

Os estudos recentes de genômica comparativa têm fornecido novas evidências quanto à determinação do sexo em vertebrados. E as sequências repetitivas têm sido amplamente utilizadas como objeto de estudo, uma vez que podem estar associadas a processos de diferenciação sexual.

Conforme evidenciado acima o estudo citogenético da família Picidae, apesar das suas características cromossômicas peculiares, é incipiente, com isso pouco se sabe sobre o estado da evolução cariotípica e a diferenciação dos cromossomos sexuais nesse grupo.

# 3 OBJETIVO

## 3.1 Objetivo geral

Determinar a distribuição de diferentes tipos de sequências repetitivas nos genomas dos pica-paus, *Colaptes campestris*, *Colaptes melanochloros* e *Melanerpes candidus*, e compreender a evolução e diferenciação dos cromossomos sexuais.

## 3.2 Objetivos específicos

- Determinar o cariótipo e número diplóide de três espécies da família Picidae, *C. campestris, C. melanochloros, M. candidus*, através da coloração convencional;

- Determinar o padrão de heterocromatina constitutiva e análises comparativas subsequentes;

- Realizar o mapeamento cromossômico das espécies utilizando diferentes classes de sequências repetitivas;

- Comparar os padrões de organização cromossômica dos DNAs repetitivos entre as três espécies.

# 4 MATERIAL E MÉTODOS

## 4.1 Coleta de espécimes

Foram coletados com redes de neblina espécimes de pica-paus, *Colaptes campestris*, *Colaptes melanochloros* e *Melanerpes candidus* (Figura 1a-c) conforme indicado na Tabela 1, em municípios do estado do Rio Grande do Sul (Figura 3) de acordo com a autorização do IBAMA/SISBIO nº 33860-1 e 44173-1 e Comissão de Ética no uso de Animais - CEUA/UNIPAMPA.

**Tabela 1** - Informações sobre os espécimes, número de exemplares e locais de coleta.

| Espécies | Número de indivíduos e sexo | Municípios |
|---|---|---|
| *Colaptes campestris* | 3 ♂ and 1 ♀ | Dom Pedrito e São Gabriel |
| *Colaptes melanochloros* | 5 ♀ | Dom Pedrito e São Gabriel |
| *Melanerpes candidus* | 1 ♂ | Porto Vera Cruz |

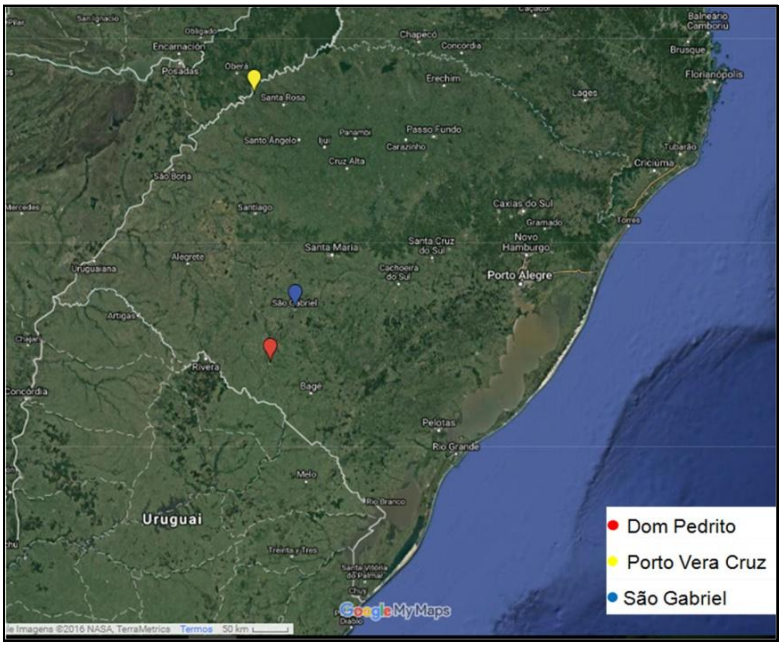


**Figura 3** - Mapa do Rio Grande do Sul evidenciando os locais de coleta dos espécimes. Adaptado de Google maps.

## 4.2 Preparações cromossômicas

As metáfases foram obtidas através da cultura de fibroblastos e cultura de medula óssea de curta duração.

A cultura de fibroblastos foi realizada utilizando uma biopsia de tecido dos espécimes segundo SASAKI; IKEUCHI; MAKINO (1968), onde as amostras foram lavadas com solução Hank's e fracionadas em uma placa de *petri* e em seguida incubadas em uma solução de colagenase (0,186g em 4mL de meio DMEN), por um período de 1 hora, em estufa a 37°C, para ocorrer a total dissociação das células. Após, foi acrescentado 5 mL de meio DMEN e centrifugado por 8 minutos a 800 – 1000 rpm. O material sedimentado foi transferido para garrafas de cultura contendo 5mL de meio DMEN enriquecido com antibióticos e soro bovino fetal. Quando as garrafas apresentavam um nível ótimo de mitoses, a partir de análises no invertoscópio, foram adicionados 100µL de colcemid. Após incubação por 1 hora em estufa a 37°C, as células foram soltas da garrafa com auxilio de solução de tripsina EDTA 0,25% e transferidas para um tubo de centrífuga. Posteriormente, o material foi centrifugado por 8 minutos a 800 – 1000 rpm, descartou-se o sobrenadante e as células sedimentadas foram tratadas com solução hipotônica (KCl, 0,075M) por 10 minutos a 37ºC, em seguida foram acrescentadas 4-5 gotas de fixador (metanol e ácido acético na proporção 3:1) ao tubo. O material foi ressuspendido cuidadosamente e centrifugado por 8 minutos a 800 – 1000 rpm, e o sobrenadante foi retirado, acrescentou-se 5-8mL de fixador e após ressuspensão o material foi centrifugado por 8 minutos a 800 – 1000 rpm, este processo foi realizado 3 vezes e o *pellet* armazenado em freezer até o momento do uso.

A cultura de medula óssea de curta duração foi realizada de acordo com GARNERO; GUNSKI (2000), foi retirada a medula óssea do fêmur do exemplar com o auxilio de uma seringa hipodérmica com agulha, em meio RPMI, em uma placa de *Petri*, a medula foi dissociada com a seringa sem a agulha, o material foi transferido para um tubo de centrífuga e incubadas com colchicina (0,005%) por 1h em banho maria a 37ºC, logo após o material foi centrifugado por 8 minutos a 800 – 1000 rpm, descartou-se o sobrenadante e as células sedimentadas foram tratadas com solução hipotônica (KCl, 0,075M) por 10 minutos a 37ºC, em seguida foram acrescentadas algumas (4-5) gotas de fixador (metanol e ácido acético na proporção 3:1) ao tubo. O material foi ressuspendido cuidadosamente e centrifugado por 8 minutos a 800 – 1000 rpm, o sobrenadante foi retirado, acrescentou-se 5-8mL de fixador e ressuspendeu-se o material seguido de centrifugação por 8 minutos a 800 – 1000

rpm, este processo foi realizado 3 vezes e o *pellet* armazenado em freezer até o momento do uso.

### 4.3 Citogenética clássica

As lâminas foram coradas com Giemsa 5% em tampão fosfato por 7 minutos, lavadas com água destilada e secadas ao ar, foram observadas em microscópio óptico, desenhadas cerca de 30 metafáses de cada exemplar e as melhores metáfases fotografadas. A partir destas imagens foi realizada a biometria cromossômica com o auxilio do *software* Micromeasure e realizada a montagem dos cariótipos com o *software* Corel PhotoPaint.

A detecção das regiões de heterocromatina constitutiva foi realizada de acordo com (FERNÁNDEZ et al., 2002), com algumas modificações. As lâminas foram envelhecidas por 48 horas em estufa a 65ºC e desnaturadas na presença de formamida 50% por 2 minutos a 70ºC, logo após as lâminas foram lavadas em 2xSSC e contracoradas com 20µL de DAPI+anti-fade. As metáfases foram fotografadas e convertidas para preto e branco com o *software* Corel PhotoPaint.

### 4.4 Preparação das sondas

Na preparação da sonda utilizada para detectar a localização do cluster do gene ribossomal 18S para o experimento de FISH, foram utilizados os *primers* NS1 5'-GTA GTC ATA TGC TTG TCT C-3', NS8 5'-TCC GCA GGT TCA CCT ACG GA-3' e DNA genômico de *Ocyurus chrysurus* (Perciformes, Lutjanidae) para amplificação por PCR (reação em cadeia da polimerase) (WHITE et al., 1990). Posteriormente, os fragmentos amplificados foram marcados com digoxigenina by nick translation (Roche) e detectados com Anti-Digoxigenina-Rhodamina, seguindo as instruções do fabricante.

A sonda de DNA telomérico $(TTAGGG)_n$ foi obtida e marcada diretamente por PCR com biotina na ausência de um DNA molde, utilizando os *primers* $(TTAGGG)_5$ e $(CCCTAA)_5$ na concentração de 10µM e detectadas com Cy3 (IJDO et al., 1991).

As sondas contendo os oligonucleotídeos enriquecidos com sequências microssatélites $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CG)_{15}$, $(CGG)_{10}$, $(GA)_{15}$,

$(GAA)_{10}$, $(GAG)_{10}$ e $(TA)_{15}$ foram marcadas diretamente durante a síntese com avidina-Cy3 (Sigma, St. Louis, MO, USA), como descrito por KUBAT et al. (2008).

**4.5 Hibridização in situ fluorescente (FISH)**

Nos experimentos com sonda ribossomal foi utilizado o protocolo de DANIELS; DELANY (2003) com modificações, as lâminas com os cromossomos fixados foram tratadas com ribonuclease e incubadas em solução de pepsina 1% por 5 minutos e 2 vezes em 2xSSC por 5 minutos cada, em seguida foram desidratadas em uma série de etanol 70%, 90% por 2 minutos e 100% por 4 minutos. Após as lâminas foram envelhecidas em estufa a 37ºC *overnight*.

As lâminas foram desnaturadas em formamida 70% a 70ºC por 1 minuto e 20 segundos, transferidas imediatamente para etanol 70% a -4ºC por 4 minutos, após incubadas em etanol 70%, 90% por 2 minutos e 100% por 4 minutos todos em temperatura ambiente.

As sondas foram desnaturadas por 10 minutos a 72ºC e aplicadas sobre as lâminas, em seguida foram incubadas em câmera úmida e escura a 37ºC *overnight*.

Para a detecção as lâminas foram incubadas 2 vezes em formamida 50% a 40ºC por 5 minutos e depois 2 vezes em 2xSSC a 40ºC por 5 minutos, com a lâmina ainda molhada foi adicionada a solução de detecção (marcador fluorescente) e incubadas em estufa por 30 minutos a 37ºC. Logo após as lâminas foram incubadas 3 vezes com 4xSSC+Tween por 5 minutos e as lâminas foram montadas com 15µL de DAPI+anti-fade.

Para os experimentos com sonda telomérica o mesmo protocolo descrito acima foi utilizado, porém sem o tratamento das lâminas com ribonuclease.

Para os experimentos de microssatélites foi utilizado o protocolo de KUBAT et al. (2008) com modificações. As lâminas com os cromossomos fixados foram envelhecidas em estufa a 37ºC por 1 hora e em estufa a 60ºC por 30 minutos. Logo após as lâminas foram tratadas com 200µL de ribonuclease (10µg/mL) em câmera úmida por 1 hora a 37ºC e incubadas 3 vezes em 2xSSC por 5 minutos cada, em seguida incubadas em pepsina 0,005% por 10 minutos em temperatura ambiente repetindo-se o tratamento em 2xSSC. Prontamente foram incubadas em formaldeído 1% por 10 minutos e novamente 3 vezes em 2xSSC. Posteriormente foram desidratadas em uma série de etanol 70%, 90% e 100% por 2 minutos em cada,

foram secas em temperatura ambiente. Depois de secas foram desnaturadas em formamida 70% por 3 minutos a 72ºC, imediatamente desidratadas em etanol 70%, 90% e 100% por 2 minutos cada.

As sondas foram desnaturadas à 80ºC por 10 minutos e transferidas imediatamente para o gelo. Após aspergiu-se 15µL de sonda sobre cada lâmina, foram vedadas com lamínula e cola sintética e incubou-se em câmera úmida e escura em estufa a 37ºC *overnight*.

Foi retirada cuidadosamente a lamínula e as lâminas foram incubadas 2 vezes em 2xSSC por 5 minutos e 2 vezes em 1xSSC por 5 minutos, logo após lavadas em PBS e desidratadas em etanol 70%, 90% e 100% por 2 minutos cada. Após as lâminas foram montadas com 20µL de DAPI+anti-fade.

## 5 RESULTADO E DISCUSSÃO

Os resultados obtidos no presente trabalho e a sua discussão encontram-se no capítulo 1 que corresponde ao artigo científico publicado na revista Plos One em 12 de janeiro de 2017, doi: 10.1371/journal.pone.0169987, disponível em http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0169987.

## 6 CAPÍTULO 1

# Genomic organization of repetitive DNA in Woodpeckers (Aves, Piciformes): Implications for karyotype and ZW sex chromosome differentiation

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## Abstract

Birds are characterized by a low proportion of repetitive DNA in their genome when compared to other vertebrates. Among birds, species belonging to Piciformes order, such as woodpeckers, show a relatively higher amount of these sequences. The aim of this study was to analyze the distribution of different classes of repetitive DNA – including microsatellites, telomere sequences and 18S rDNA – in the karyotype of three Picidae species (Aves, Piciformes) - *Colaptes melanochloros* (2n=84), *Colaptes campestris* (2n=84) and *Melanerpes candidus* (2n=64) – by means of fluorescence *in situ* hybridization. Clusters of 18S rDNA were found in one microchromosome pair in each of the three species, coinciding to a region of $(CGG)_{10}$ sequence accumulation. Interstitial telomeric sequences were found in some macrochromosomes pairs, indicating possible regions of fusions, which can be related to variation of diploid number in the family. Only one, from the 11 different microsatellite sequences used, did not produce any signals. Both species of genus *Colaptes* showed a similar distribution of microsatellite sequences, with some difference when compared to *M. candidus*. Microsatellites were found preferentially in the centromeric and telomeric regions of micro and macrochromosomes. However, some sequences produced patterns of interstitial bands in the Z chromosome, which corresponds to the largest element of the karyotype in all three species. This was not observed in the W chromosome of *Colaptes melanochloros*, which is heterochromatic in most of its length, but was not hybridized by any of the sequences used. These results highlight the importance of microsatellite sequences in differentiation of sex chromosomes, and the accumulation of these sequences is probably responsible for the enlargement of the Z chromosome.

Keywords: Birds; Sex chromosomes; microsatellites; telomeric; rDNA.

## Introduction

It is known that the avian genome is extremely compact, corresponding to approximately one third of the typical mammalian DNA content [1]. This decrease occurred due to the loss of many genes and, in a higher proportion, DNA repetitive sequences [2]. Recently, published results of genome sequences from 48 bird species showed that their amount of repetitive sequences is much smaller than other

groups of Tetrapods, corresponding to 4-10%, while in mammals the percentage of these sequences can reach up to 52% from the genome [3]. Among different classes of repetitive sequences we can find satellites, microsatellites, multigenic families, and transposable elements [3, 4]. Repetitive DNA plays an important role in genetic variation within populations, as well as in gene expression, recombination, genome structural organization, chromosomal instability and sex chromosome differentiation [5-9].

So far, the Picidae family (woodpeckers) shows the highest proportion of repetitive sequences in genomes among birds, reaching up to 22% of total DNA amount in the species *Picoides pubescens*, where the transposable element CR1 is one of the most important components [3]. In addition, karyotype analyses showed that woodpeckers have some distinctive features, such as high diploid numbers (2n), with some species possessing more than 100 chromosomes, and a large Z chromosome, the largest element of the karyotype [10-15].

In other groups of organisms, analyses of repetitive sequences have related their accumulation to the process of sex chromosomes differentiation, as in plants, reptiles and, in a greater proportion, fish [16-18]. In this regard, Matsubara and co-workers [19] observed that in *Gallus gallus* motifs $(GA)_{15}$ and $(GAG)_{10}$ were detected mainly in the W chromosome and, at low frequency, in the Z and autosomes. However, the organization of repetitive sequences in the genome of birds, especially their chromosomal location, is practically unknown.

The Picidae family showed a large karyotype variation in their 2n and a high amount of repetitive sequences in their genomes in comparison with other birds. Therefore, in this study we aim to characterize the chromosomal distribution of 13 classes of repetitive DNA sequences in three Picidae species, focusing on the association of these sequences with karyotype evolution and the ZW sex system differentiation.

## Material and Methods

### Animals

Ten individuals belonging to three different Picidae species (Aves, Piciformes) were used in this study: three male and one female *Colaptes campestris*, five female

*Colaptes melanochloros* and one male *Melanerpes candidus* (Table 1). Animals were collected in Rio Grande do Sul State (Brazil) using mist nets (permissions SISBIO 33860-1 and 44173-1). The experiments followed protocols approved by the Ethics Committee on the Use of Animals (CEUA - Universidade Federal do Pampa, 026/2012).

**Table 1. Specimen information and number of samples used in this study.**

| Species | Number of individuals and sex | City | Geographic coordinate |
|---|---|---|---|
| *C. campestris* | 3 ♂ and 1 ♀ | Dom Pedrito and São Gabriel | 31°00'37.68" S; 054°36'54.29" W and 30°20'05.93" S; 054°21'47.93" W |
| *C. melanochloros* | 5 ♀ | Dom Pedrito and São Gabriel | 31°00'37.68" S; 054°36'54.29" W and 30°20'05.93" S; 054°21'47.93" W |
| *M. candidus* | 1 ♂ | Porto Vera Cruz | 27°42'33" S; 054°53'29" W |

## Chromosome preparation

Chromosome preparations were obtained using short term cultivation of bone marrow [20] or fibroblast culture [21]. Both methods included colcemid incubation, hypotonic treatment and fixation with methanol: acetic acid (3:1).

## Classical Cytogenetics

Chromosome biometry was performed using the software Micromeasure 3.3 [22]. C-banding was performed according to Fernández et al. [23] with modifications.

## Probe Preparation and Fluorescent *in situ* hybridization (FISH)

18S rDNA fragments were amplified by PCR using *primers* NS1 5′-GTA GTC ATA TGC TTG TCT C-3′, NS8 5′-TCC GCA GGT TCA CCT ACG GA-3′ and nuclear DNA of *Ocyurus chrysurus* (Perciformes, Lutjanidae) [24]. Afterwards, fragments were labeled with digoxigenin by nick translation (Roche) and detected with Anti-Digoxigenin-Rhodamine, following manufacturer´s instructions. Telomeric probes $(TTAGGG)_n$ were obtained by PCR in the absence of a DNA template, using *primers* $(TTAGGG)_5$ and $(CCCTAA)_5$ [25], labeled with biotin and detected with avidin-Cy3.

Preparation of slides, hybridization and washes were performed according to Daniels and Delany [26].

Oligonucleotide probes containing microsatellite sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CG)_{15}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(TA)_{15}$ were directly labeled with Cy3 during synthesis (Sigma, St. Louis, MO, USA), as described by Kubat et al. [16].

### Microscopy

At least 30 metaphase spreads per individual were analyzed to confirm the 2n, karyotype structure and FISH results. Metaphases were analyzed in an epifluorescent microscope (Imager Z2, Zeiss, Germany), and images were captured with the software Axiovision 4.8 (Zeiss, Germany). Final editing of images used Corel Photo Paint X5.

## Results

### Karyotyping and C-banding

We found 2n=84 in both species of genus *Colaptes*, while *Melanerpes candidus* showed 2n=64 (Figs 1A, 1B and 1C). *C. campestris* and *C. melanochloros* also showed chromosomes with the same morphology, with 14 pairs of macrochromosomes, including the Z and W sex chromosomes. Chromosomal morphology of each species is described in Table 2. In all these species, the Z chromosome was acrocentric and was the largest element of the karyotype. The W was smaller, with size between the sixth and seventh pairs in *Colaptes*.

**Figure 1. Partial karyotypes of a female *Colaptes campestris* (A), a female *Colaptes melanochloros* (B) and a male *Melanerpes candidus* (C).** Bar = 5µm.

***Table 2. Chromosomal morphology of Picidae species included in this study.***

| | *Colaptes campestris* | | | | *Colaptes melanochloros* | | | | *Melanerpes candidus* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Chromosomes** | Long arm | Short arm | CI | Morphology | Long arm | Short arm | CI | Morphology | Long arm | Short arm | CI | Morphology |
| **1** | 1,77 | 1,25 | 41,391 | M | 1,72 | 1,18 | 40,690 | M | 1,722 | 1,722 | 50,000 | M |
| **2** | 1,59 | 0,96 | 37,647 | SM | 1,62 | 0,97 | 37,452 | SM | 1,353 | 1,312 | 49,231 | M |
| **3** | 1,6 | 0,53 | 24,883 | SM | 1,7 | 0,73 | 30,041 | SM | 1,64 | 0,656 | 28,571 | SM |
| **4** | 1,79 | 0,15 | 7,732 | A | 1,71 | 0,17 | 9,043 | A | 1,886 | 0,369 | 16,364 | A |
| **5** | 1,14 | 0,55 | 32,544 | SM | 1,13 | 0,75 | 39,894 | SM | 0,861 | 0,82 | 48,780 | M |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **6** | 1,47 | 0,15 | 9,259 | A | 1,61 | 0,14 | 8,000 | A | 0,943 | 0,492 | 34,286 | SM |
| **7** | 1,26 | 0,17 | 11,888 | A | 1,38 | 0,14 | 9,211 | A | 1,312 | 0,246 | 15,789 | A |
| **8** | 1,37 | 0,09 | 6,164 | A | 1,47 | 0,1 | 6,369 | A | 1,23 | 0,328 | 21,053 | A |
| **9** | 1,09 | 0,1 | 8,403 | A | 1,17 | 0,1 | 7,874 | A | 1,189 | 0,164 | 12,121 | A |
| **10** | 1,15 | 0,13 | 10,156 | A | 1,26 | 0,1 | 7,353 | A | 1,107 | 0,328 | 22,857 | A |
| **11** | 1,3 | 0 | 0,000 | T | 1,34 | 0 | 0,000 | T | 1,107 | 0,246 | 18,182 | A |
| **12** | 1,1 | 0 | 0,000 | T | 1,16 | 0 | 0,000 | T | 1,025 | 0,287 | 21,875 | A |
| **13** | 1,15 | 0 | 0,000 | T | 0,83 | 0 | 0,000 | T | 1,025 | 0,205 | 16,667 | A |
| **Z** | 2,43 | 0,59 | 19,536 | A | 2,45 | 0,69 | 21,975 | A | 3,239 | 0,82 | 20,202 | A |
| **W** | 1,43 | 0,21 | 12,805 | A | 1,33 | 0,17 | 11,333 | A | - | - | - | - |

CI = Centromeric Index, M = metacentric, SM = submetacentric, A = acrocentric, T = telocentric

Blocks of constitutive heterochromatin were seen in the pericentromeric region of macrochromosomes, including the Z chromosome, and some microchromosomes, in all the species analyzed (Figs 2A, 2B and 2C). The W chromosome was heterochromatic in most of its length in both *Colaptes* species (Figs 2A and 2B).

**Figure 2. C-banded chromosomes in mitotic metaphase of *Colaptes campestris* (A), *Colaptes melanochloros* (B) and *Melanerpes candidus* (C).** Chromosomes were stained with DAPI and converted to black and white with Corel® photo editor. Sex chromosomes are indicated in each metaphase. Bar = 5µm.

**Chromosomal mapping of repetitive elements**

Clusters of 18S rDNA were found in only one microchromosome pair in all species analyzed (Figs 3A and 4A). According to chromosomal biometry, this chromosome correspondeds to pair 13 in both *Colaptes*, which exhibits a secondary constriction, and to pair 18 in *M. candidus*.

**Figure 3. Metaphase chromosomes of a female *Colaptes melanochloros* hybridized with: 18S rDNA (A), telomeric DNA (B) and microsatellites DNA (C-G).** Chromosomes were counterstained with DAPI (blue), and microsatellite probes were labeled directly during synthesis with Cy3 (red). Probes used are indicated in the lower left corner of the images. Sex chromosomes are indicated in each metaphase. Bar = 5µm.

**Figure 4**. **Metaphase chromosomes of a male *Melanerpes candidus* hybridized with: 18S rDNA (A), telomeric DNA (B) and microsatellites DNA (C-G).** Chromosomes were counterstained with DAPI (blue) and microsatellite probes were labeled directly during synthesis with Cy3 (red). Probes used are indicated in the lower left corner of the images. Sex chromosomes are indicated in each metaphase. Bar = 5µm.

Telomeric probes produced signals only in the terminal region of all chromosomes, except for the interstitial telomere sequences (ITS) observed in the centromeric region of pairs 1-3 in both *Colaptes species* (Fig 3B) and pairs 1-2 and 5 in *M. candidus* (Fig 4B).

Among the 11 distinct microsatellite sequences used in this study, only $(TA)_{15}$ did not produce signals in any of the species analyzed. The same hybridization patterns were observed in both *Colaptes* species, while *M. candidus* showed a distinct pattern (Table 3, Figs 3C-L and Figs 4C-L). Accumulations of microsatellites were found mainly in centromeric and telomeric regions of the chromosomes, although some sequences produced signals in interstitial blocks.

**Table 3. Hybridization of repetitive sequences in Picidae.**

| **Probes** | **Chromosomes** | | | | | |
|---|---|---|---|---|---|---|
| | Macrochromosomes | | Microchromosomes | | Z | |
| | **COL** | **MCA** | **COL** | **MCA** | **COL** | **MCA** |
| $(GAA)_{10}$ | + | + | + | + | + | + |
| $(GAG)_{10}$ | + | + | + | + | + | + |
| $(GA)_{15}$ | + | + | + | - | + | + |
| $(CG)_{15}$ | + | + | + | + | + | + |
| $(CAG)_{10}$ | + | - | + | + | - | - |
| $(CGG)_{10}$ | + | + | + | + | - | + |
| $(CAC)_{10}$ | + | + | + | + | + | + |
| $(CAT)_{10}$ | + | + | + | + | - | + |
| $(CA)_{15}$ | + | + | + | + | - | + |
| $(CAA)_{10}$ | + | + | + | + | + | + |

(+) positive hybridization signals; (-) no hybridization signals; (COL) Genus *Colaptes*; (MCA) *Melanerpes candidus*.

Macrochromosomes showed a preferential accumulation of microsatellites in centromeric and telomeric regions in pairs 1, 2, 3 and 5 in all species, while pair 4 showed signals only in *Colaptes* (Figs 5A and 5B). At interstitial sites, pairs 1 and 3 showed accumulation of $(CAC)_{10}$ and $(CAG)_{10}$ in *Colaptes*, while *M. candidus* showed bands with $(CAC)_{10}$, $(GC)_{15}$ and $(GAA)_{10}$ in chromosome 2. For microchromosomes, two different patterns of microsatellite distribution were observed: some accumulated along the total length of the microchromosomes (such as $(CA)_{15}$ and $(CGG)_{10}$), and the rest were observed in the terminal region of the chromosome arms (such as $(GAA)_{10}$, $(CG)_{15}$ and $(CAT)_{10}$).

**Figure 5. Distribution and localization of microsatellite sequences in chromosomes 1-5 and Z in *Colaptes* (A) and *M. candidus* (B).**

Interstitial hybridization signals were observed in the long arms of the Z chromosomes. Different patterns of distribution of $(GAA)_{10}$, $(GAG)_{10}$ and $(GA)_{15}$ sequences were found at the three species analyzed, showing three different bands along this chromosome: $(GAG)_{10}$ and $(GA)_{15}$ were found in three bands in *Colaptes*, while in *M. candidus* they formed four distinct bands in the Z chromosome. And $(CGG)_{10}$, $(CAT)_{10}$ and $(CA)_{15}$ were found only in *M. candidus*, in the terminal region of the Z chromosome (Figs 5A and 5B).

## Discussion

Birds belonging to the Picidae family showed an interesting chromosomal variation, with 2n ranging from 64 to 108 (Table 4). This, along with their large Z chromosome [11], makes them an interesting group for cytogenetic analyses, especially for studies involving the distribution of repetitive sequences, usually associated with morphological differentiation of sex chromosomes [9].

**Table 4. Diploid number of Picidae species.**

| Species | 2n | References |
|---|---|---|
| *Dryocopus martuis* | 88 | [11] |
| *Colaptes campestris* | 84 | This paper |
| *Colaptes melanochloros* | 84 | This paper |

| | | |
|---|---|---|
| *Colaptes auratus* | 90 | [11] |
| *Picus canus* | 92 | [13] |
| *Picus viridis* | 94 | [12] |
| *Dinopium benghalense* | 92 | [10] |
| *Melanerpes candidus* | 64 | This paper |
| *Sphyrapicus varius* | 92 | [11] |
| *Dendrocopos minor* | 108 | [11] |
| *Dendrocopos major* | 108 | [11] |
| *Dendrocopos hyperythrus* | 92 | [13] |
| *Dendrocopos kizuki* | 90 | [13] |
| *Dendrocopos leucotos* | 92 | [13] |
| *Picoides mahrattensis* | 84 | [10] |
| *Picoides villosus* | 92 | [11] |
| *Picoides pubescens* | 92 | [11] |
| *Jynx torquilla* | 90 | [13] |

The species are ordered from the most derived to most basal, following the phylogeny proposed by Benz et al. [27].

Our data suggest that *C*. *campestris* and *C*. *melanochloros* have similar karyotypes in both, morphology and number of chromosomes, with 2n=84 each. This same 2n has also been found in another species of this family, *Picoides mahrattensis*, although the only other species from the same genus, *Colaptes auratus*, showed 2n=90 [11]. Considering molecular phylogeny, it is indicated that *C. auratus* is more basal than both, *C. campestris* and *C. melanochloros* [28], thus, taking into account other 2n in the Picidae family, our findings indicate that the increase or decrease of chromosomes in this family occurs randomly, without a phylogenetic tendency [27].

Although in birds, as in other groups, the occurrence of ITS in the centromeric region may represent the accumulation of repetitive sequences, coincidently similar to telomeres [29], it may also represent evidence of chromosomal fusions, as already documented in other bird species [30]. If this is the case in *Colaptes*, and considering that *C. auratus* (2n=90) is placed in a more basal position in relation to *C. campestris* and *C. melanochloros*, both with 2n=84, it can be argued that ITS found in the centromeric region of submetacentric pairs 1, 2 and 3 in species with 2n=84 confirm the occurrence of three centric fusions, which would decrease the hypothetical basal

2n from 90 to 84. However, we also need the occurrence of a pericentric inversion to explain the difference in the number of arms (92 in *C. auratus* to 90 in *C. campestris* and *C. melanochoros*), but all of these rearrangements can be confirmed only by comparative chromosome painting.

Despite their phylogenetic position, the species analyzed in this study retained the plesiomorphic character of showing 18S rDNA clusters in only one microchromosome pair, as it is in the majority of the bird species analyzed so far, including some basal groups such as Paleognathas *Rhea americana*, *Crypturellus tataupa*, *Tinamus solitarius* and *Pterocnemia pennata* [20, 31, 32]. Interestingly, microsatellite sequence $(CGG)_{10}$ was found in chromosomes possessing the secondary constriction, bearers of 18S rDNA clusters. A similar result was found in the fish *Triportheus trifurcatus* (Characiformes, Characidae), where this sequence exist in the W chromosome, which also bears 18S rDNA [9].

Microsatellite sequences were found in both, macrochromosomes, including the Z and in microchromosomes, with some differences between species and in each of the sequences. Despite the existence of interstitial blocks of repetitive DNA, accumulations of microsatellites were also found in centromeric and telomeric regions of the chromosomes.

Repetitive sequences play an important role in the differentiation of sex chromosomes in different groups, including birds, despite the small amount of these classes of DNA in their genome. For instance, it was found that the sequence $(GAG)_{10}$ is accumulated in the W chromosome of *Gallus gallus* [19]. However, none of the microsatellite sequences used in this study hybridized in the W chromosome of *Colaptes.* Instead, some of these sequences were found accumulated in both Z chromosome arms which could explain the fact that it is the largest chromosome in these species. It represents an unusual example of the Z accumulating more repetitive DNAs than the W chromosome. Similar results have been found in the fish *Hoplias malabaricus,* in which the X chromosome was the preferred site for repetitive DNA accumulation in comparison with the Y [33]. Thus, taking into account that the suppression of recombination between the sex chromosome pair is a prerequisite during the evolution of sex chromosomes and that the accumulation of repetitive sequences usually occurs in non- recombining regions, it is possible to track a close relationship between accumulation of different kinds of microsatellite motifs and the physical differentiation of these chromosomes. Probably this is also the situation for

other Picidae species, where the Z chromosome is the largest element of the karyotype.

Overall, it may be said that our FISH analysis showed that several microsatellite sequences are found amplified on the Z chromosome of three species belonging to the Picidae family. This may explain the fact that the Z is the largest element of the karyotype, and that their genome contains the highest number of repetitive sequences compared to other groups of birds. Interestingly, none of the sequences were found accumulated on the W chromosome, although they play an important role in the differentiation of sex chromosomes, and are usually found amplified on Y/W chromosomes. These results suggest that, despite the common origin proposed for the ZW sex system in birds, these chromosomes followed different evolutionary trajectories in each species, indicating a high plasticity for sex chromosome differentiation in this group. Amplifications of microsatellite motifs were also found in macrochromosomes and microchromosomes. However, considering the lack of information concerning the genomic distribution of these sequences, it is not yet possible to make a comparison with other birds.

This work is the first step towards clarifying the role of satellites and microsatellite sequences in the differentiation of sex chromosomes. Future studies involving other groups of birds are needed to increase our knowledge in processes of evolution and differentiation of these chromosomes.

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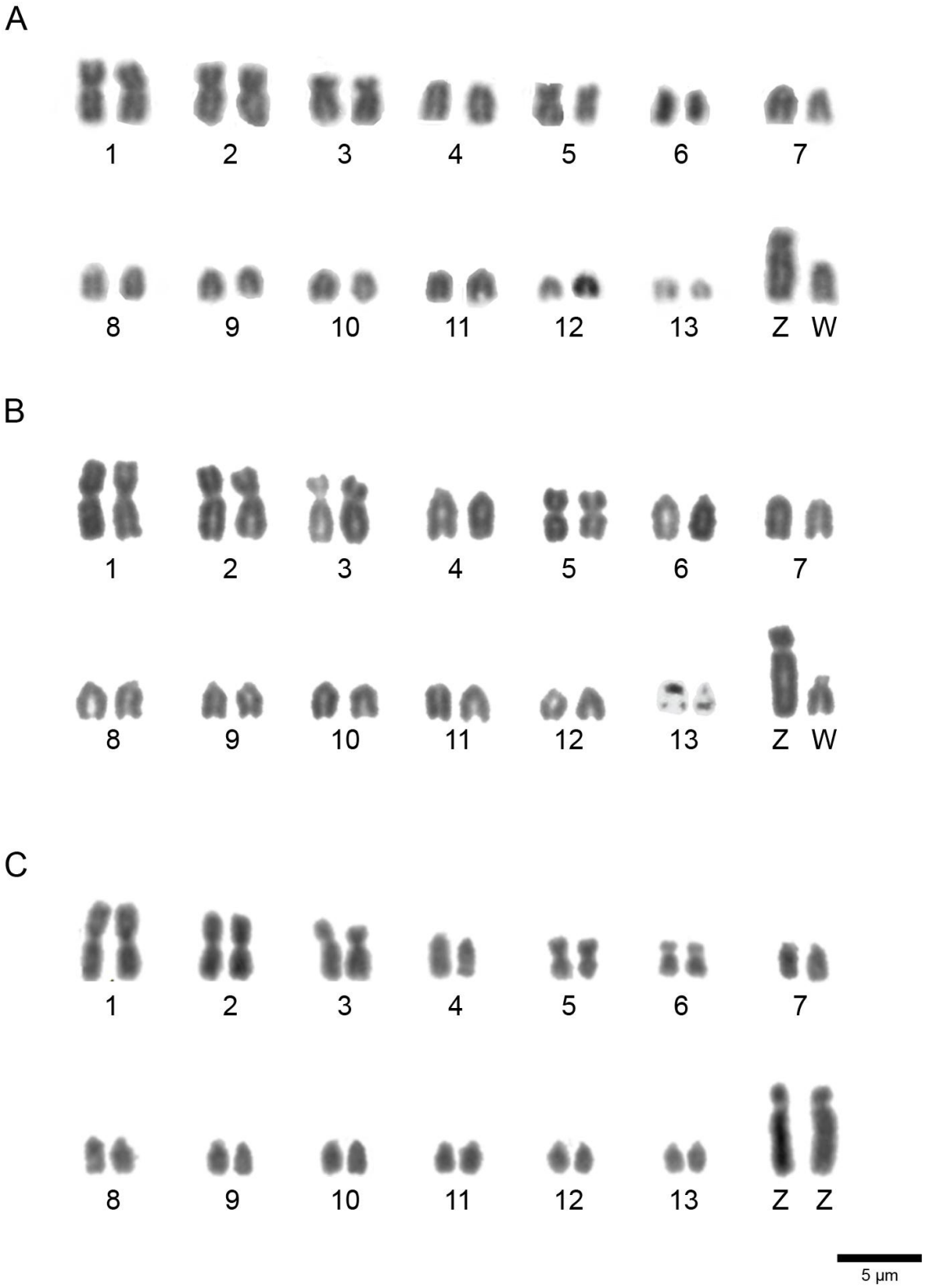
**Figure 1**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1 2 3 4 5 6 7
8 9 10 11 12 13 Z W
B
1 2 3 4 5 6 7
8 9 10 11 12 13 Z W
C
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8 9 10 11 12 13 Z Z
5 µm

**Figure 2**

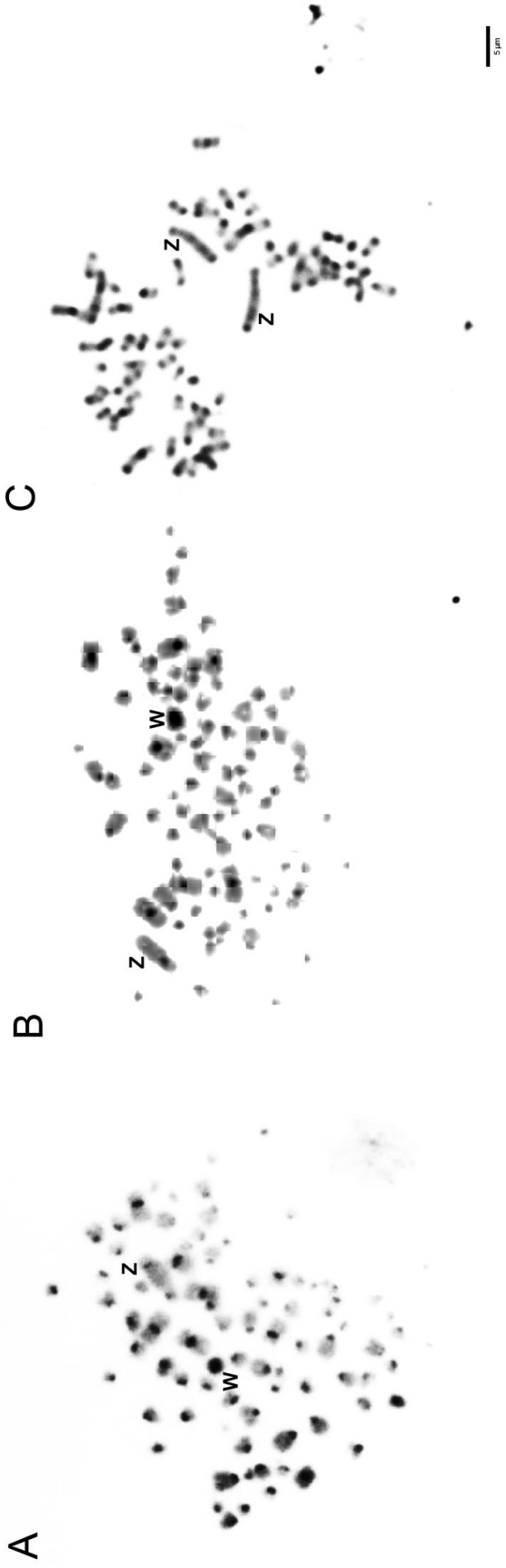
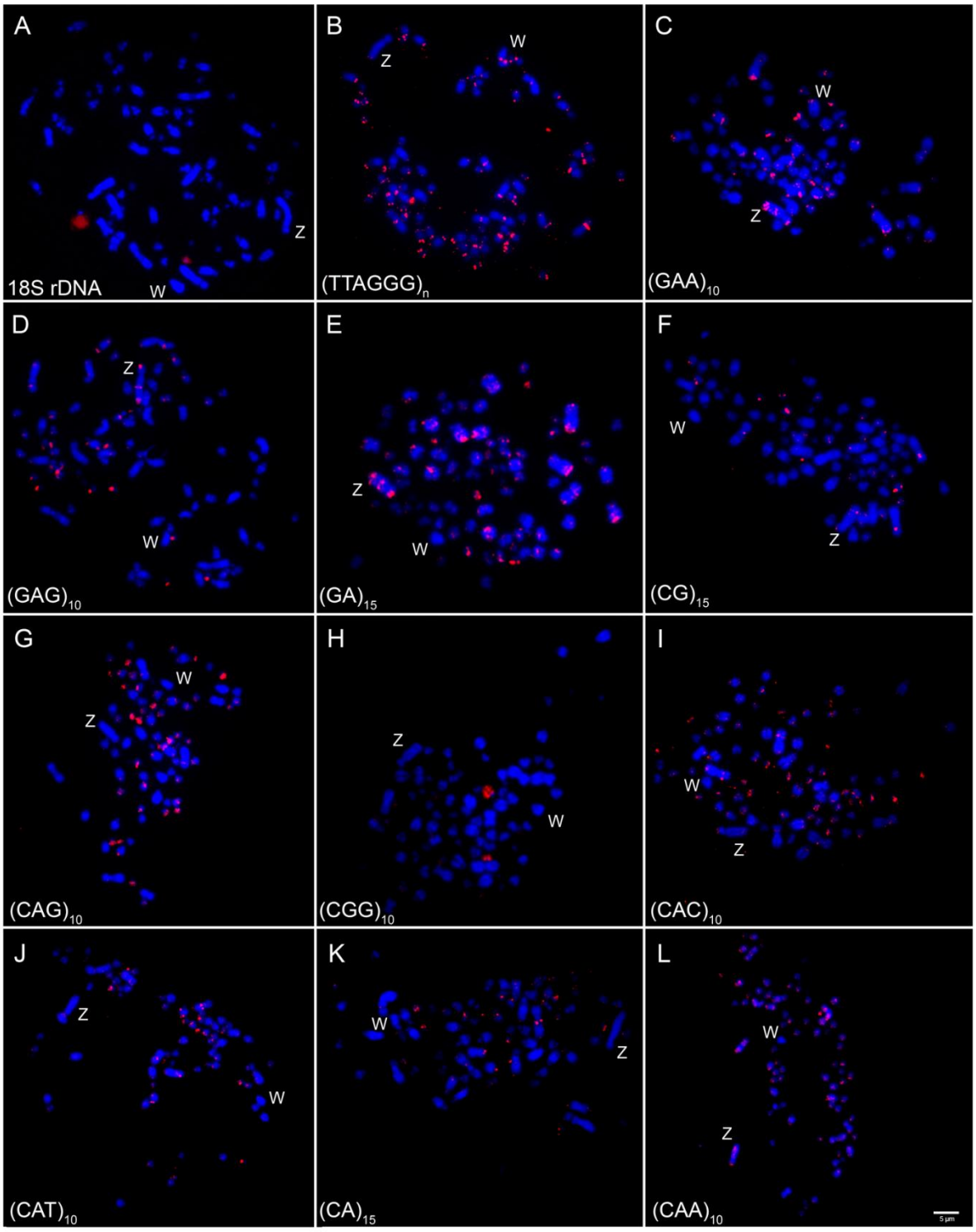


**Figure 3**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Z
W
B
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Z
W
C
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Z
D
(GAG)10
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W
E
(GA)15
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F
(CG)15
W
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(CAG)10
W
Z
H
(CGG)10
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W
I
(CAC)10
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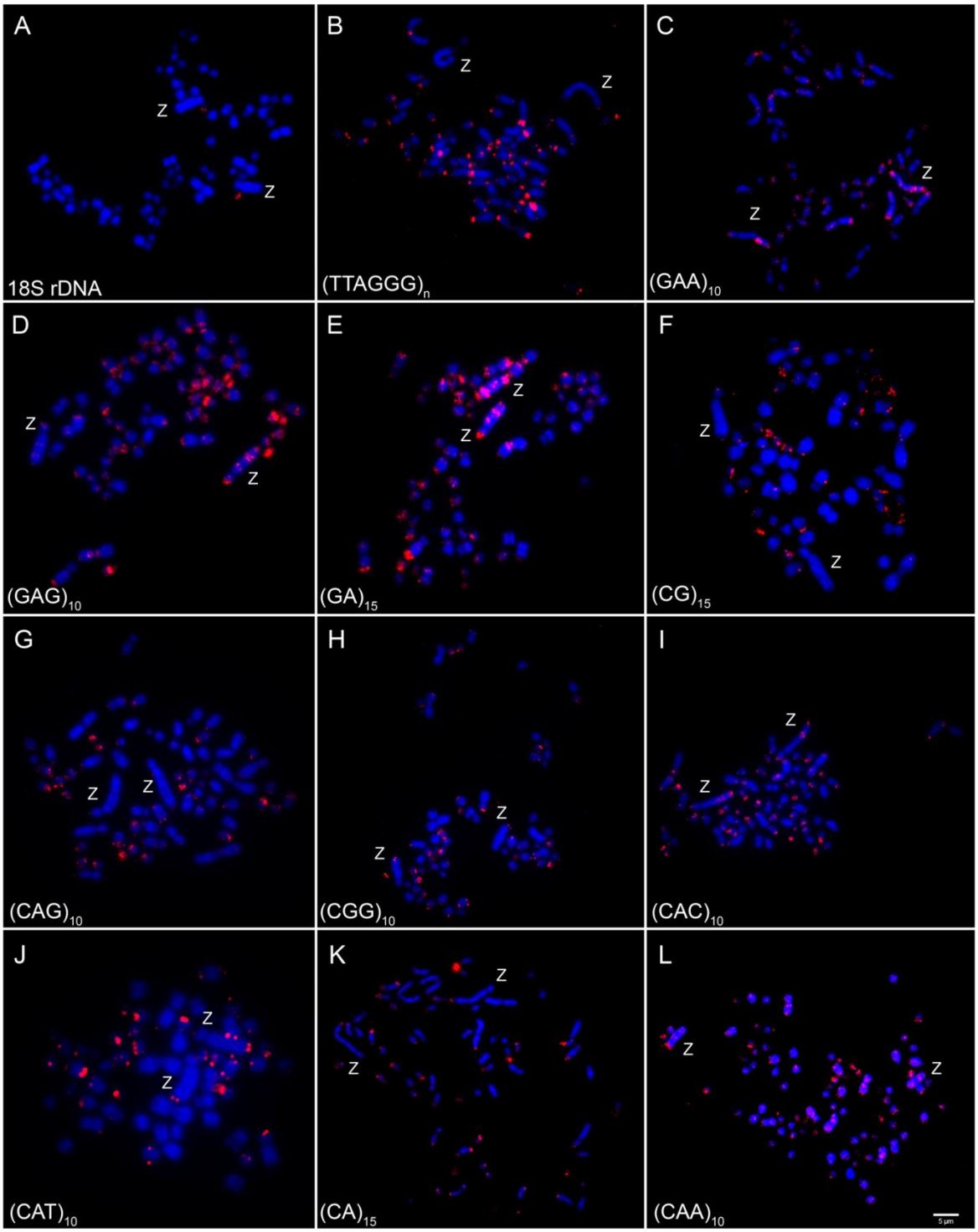
5 µm

**Figure 4**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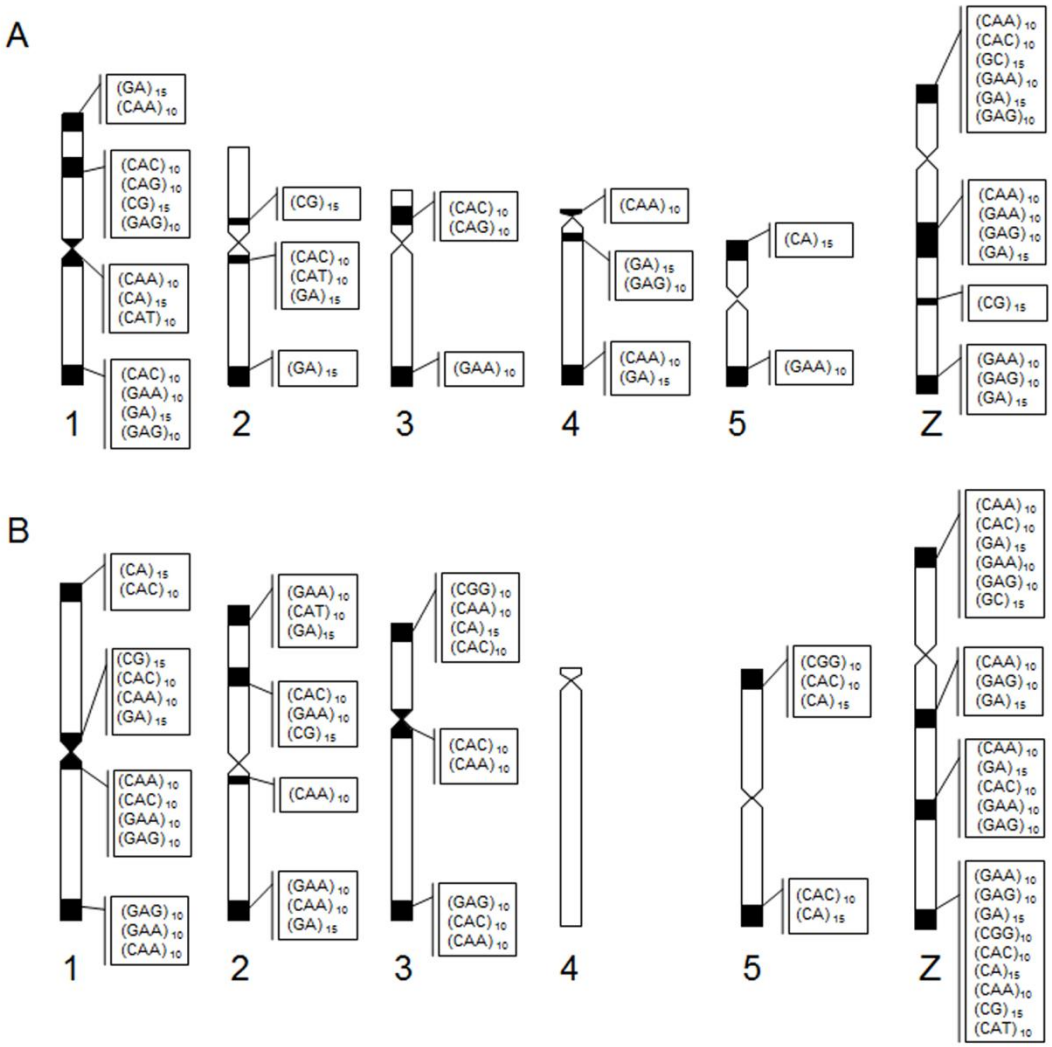
5 µm

**Figure 5**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1
(CG)15
(CAC)10 (CAT)10 (GA)15
(GA)15
2
(CAC)10 (CAG)10
(GAA)10
3
(CAA)10
(GA)15 (GAG)10
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4
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(CG)15
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Z
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Z

## 7 CONCLUSÃO

A grande acumulação de sequências repetitivas no cromossomo Z e nula no W evidencia que, apesar da origem comum proposta para o sistema sexual ZW em Aves, esses cromossomos seguiram diferentes trajetórias evolutivas em cada espécie, indicando uma alta plasticidade para a diferenciação cromossômica sexual neste grupo. Este trabalho é o primeiro passo para esclarecer o papel das sequências satélites e microssatélites na diferenciação de cromossomos sexuais. Estudos futuros envolvendo outros grupos de aves são necessários para aumentar nosso conhecimento dos processos de evolução e diferenciação desses cromossomos.

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# APÊNDICE

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RESEARCH ARTICLE

# Genomic Organization of Repetitive DNA in Woodpeckers (Aves, Piciformes): Implications for Karyotype and ZW Sex Chromosome Differentiation

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## Abstract

Birds are characterized by a low proportion of repetitive DNA in their genome when com-pared to other vertebrates. Among birds, species belonging to Piciformes order, such as woodpeckers, show a relatively higher amount of these sequences. The aim of this study was to analyze the distribution of different classes of repetitive DNAÐincluding microsatel-lites, telomere sequences and 18S rDNAÐin the karyotype of three Picidae species (Aves, Piciformes)Ð*Colaptes melanochloros* (2n = 84), *Colaptes campestris* (2n = 84) and *Mela-nerpes candidus* (2n = 64)±by means of fluorescence *in situ* hybridization. Clusters of 18S rDNA were found in one microchromosome pair in each of the three species, coinciding to a region of $(CGG)_{10}$ sequence accumulation. Interstitial telomeric sequences were found in some macrochromosomes pairs, indicating possible regions of fusions, which can be related to variation of diploid number in the family. Only one, from the 11 different microsatel-lite sequences used, did not produce any signals. Both species of genus *Colaptes* showed a similar distribution of microsatellite sequences, with some difference when compared to *M*. *candidus*. Microsatellites were found preferentially in the centromeric and telomeric regions of micro and macrochromosomes. However, some sequences produced patterns of intersti-tial bands in the Z chromosome, which corresponds to the largest element of the karyotype in all three species. This was not observed in the W chromosome of *Colaptes melano-chloros*, which is heterochromatic in most of its length, but was not hybridized by any of the sequences used. These results highlight the importance of microsatellite sequences in dif-ferentiation of sex chromosomes, and the accumulation of these sequences is probably responsible for the enlargement of the Z chromosome.

## Introduction

It is known that the avian genome is extremely compact, corresponding to approximately one third of the typical mammalian DNA content [1]. This decrease occurred due to the loss of many genes and, in a higher proportion, DNA repetitive sequences [2]. Recently, published results of genome sequences from 48 bird species showed that their amount of repetitive sequences is much smaller than other groups of Tetrapods, corresponding to 4±10%, while in mammals the percentage of these sequences can reach up to 52% from the genome [3]. Among different classes of repetitive sequences we can find satellites, microsatellites, multi-genic families, and transposable elements [3, 4]. Repetitive DNA plays an important role in genetic variation within populations, as well as in gene expression, recombination, genome structural organization, chromosomal instability and sex chromosome differentiation [5±9].

So far, the Picidae family (woodpeckers) shows the highest proportion of repetitive sequences in genomes among birds, reaching up to 22% of total DNA amount in the species *Picoides pubescens*, where the transposable element CR1 is one of the most important compo-nents [3]. In addition, karyotype analyses showed that woodpeckers have some distinctive features, such as high diploid numbers (2n), with some species possessing more than 100 chro-mosomes, and a large Z chromosome, the largest element of the karyotype [10±15].

In other groups of organisms, analyses of repetitive sequences have related their accumula-tion to the process of sex chromosomes differentiation, as in plants, reptiles and, in a greater proportion, fish [16±18]. In this regard, Matsubara and co-workers [19] observed that in *Gal-lus gallus* motifs $(GA)_{15}$ and $(GAG)_{10}$ were detected mainly in the W chromosome and, at low frequency, in the Z and autosomes. However, the organization of repetitive sequences in the genome of birds, especially their chromosomal location, is practically unknown.

The Picidae family showed a large karyotype variation in their 2n and a high amount of repetitive sequences in their genomes in comparison with other birds. Therefore, in this study we aim to characterize the chromosomal distribution of 13 classes of repetitive DNA sequences in three Picidae species, focusing on the association of these sequences with karyotype evolu-tion and the ZW sex system differentiation.

## Material and Methods

### Animals

Ten individuals belonging to three different Picidae species (Aves, Piciformes) were used in this study: three male and one female *Colaptes campestris*, five female *Colaptes melanochloros* and one male *Melanerpes candidus* (Table 1). Animals were collected in Rio Grande do Sul State (Brazil) using mist nets (permissions SISBIO 33860±1 and 44173±1). The experiments followed protocols approved by the Ethics Committee on the Use of Animals (CEUAÐUni-versidade Federal do Pampa, 026/2012).

### Chromosome preparation

Chromosome preparations were obtained using short term cultivation of bone marrow [20] or fibroblast culture [21]. Both methods included colcemid incubation, hypotonic treatment and fixation with methanol: acetic acid (3:1).

### Classical cytogenetics

Chromosome biometry was performed using the software Micromeasure 3.3 [22]. C-banding was performed according to FernaÂndez et al. [23], with modifications.

**Table 1. Specimen information and number of samples used in this study.**

| Species | Number of individuals and sex | City | Geographic coordinate |
|---|---|---|---|
| *C. campestris* | 3 ♂ and 1 ♀ | Dom Pedrito and São Gabriel | 31Ê00'37.68º S; 054Ê36'54.29º W and 30Ê20'05.93º S; 054Ê21'47.93º W |
| *C. melanochloros* | 5 ♀ | Dom Pedrito and São Gabriel | 31Ê00'37.68º S; 054Ê36'54.29º W and 30Ê20'05.93º S; 054Ê21'47.93º W |
| *M. candidus* | 1 ♂ | Porto Vera Cruz | 27Ê42'33º S; 054Ê53'29º W |

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## Probe Preparation and Fluorescent *in situ* hybridization (FISH)

18S rDNA fragments were amplified by PCR using primers NS1 $5^0$-GTA GTC ATA TGC TTG TCT C-$3^0$, NS8 $5^0$-TCC GCA GGT TCA CCT ACG GA-$3^0$ and nuclear DNA of *Ocyurus chry-surus* (Perciformes, Lutjanidae) [24]. Afterwards, fragments were labeled with digoxigenin by nick translation (Roche) and detected with Anti-Digoxigenin-Rhodamine, following manufac-turer's instructions. Telomeric probes $(TTAGGG)_n$ were obtained by PCR in the absence of a DNA template, using primers $(TTAGGG)_5$ and $(CCCTAA)_5$ [25], labeled with biotin and detected with avidin-Cy3. Preparation of slides, hybridization and washes were performed according to Daniels and Delany [26].

Oligonucleotide probes containing microsatellite sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CG)_{15}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(TA)_{15}$ were directly labeled with Cy3 during synthesis (Sigma, St. Louis, MO, USA), as described by Kubat et al. [16].

## Microscopy

At least 30 metaphase spreads per individual were analyzed to confirm the 2n, karyotype struc-ture and FISH results. Metaphases were analyzed in an epifluorescent microscope (Imager Z2,

Zeiss, Germany), and images were captured with the software Axiovision 4.8 (Zeiss, Ger-many). Final editing of images used Corel Photo Paint X5.

# Results

## Karyotyping and C-banding

We found 2n = 84 in both species of genus *Colaptes*, while *Melanerpes candidus* showed 2n = 64 (Fig 1A, 1B and 1C). *C. campestris* and *C. melanochloros* also showed chromosomes with the same morphology, with 14 pairs of macrochromosomes, including the Z and W sex chromosomes. Chromosomal morphology of each species is described in Table 2. In all these species, the Z chromosome was acrocentric and was the largest element of the karyotype. The W was smaller, with size between the sixth and seventh pairs in *Colaptes*.

Blocks of constitutive heterochromatin were seen in the pericentromeric region of macro-chromosomes, including the Z chromosome, and some microchromosomes, in all the species analyzed (Fig 2A, 2B and 2C). The W chromosome was heterochromatic in most of its length in both *Colaptes* species (Fig 2A and 2B).

## Chromosomal mapping of repetitive elements

Clusters of 18S rDNA were found in only one microchromosome pair in all species analyzed

(Figs 3A and 4A). According to chromosomal biometry, this chromosome correspondeds to pair 13 in both *Colaptes*, which exhibits a secondary constriction, and to pair 18 in *M. candidus*.

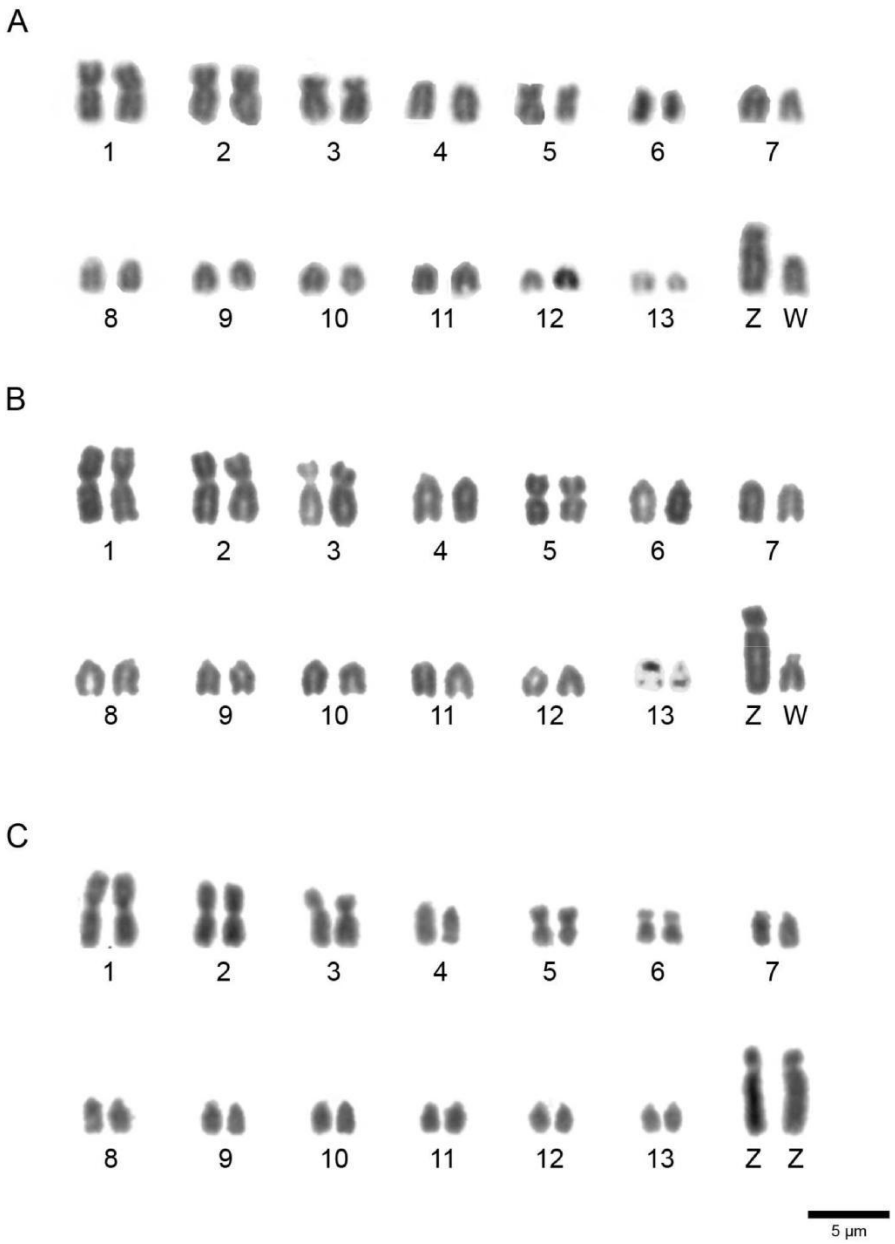


**Fig 1. Partial karyotypes of a female *Colaptes campestris* (A), a female *Colaptes melanochloros* (B) and a male *Melanerpes candidus* (C).** Bar = 5µm.

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Telomeric probes produced signals only in the terminal region of all chromosomes, except for the interstitial telomere sequences (ITS) observed in the centromeric region of pairs 1±3 in both *Colaptes species* (Fig 3B) and pairs 1±2 and 5 in *M*. *candidus* (Fig 4B).

Among the 11 distinct microsatellite sequences used in this study, only $(TA)_{15}$ did not pro-duce signals in any of the species analyzed. The same hybridization patterns were observed in both *Colaptes* species, while *M*. *candidus* showed a distinct pattern (Table 3, Figs 3C, 3D, 3E, 3F, 3G, 3H, 3I, 3J, 3K, 3L, 4C, 4D, 4E, 4F, 4G, 4H, 4I, 4J, 4K and 4L). Accumulations of micro-satellites were found mainly in centromeric and telomeric regions of the chromosomes, although some sequences produced signals in interstitial blocks.

**Table 2. Chromosomal morphology of Picidae species included in this study.**

| Chromosomes | *Colaptes campestris* | | | | *Colaptes melanochloros* | | | | *Melanerpes candidus* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Long arm | Short arm | CI | Morphology | Long arm | Short arm | CI | Morphology | Long arm | Short arm | CI | Morphology |
| **1** | 1,77 | 1,25 | 41,391 | M | 1,72 | 1,18 | 40,690 | M | 1,722 | 1,722 | 50,000 | M |
| **2** | 1,59 | 0,96 | 37,647 | SM | 1,62 | 0,97 | 37,452 | SM | 1,353 | 1,312 | 49,231 | M |
| **3** | 1,6 | 0,53 | 24,883 | SM | 1,7 | 0,73 | 30,041 | SM | 1,64 | 0,656 | 28,571 | SM |
| **4** | 1,79 | 0,15 | 7,732 | A | 1,71 | 0,17 | 9,043 | A | 1,886 | 0,369 | 16,364 | A |
| **5** | 1,14 | 0,55 | 32,544 | SM | 1,13 | 0,75 | 39,894 | SM | 0,861 | 0,82 | 48,780 | M |
| **6** | 1,47 | 0,15 | 9,259 | A | 1,61 | 0,14 | 8,000 | A | 0,943 | 0,492 | 34,286 | SM |
| **7** | 1,26 | 0,17 | 11,888 | A | 1,38 | 0,14 | 9,211 | A | 1,312 | 0,246 | 15,789 | A |
| **8** | 1,37 | 0,09 | 6,164 | A | 1,47 | 0,1 | 6,369 | A | 1,23 | 0,328 | 21,053 | A |
| **9** | 1,09 | 0,1 | 8,403 | A | 1,17 | 0,1 | 7,874 | A | 1,189 | 0,164 | 12,121 | A |
| **10** | 1,15 | 0,13 | 10,156 | A | 1,26 | 0,1 | 7,353 | A | 1,107 | 0,328 | 22,857 | A |
| **11** | 1,3 | 0 | 0,000 | T | 1,34 | 0 | 0,000 | T | 1,107 | 0,246 | 18,182 | A |
| **12** | 1,1 | 0 | 0,000 | T | 1,16 | 0 | 0,000 | T | 1,025 | 0,287 | 21,875 | A |
| **13** | 1,15 | 0 | 0,000 | T | 0,83 | 0 | 0,000 | T | 1,025 | 0,205 | 16,667 | A |
| **Z** | 2,43 | 0,59 | 19,536 | A | 2,45 | 0,69 | 21,975 | A | 3,239 | 0,82 | 20,202 | A |
| **W** | 1,43 | 0,21 | 12,805 | A | 1,33 | 0,17 | 11,333 | A | - | - | - | - |

CI = Centromeric Index, M = metacentric, SM = submetacentric, A = acrocentric, T = telocentric

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Macrochromosomes showed a preferential accumulation of microsatellites in centromeric and telomeric regions in pairs 1, 2, 3 and 5 in all species, while pair 4 showed signals only in *Colaptes* (Fig 5A and 5B). At interstitial sites, pairs 1 and 3 showed accumulation of $(CAC)_{10}$ and $(CAG)_{10}$ in *Colaptes*, while *M*. *candidus* showed bands with $(CAC)_{10}$, $(GC)_{15}$ and $(GAA)_{10}$ in chromosome 2. For microchromosomes, two different patterns of microsatellite distribu-tion were observed: some accumulated along the total length of the microchromosomes (such as $(CA)_{15}$ and $(CGG)_{10}$), and the rest were observed in the terminal region of the chromosome arms (such as $(GAA)_{10}$, $(CG)_{15}$ and $(CAT)_{10}$).

Interstitial hybridization signals were observed in the long arms of the Z chromosomes. Dif-ferent patterns of distribution of $(GAA)_{10}$, $(GAG)_{10}$ and $(GA)_{15}$ sequences were found at the three species analyzed, showing three different bands along this chromosome: $(GAG)_{10}$ and $(GA)_{15}$ were found in three bands in *Colaptes*, while in *M*. *candidus* they formed four distinct

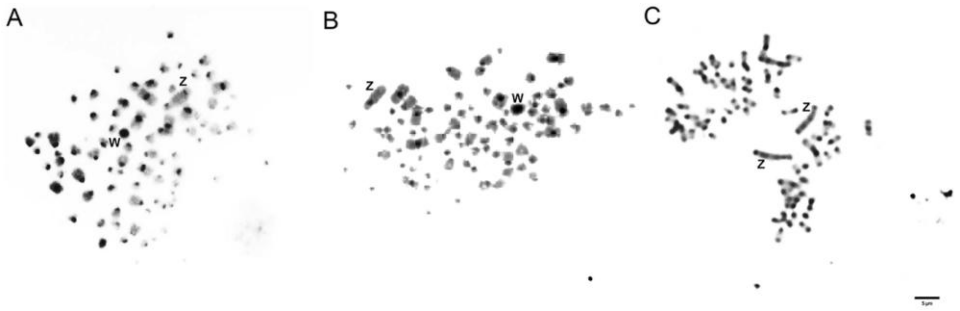


**Fig 2. C-banded chromosomes in mitotic metaphase of *Colaptes campestris* (A), *Colaptes melanochloros* (B) and *Melanerpes candidus* (C).** Chromosomes were stained with DAPI and converted to black and white with Corel® photo editor. Sex chromosomes are indicated in each metaphase. Bar = 5μm.

doi:10.1371/journal.pone.0169987.g002

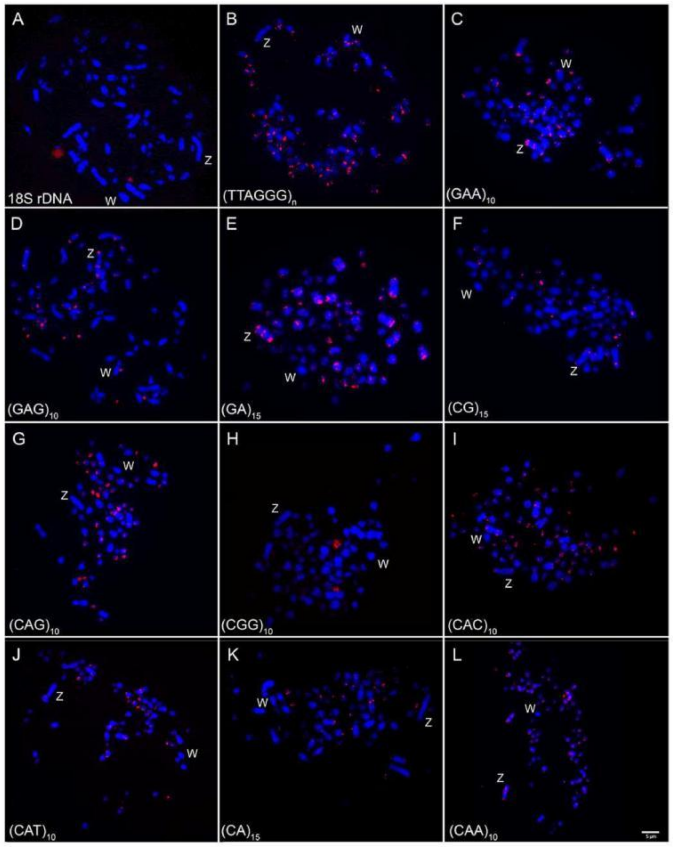


**Fig 3. Metaphase chromosomes of a female *Colaptes melanochloros* hybridized with: 18S rDNA (A), telomeric DNA (B) and microsatellites DNA (C-G).** Chromosomes were counterstained with DAPI (blue), and microsatellite probes were labeled directly during synthesis with Cy3 (red). Probes used are indicated in the lower left corner of the images. Sex chromosomes are indicated in each metaphase. Bar = 5μm.

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bands in the Z chromosome. And $(CGG)_{10}$, $(CAT)_{10}$ and $(CA)_{15}$ were found only in *M*. *candidus*, in the terminal region of the Z chromosome (Fig 5A and 5B).

## Discussion

Birds belonging to the Picidae family showed an interesting chromosomal variation, with 2n ranging from 64 to 108 (Table 4). This, along with their large Z chromosome [11], makes them an interesting group for cytogenetic analyses, especially for studies involving the distribution of repetitive sequences, usually associated with morphological differentiation of sex chromosomes [9].

Our data suggest that *C*. *campestris* and *C*. *melanochloros* have similar karyotypes in both, morphology and number of chromosomes, with 2n = 84 each. This same 2n has also been found in another species of this family, *Picoides mahrattensis*, although the only other species from the same genus, *Colaptes auratus*, showed 2n = 90 [11]. Considering molecular phylog-eny, it is indicated that *C*. *auratus* is more basal than both, *C*. *campestris* and *C*. *melanochloros* [28], thus, taking into account other 2n in the Picidae family, our findings indicate that the increase or decrease of chromosomes in this family occurs randomly, without a phylogenetic tendency [27].

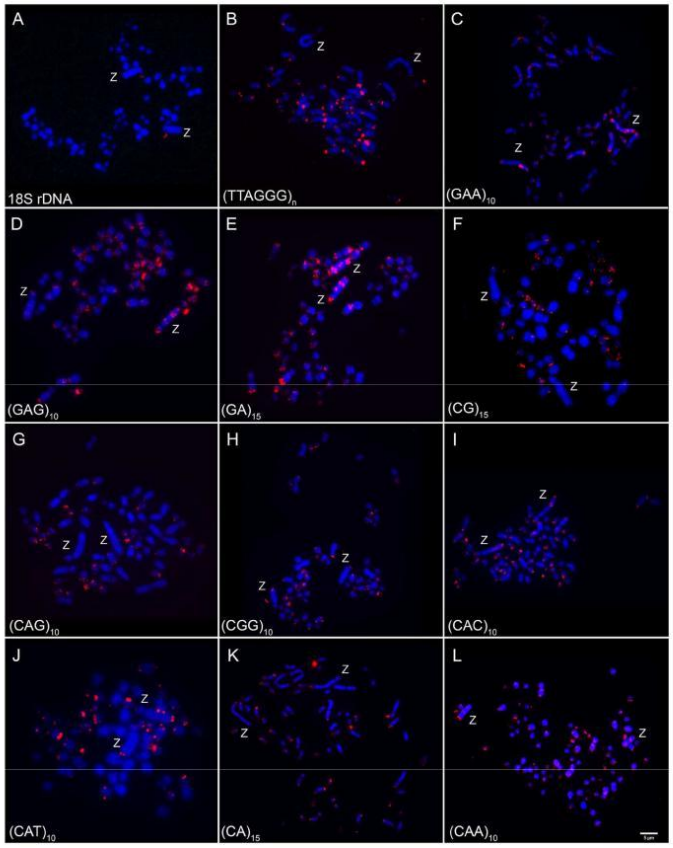


**Fig 4. Metaphase chromosomes of a male *Melanerpes candidus* hybridized with: 18S rDNA (A), telomeric DNA (B) and microsatellites DNA (C-G).** Chromosomes were counterstained with DAPI (blue) and microsatellite probes were labeled directly during synthesis with Cy3 (red). Probes used are indicated in the lower left corner of the images. Sex chromosomes are indicated in each metaphase. Bar = 5μm.

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**Table 3. Hybridization of repetitive sequences in Picidae.**

| Probes | Chromosomes | | | | | |
|---|---|---|---|---|---|---|
| | Macrochromosomes | | Microchromosomes | | Z | |
| | COL | MCA | COL | MCA | COL | MCA |
| (GAA)10 | + | + | + | + | + | + |
| (GAG)10 | + | + | + | + | + | + |
| (GA)15 | + | + | + | - | + | + |
| (CG)15 | + | + | + | + | + | + |
| (CAG)10 | + | - | + | + | - | - |
| (CGG)10 | + | + | + | + | - | + |
| (CAC)10 | + | + | + | + | + | + |
| (CAT)10 | + | + | + | + | - | + |
| (CA)15 | + | + | + | + | - | + |
| (CAA)10 | + | + | + | + | + | + |

(+) positive hybridization signals; (-) no hybridization signals; (COL) Genus *Colaptes*; (MCA) *Melanerpes candidus*.

doi:10.1371/journal.pone.0169987.t003

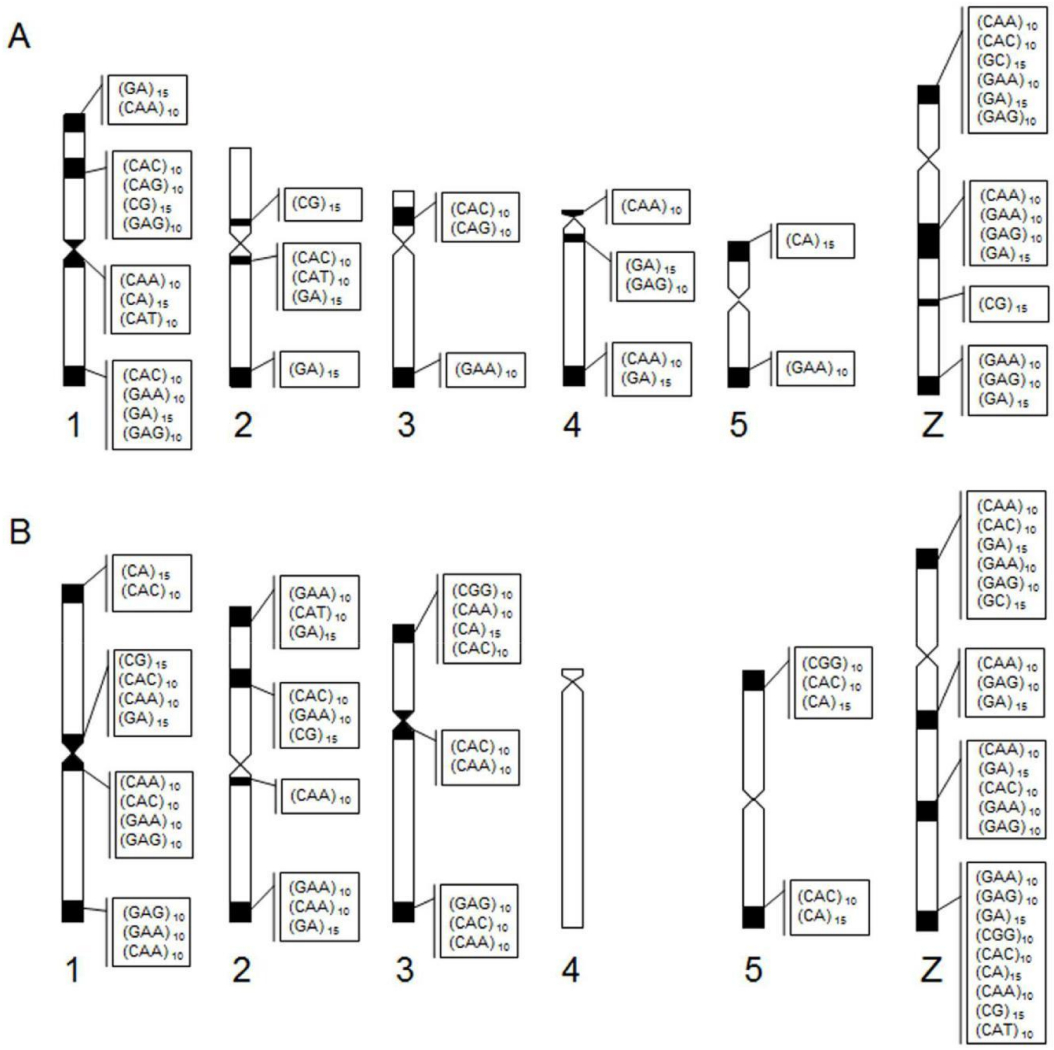


**Fig 5. Distribution and localization of microsatellite sequences in chromosomes 1±5 and Z in *Colaptes* (A) and *M. candidus* (B).**

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Although in birds, as in other groups, the occurrence of ITS in the centromeric region may represent the accumulation of repetitive sequences, coincidently similar to telomeres [29], it may also represent evidence of chromosomal fusions, as already documented in other bird species [30]. If this is the case in *Colaptes*, and considering that *C. auratus* (2n = 90) is placed in a more basal position in relation to *C. campestris* and *C. melanochloros*, both with 2n = 84, it can be argued that ITS found in the centromeric region of submetacentric pairs 1, 2 and 3 in species with 2n = 84 confirm the occurrence of three centric fusions, which would decrease the hypothetical basal 2n from 90 to 84. However, we also need the occurrence of a pericentric inversion to explain the difference in the number of arms (92 in *C. auratus* to 90 in *C. campes-tris* and *C. melanochoros*), but all of these rearrangements can be confirmed only by compara-tive chromosome painting.

Despite their phylogenetic position, the species analyzed in this study retained the plesio-morphic character of showing 18S rDNA clusters in only one microchromosome pair, as it is in the majority of the bird species analyzed so far, including some basal groups such as

**Table 4. Diploid number of Picidae species.**

| Species | 2n | References |
|---|---|---|
| *Dryocopus martuis* | 88 | [11] |
| *Colaptes campestris* | 84 | This paper |
| *Colaptes melanochloros* | 84 | This paper |
| *Colaptes auratus* | 90 | [11] |
| *Picus canus* | 92 | [13] |
| *Picus viridis* | 94 | [12] |
| *Dinopium benghalense* | 92 | [10] |
| *Melanerpes candidus* | 64 | This paper |
| *Sphyrapicus varius* | 92 | [11] |
| *Dendrocopos minor* | 108 | [11] |
| *Dendrocopos major* | 108 | [11] |
| *Dendrocopos hyperythrus* | 92 | [13] |
| *Dendrocopos kizuki* | 90 | [13] |
| *Dendrocopos leucotos* | 92 | [13] |
| *Picoides mahrattensis* | 84 | [10] |
| *Picoides villosus* | 92 | [11] |
| *Picoides pubescens* | 92 | [11] |
| *Jynx torquilla* | 90 | [13] |

The species are ordered from the most derived to most basal, following the phylogeny proposed by Benz et al. [27].

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Paleognathas *Rhea americana*, *Crypturellus tataupa*, *Tinamus solitarius* and *Pterocnemia pen-nata* [20, 31, 32]. Interestingly, microsatellite sequence $(CGG)_{10}$ was found in chromosomes possessing the secondary constriction, bearers of 18S rDNA clusters. A similar result was found in the fish *Triportheus trifurcatus* (Characiformes, Characidae), where this sequence exist in the W chromosome, which also bears 18S rDNA [9].

Microsatellite sequences were found in both, macrochromosomes, including the Z and in microchromosomes, with some differences between species and in each of the sequences. Despite the existence of interstitial blocks of repetitive DNA, accumulations of microsatellites were also found in centromeric and telomeric regions of the chromosomes.

Repetitive sequences play an important role in the differentiation of sex chromosomes in different groups, including birds, despite the small amount of these classes of DNA in their genome. For instance, it was found that the sequence $(GAG)_{10}$ is accumulated in the W chro-mosome of *Gallus gallus* [19]. However, none of the microsatellite sequences used in this study hybridized in the W chromosome of *Colaptes*. Instead, some of these sequences were found accumulated in both Z chromosome arms which could explain the fact that it is the largest chromosome in these species. It represents an unusual example of the Z accumulating
more repetitive DNAs than the W chromosome. Similar results have been found in the fish *Hoplias malabaricus*, in which the X chromosome was the preferred site for repetitive DNA accumulation in comparison with the Y [33]. Thus, taking into account that the suppression of recombination between the sex chromosome pair is a prerequisite during the evolution of sex chromosomes and that the accumulation of repetitive sequences usually occurs in non-recom-bining regions, it is possible to track a close relationship between accumulation of different kinds of microsatellite motifs and the physical differentiation of these chromosomes. Probably this is also the situation for other Picidae species, where the Z chromosome is the largest ele-ment of the karyotype.

Overall, it may be said that our FISH analysis showed that several microsatellite sequences are found amplified on the Z chromosome of three species belonging to the Picidae family. This may explain the fact that the Z is the largest element of the karyotype, and that their genome contains the highest number of repetitive sequences compared to other groups of birds. Interestingly, none of the sequences were found accumulated on the W chromosome, although they play an important role in the differentiation of sex chromosomes, and are usually found amplified on Y/W chromosomes. These results suggest that, despite the common origin proposed for the ZW sex system in birds, these chromosomes followed different evolutionary trajectories in each species, indicating a high plasticity for sex chromosome differentiation in this group. Amplifications of microsatellite motifs were also found in macrochromosomes and microchromosomes. However, considering the lack of information concerning the genomic distribution of these sequences, it is not yet possible to make a comparison with other birds.

This work is the first step towards clarifying the role of satellites and microsatellite sequences in the differentiation of sex chromosomes. Future studies involving other groups of birds are needed to increase our knowledge in processes of evolution and differentiation of these chromosomes.

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