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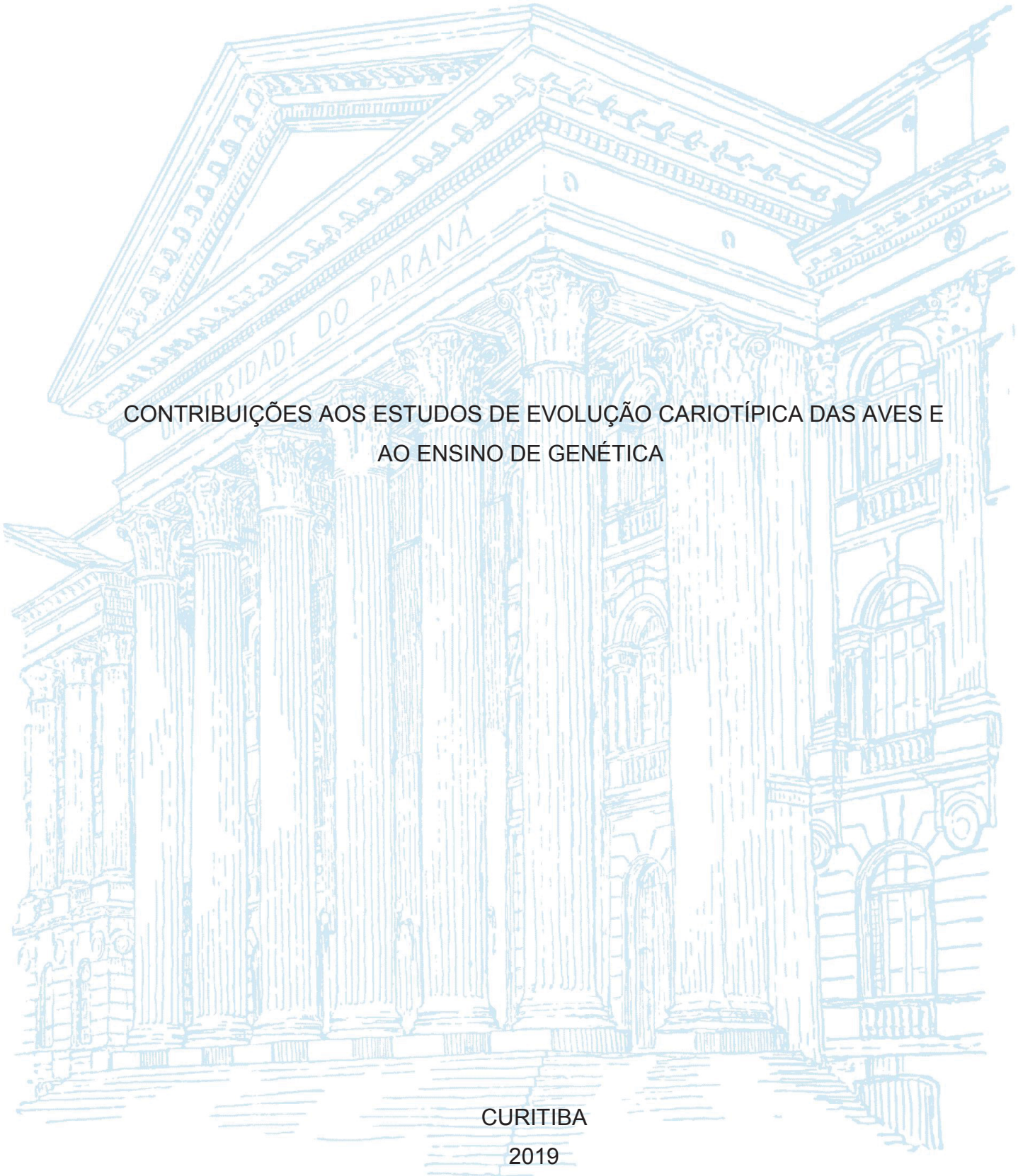

CONTRIBUIÇÕES AOS ESTUDOS DE EVOLUÇÃO CARIOTÍPICA DAS AVES E AO ENSINO DE GENÉTICA

CURITIBA

2019

TIAGO MARAFIGA DEGRANDI

CONTRIBUIÇÕES AOS ESTUDOS DE EVOLUÇÃO CARIOTÍPICA DAS AVES E AO ENSINO DE GENÉTICA

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Genética, no Curso de Pós-Graduação em Genética, Setor de Ciências Biológicas, da Universidade Federal do Paraná.

Orientadora: Drª. Iris Hass
Coorientator: Dr. Ricardo José Gunski

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em GENÉTICA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **TIAGO MARAFIGA DEGRANDI** intitulada: **Contribuições aos estudos de evolução cariotípica das aves e ao ensino de genética.** , após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua aprovação no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 22 de Março de 2019.

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Dedico esta tese ao professor Ricardo José Gunski, que sempre confiou no meu trabalho, me incentivou e trilhou este caminho comigo. És um exemplo de professor, pesquisador e um grande amigo.

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“Insanidade é continuar fazendo a mesma coisa sempre e esperar resultados diferentes”.

(Rita Mae Brown, 1983)

RESUMO

A citogenética é a ciência que estuda os cromossomos. Nela, as caraterísticas como o número diploide (2n), morfologia, padrões de bandas, e homologias dos cromossomos, podem ter valiosas aplicações sejam em diagnósticos clínicos ou para propor relações evolutivas entre as espécies. A grande diversidade da classe Aves e também o encanto pelo estudo dos cromossomos foram os grandes motivadores no desenvolvimento desta tese. No capítulo I, é apresentado cariótipo de *Megaceryle torquata* e *Chloroceryle americana* (Coraciformes, Alcedinidae). Foi observado que *M. torquata* e *C. americana*, divergem no número de cromossomos de 2n= 84 para 94, respectivamente. Nossa hipótese é que esta variação teve sua origem em fissões cêntricas, que ocorreram nos macrocromossomos. No capítulo II, é apresentado o cariótipo da espécie *Trogon surrucura surrucura* (Trogoniformes, Trogonidae) 2n= 82. A pintura cromossômica com as sondas cromossômicas do *Gallus gallus* e *Leucopternis albicollis*, evidenciaram que o cariótipo de *T. s. surrucura* foi derivado por fissões, fusões e inversões intracromossômicas. No capítulo III, é analisada a distribuição cromossômica dos sítios do 45S rDNA em 72 espécies de aves. Embora tenha sido observado que a maioria das espécies preserva o 45S rDNA em um par de microcromossomos. Foi observado que o 45S rDNA também ocorre em macrocromossomos e assim como em múltiplos microcromossomos. As origens destas variações são discutidas através de comparações que foram ancoradas em uma filogenia existente. É sugerido que processos recorrentes de duplicação resultaram em variações do número de cromossomos portadores do cluster 45S rDNA. Enquanto que fusões foram responsáveis pela redistribuição do cluster 45S rDNA de um ancestral localizado em microcromossomos para diferentes macrocromossomos. No capítulo IV, é apresentado o banco de dados citogenéticos para as aves, que foi elaborado nesta tese. Nele são disponibilizados os dados do número diploide para 1032 espécies e de homologia cromossômica com o cariótipo do *Gallus gallus* para 83 espécies. Assim, espera-se que as informações disponibilizadas no *database* possam estimular e guiar o desenvolvimento de novos trabalhos. E por último, no capítulo V, é apresentada uma alternativa para o ensino das alterações cromossômicas numéricas, as aneuploidias. A atividade é proposta na forma de um jogo de cartas, que são combinadas aos pares durante a montagem do cariótipo, originando assim diferentes aneuploidias.

Palavras-chave: Cromossomo. Jogo. Genética. Aves. 45S rDNA.

ABSTRACT

Cytogenetics is the science that studies the chromosomes. In it, features such as diploid number (2n), morphology, band patterns, and chromosome homologies may have valuable applications in clinical diagnostics or to propose evolutionary relationships between species. The great diversity of the class Aves and also the charm by the study of the chromosomes were the great motivators in the development of this thesis. In chapter I, it presents karyotype of *Megaceryle torquata* and *Chloroceryle americana* (Coraciformes, Alcedinidae). It was observed that *M. torquata* and *C. americana*, differ in the number of chromosomes from 2n = 84 to 94, respectively. Our hypothesis is that this variation had its origin in centric fissions, which occurred in macrochromosomes. In chapter II, the karyotype of the species *Trogon surrucura surrucura* (Trogoniformes, Trogonidae) 2n = 82 is presented. The chromosome painting with the chromosome probes of *Gallus gallus* and *Leucopternis albicollis*, showed that the karyotype of *T. s. surrucura* was derived by intrachromosomal fissions, fusions and inversions. In Chapter III, the chromosomal distribution of the 45S rDNA sites in 72 species of birds is analyzed. Although it has been observed that most species preserve the 45S rDNA in a pair of microchromosomes. It has been observed that 45S rDNA also occurs in macrochromosomes and as in multiple microchromosomes. The origins of these variations are discussed through comparisons that were anchored in an existing phylogeny. It is suggested that recurring duplication processes resulted in variations in the number of chromosomes carrying the 45S rDNA cluster. While fusions were responsible for the redistribution of the 45S rDNA cluster from an ancestor located in microchromosomes to different macrochromosomes. In Chapter IV, the cytogenetic database for birds is presented, which was elaborated in this thesis. The data of the diploid number for 1032 species and chromosome homology with the *G. gallus* karyotype for 83 species are available. Thus, it is expected that the information available in the database can stimulate and guide the development of new works. Finally, in Chapter V, an alternative for the teaching of numerical chromosomal alterations, aneuploidies, is presented. The activity is proposed in the form of a card game, which are combined in pairs during the assembly of the karyotype, thus giving rise to different aneuploidies.

Key- words: Chromosome. Game. Genetics. Birds. 45S rDNA.

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1. INTRODUÇÃO

As aves são o grupo de vertebrados terrestres, que compreende uma grande riqueza de espécies. De acordo com a comunidade internacional de ornitologistas, o grupo inclui 10.857 espécies, e mais de 20 mil subespécies (GILL e DONSKER, 2018). Na última década houve um grande esforço para reconstruir a filogenia das aves. Os maiores avanços neste sentido foram obtidos no “Avian Phylogenomic Project”, onde mais de 50 espécies de aves tiveram seu genoma completamente sequenciado (JARVIS *et al*., 2014). As análises destes genomas permitiram revisar as relações filogenéticas entre os principais grupos desde a clássica filogenia proposta por SIBLEY e AHLQUIST (1990). Hoje, a origem monofilética das aves é corroborada por análises do DNA e duas infraclasses são definidas: as Paleognatas, que inclui as ordens Casuariformes, Rheiformes, Struthioniformes e Tinamiformes, e as Neognatas, que inclui todas as outras ordens de aves modernas (JARVIS *et al*., 2014).

Apesar destes grandes avanços no conhecimento evolutivo das aves, estamos muito longe de conhecer uma pequena fração de espécies do ponto de vista genômico. Assim, permanecem muitas lacunas nas relações filogenéticas aos níveis mais inferiores como entre famílias, gêneros e espécies, que ainda não foram estudadas (PRUM *et al*., 2015). Em vista disso, os pesquisadores têm utilizado outras abordagens como, por exemplo, a citogenética. Nesta área da genética, o foco é o estudo dos cromossomos e cariótipos, onde as variações no número, morfologia, padrões de bandas e homologias cromossômicas são características que podem ser facilmente obtidas e que historicamente são exploradas para diferenciar espécies e inferir as relações evolutivas (DOBIGNY *et al*., 2004).

No início, a citogenética das aves enfrentou muitas dificuldades, principalmente para a obtenção de metáfases de boa qualidade e para determinar o número diploide, devido ao elevado número de microcromossomos. Estes fatores mais tarde somaram-se a um pressuposto teórico de conservatividade do cariótipo das aves (OHNO *et al*., 1964) e contribuíram para que as aves permanecessem como um dos grupos menos estudados do ponto de vista citogenético (CAMPANNA *et al*., 1987). No entanto, hoje as melhorias nos procedimentos de cultivo celular e o uso da técnica de hibridização *in situ* fluorescente (FISH) têm revelado uma nova história a respeito da estrutura do genoma e evolução do cariótipo das aves. Dada esta utilidade, o número de espécies analisadas por técnicas citogenéticas vem

crescendo a cada ano (KRETSCHMER *et al*., 2018a). Contudo, é observado que a distribuição das espécies analisadas compreende poucas ordens (Struthioniformes, Rheiformes, Casuariiformes, Tinamiformes, Anseriformes, Galliformes, Accipitriformes, Eurypygiformes, Gruiformes, Charadriiformes, Opisthocomiformes, Columbiformes, Strigiformes, Falconiformes, Pisittaciformes e Passeriformes) da classe Aves, e novamente esta abordagem recai-se sobre falhas nos grupos amostrados (KRETSCHMER *et al*., 2018a).

2. OBJETIVOS

1.1 OBJETIVO GERAL

Esta tese teve como objetivo entender os processos de diversificação cariotipica em aves e as relações evolutivas entre as espécies.

1.2 OBJETIVOS ESPECÍFICOS

- Ampliar o estudo cariotípico de espécies de aves pertencentes a Ordens pouco estudadas;

-Analisar a distribuição cromossômica do sitio 45S rDNA e correlacionar estas informações com a mais recente filogenia das aves;

-Promover o interesse da comunidade cientifica para o conhecimento citogenético das aves através da criação e divulgação de um banco de dados;

-Desenvolver um material didático para o ensino de genética envolvendo o tema cromossomos e cariótipo;

3. REVISÃO DE LITERATURA

3.1 Citogenética clássica das Aves

A citogenética compreende todo e qualquer tipo de estudo relativo aos cromossomos, isolados ou em conjunto, condensados ou distendidos, tanto no que diz respeito a morfologia, organização, função, replicação e quanto sua variação e evolução (GUERRA, 1988). Os estudos citogenético em aves já vem sendo empregados a mais de um século. O primeiro estudo data de 1902, e foi realizado por Guyer em pombos normais e híbridos. Nesta época as preparações cromossômicas eram obtidas de cortes histológicos do tecido gonadal, os quais não ofereciam qualidade suficiente para uma identificação precisa do número e da morfologia cromossômica. Mesmo assim, estima-se que deste período até 1950, pelo menos 107 espécies de aves foram analisadas do ponto de vista citogenético (SANTOS e GUSNKI, 2006).

A partir da segunda metade do século XX, com desenvolvimento das técnicas de cultivo celular e os tratamentos com solução hipotônica e colchicina, foi possível melhorar consideravelmente a qualidade das preparações cromossômicas. A partir disso, o número de espécies aviárias cariotipadas começou a ser ampliado. No entanto logo nas primeiras observações o elevado número diploide, o grande número de microcromossomos, e um pressuposto teórico de conservatividade do cariótipo, contribuíram para que as Aves permanecessem um grupo muito pouco estudado do ponto de vista citogenético (BOER, 1984)

Atualmente estima-se que aproximadamente 12% das aves foram estudadas por métodos citogenéticos (KRETSCHMER *et al*., 2018a). Em geral os cariótipos aviários são caracterizados pela variação interna no tamanho dos cromossomos, apresentando dois grupos distintos, os macrocromossomos (6µm- 2,5 µm) e microcromossomos (menores de 2,5 µm) (RODIONOV, 1996). O número diploide é considerado elevado, por volta de 80 cromossomos (TEGELSTROM e RYTTMAN, 1981). E um sistema de determinação cromossômica do sexo definido por ZZ nos machos e ZW nas fêmeas (GRAVES e SHETTY, 2001).

As técnicas de bandamento cromossômico (C, G e NOR) também tem sido utilzadas para obter marcadores cromossômicos nas aves. O bandamento C é bastante utilizado e tem especial importância para identificação do cromossomo W da fêmea, visto que este cromossomo geralmente apresenta alto conteúdo

heterocromático, distinguindo-se facilmente dos demais cromossomos do cariótipo (DE LUCCA, 1983). A banda G, é um método pouco utilizado, pois apenas os macrocromossomos apresentam um padrão de bandas bem definido, tornando limitado o uso desta técnica nos cariótipos das aves (BENMESSAOUD e MOHAMMEDI, 2018).

A técnica de Ag-NORs é amplamente utilizada, pois ela permite a identificação dos cromossomos portadores dos sítios de DNA ribossomal 45S (KRETSCHMER *et al*., 2014). Frequentemente os estudos que tem utilizado Ag-NOR em aves tem observado que as NORS encontram-se localizados em um par de microcromossomos, sendo esta uma condição ancestral no cariótipo aviário (NISHIDA-UMEHARA *et al*., 2007).

3.2 Pintura cromossômica em Aves

A técnica de *Fluorescence in situ hybridization* (FISH) revolucionou os estudos citogenéticos. Ela permite que determinadas sequências de DNA (sondas) sejam observadas diretamente no cromossomo, em seus locais de completa ou parcial homologia (PINKEL *et al*., 1986). Neste sentido tanto sondas de cromossomos inteiros, como sondas de sequências gênicas, DNA repetitivos (microssatélite e elementos transponíveis), tem sido utilizadas no objetivo de compreender a estrutura e evolução do cariótipo das aves (NISHIDA-UMEHARA *et al*., 2007; DE OLIVEIRA *et al*., 2013; DE OLIVEIRA *et al*., 2017; BERTOCCHI *et al*., 2018).

O galo doméstico (*Gallus gallus*- GGA) foi a primeira espécie utilizada para isolar sondas de cromossomos inteiros (GRIFFIN *et al*., 1999). Os macrocromossomos GGA1 ao GGA10 são os mais utilizados na pintura cromossômica comparativa. Através dela foi possível estabelecer com sucesso a homologia entre os macrocromossomos entre espécies distantemente aparentadas, pertencentes as infra classes Paleognata e Neognata (DE OLIVEIRA *et al*., 2005; NISHIDA-UMEHARA *et al*., 2007; NANDA *et al*., 2011; KRETSCHMER *et al*., 2014).

A conservação sintênica dos macrocromossomos do *Gallus* tem sido amplamente observada entre as aves (NISHIDA-UMEHARA *et al*., 2007; KRETSCHMER *et al*., 2015; DOS SANTOS *et al*., 2017). No entanto, em algumas famílias como por exemplo Accipitridae (Águias e Gaviões), foi evidenciado uma profunda reorganização cariotípica, originada por quebras e fusões cromossômicas,

envolvendo tanto os macrocromossomos quanto os microcromossomos. Estes complexos rearranjos cromossômicos tiveram como resultado, a redução do número diploide (2n=66) que é característico das espécies da família Accipitridae (DE OLIVEIRA *et al*., 2005; 2010; 2013; NISHIDA *et al*., 2013; NIE *et al*., 2015).

Além do *G. gallus*, outras espécies foram utilizadas para obtenção de sondas cromossômicas. Em 2009, Nie e colaboradores isolaram os cromossomos da espécie *Burhinus oedicnemus* 2n= 42, que pertence a ordem Charadriiformes (NIE *et al*., 2009). Em 2010, foram isolados os cromossomos de *Leucopternis albicollis* (Accipitriformes) 2n= 66 (DE OLIVEIRA *et al*., 2010). Em 2015 foi a vez de *Gyps fulvus* (Accipitriformes) (2n = 66) (NIE *et al*., 2015). E o mais recente conjunto de sondas cromossômicas isolado foi da espécie *Zenaida auriculata* (Columbiformes) 2n = 76, (KRETSCHMER *et al*., 2018b).

As sondas de *L. albicollis* (LAL), tem sido as mais utilizadas além das do *Gallus*. Através da análise combinadas das sondas destas espécies foi possível identificar a ocorrência de rearranjos intra cromossômicos como as inversões pericêntricas e paracêntricas. Estes achados deram uma nova direção para discutir o estado conservativo dos macrocromossomos das aves, pois mesmo aqueles cromossomos que eram vistos como homólogos através das sondas de *Gallus*, encontram-se diferenciados devido a ocorrência de rearranjos intra cromossômicos (KRETSCHMER *et al*., 2014; DOS SANTOS *et al*., 2015; DEGRANDI *et al*., 2017).

Com a disseminação da técnica de FISH e das sondas cromossômicas entre os laboratórios, diversas espécies começaram a ser analisadas com intuito de compreender os mecanismos de estruturação cariotipica e também para propósitos filogenéticos. Atualmente a pintura cromossômica foi empregada em mais de 70 espécies de aves que compreendem as ordens Struthioniformes, Rheiformes, Casuariiformes, Tinamiformes, Anseriformes, Galliformes, Accipitriformes, Gruiformes, Charadriiformes, Opisthocomiformes, Columbiformes, Strigiformes, Trogoniformes, Falconiformes, Psittaciformes, Passeriformes (KRETSCHMER *et al*., 2018a).

Tão logo estes dados começaram a se acumular, valiosas informações a respeito da evolução das aves puderam ser identificadas. Por exemplo, a fissão do cromossomo GGA1 é observada entre todas as espécies da ordem Passeriformes e vem sendo considerada uma assinatura cromossômica exclusiva do grupo. As fusões GGA6/GGA7 e GGA8/GGA9 é comum aos Pissitaciformes. E as fissões do

GGA2, GGA3 e GGA5 e também fusões entre microcromossomos são observadas entre aos Falconiformes. Mais detalhes sobre as assinaturas cromossômicas observadas em aves, ver revisão de KRETSCHMER *et al*., (2018a).

3.3 Distribuição cromossômica do 45S rDNA em aves

Os sítios de rDNA constituem, nas regiões cromossômicas onde encontram-se localizados os genes que codificam para o RNA ribossômico (18S, 5.8S, 28S) (DYOMIN *et al*., 2016). Estes sítios cromossômicos são formados por várias cópias dos genes ribossomais, dispostos em tandem no DNA. Cada unidade de repetição é formada pela trinca dos genes 18S, 5.8S e 28S, separados por espaçadores transcritos internos (ITS1 e ITS2) e externos (5'ETS e 3'ETS), que formam o cluster 45S rDNA (DYOMIN *et al*., 2016). Enquanto o gene 5S, um dos constituintes da subunidade maior do ribossomo, é transcrito em um outro sítio cromossômico próprio, sítio 5S rDNA (DANIELS e DELANY, 2003).

Os estudos dos sítios de rDNA em espécies da classe Aves são bastante insipientes, a maioria das informações deram-se através da identificação destes cromossomos utilizando a técnica de coloração com nitrato de prata (Ag-NOR), que foi proposta por Howel e Black (1980). Porém a Ag-NOR tende a apresentar variações intra e inter-celulares e também associações inespecíficas, pois a prata reage com as proteínas e não com o DNA, induzindo a erros na identificação destes cromossomos. Sendo assim, a técnica de FISH é mais recomendada, pois permite a identificação especifica da localização do rDNA, e pode ser feita por meio do uso de sondas dos genes (18S, 5.8S, 28S) que fazem parte do cluster 45S rDNA.

Considerando o uso do FISH para identificação dos sítios de 45S rDNA em aves, o primeiro registro foi realizado no cariótipo do *Gallus gallus* (2n=78) por DOMINGUEZ-STEGLICH *et al*., (1991). Interessantemente, foi observado que o 45S rDNA encontra-se no microcromossomo GGA16, associado com genes do complexo de histocompatibilidade (MHC). Esta associação também foi observada em *Coturnix japônica* e *Meleagris gallopavo* e foi provavelmente originada por uma fusão cromossômica, que até então é considerada como exclusiva da ordem Galliformes (MCPHERSON *et al*., 2014).

Nas aves da infra classe Paleognata, foi observado em cinco espécies (*Dromaius novaehollandiae*, *Casuarius casuarius*, *Struthio camelus*, *Rhea pennata*, *Rhea americana*) que o 45S rDNA está localizado em um par de microcromossomos,

divergindo para dois pares em *Eudromia elegans* (NISHIDA-UMEHARA *et al*., 2007). Considerando a localização do rDNA em um par de microcromossomo comum entre as aves Paleognatas e o *Gallus*, foi proposto que esta seria a condição ancestral do rDNA para as aves (NISHIDA-UMEHARA *et al*., 2007).

No entanto, tão logo mais espécies começaram a ser analisadas as variações numéricas e também cromossômicas foram identificadas. Por exemplo, em Falconiformes foi registrada uma variação no número de cromossomos portadores do cluster rDNA entre espécies filogeneticamente relacionadas. No *Falco tinnunculus* o 45S rDNA está localizado em dois pares de microcromossomos, no *Falco peregrinus* em 5 pares, e no *Falco columbarius* em 6 ou 7 pares (NISHIDA *et al*., 2008).

Em Accipitriformes, 11 espécies tiveram a localização do 45S rDNA identificada. Na família Cathartidae (*Sarcoramphus papa*, *Cathartes burrovianus*, *Cathartes aura* e *Gymnogyps californianus*) o rDNA está localizado em um único par de microcromossomos (RAUDSEPP *et al*., 2002; TAGLIARINI *et al*., 2009). Já para os Accipitridae existe uma grande variação, sendo o rDNA localizado em um par de microcromossomos em *Nisaetus n. orientalis* (NISHIDA *et al*., 2013), em um par de microcromossomos e um par de macrocromossomos na *Hapia harpyja* (TAGLIARINI, 2013) e exclusivamente em um par de macromossomos nas espécies *Pandion haliaetus*, *Buteo nitidus*, *Rupornis magnirostris*, *Buteogallus meridionalis*, *Morphnus guianensis* (DE OLIVEIRA *et al*., 2013; TAGLIARINI, 2013; NISHIDA *et al*., 2014).

Atualmente a localização do 45S rDNA em um par de microcromossomos é aceita como o estado ancestral em Aves e as variações observadas são explicadas por rearranjos cromossômicos como duplicações, translocações e fusões cromossômicas. No entanto estas observações são restritas a poucas ordens que foram estudadas até o presente (Tinamiformes, Casuariiformes, Struthioniformes, Rheiformes, Galliformes, Columbiformes, Charadriiformes, Accipitriformes, Piciformes, Pisittaciformes, Falconiformes, Passeriformes) (RAUDSEPP *et al*., 2002; NISHIDA-UMEHARA *et al*., 2007; NISHIDA *et al*., 2008; TAGLIARINI *et al*., 2009; NIE *et al*., 2009; DE OLIVEIRA *et al*., 2013; TAGLIARINI, 2013; NISHIDA *et al*., 2013; MCPHERSON *et al*., 2014; NISHIDA *et al*., 2014; KRETSCHMER *et al*., 2014; DOS SANTOS *et al*., 2015; KRETSCHMER *et al*., 2015; SEIBOLD-TORRES *et al*.,

2015; DYOMIN *et al*., 2016; DOS SANTOS *et al*., 2017; DE OLIVEIRA *et al*., 2017; DEGRANDI *et al*., 2017; KRETSCHMER *et al*., 2018).

3.4 O uso de jogos no ensino de genética

Os jogos didáticos constituem uma excelente alternativa para melhorar o desempenho dos alunos no processo de aprendizagem. Além de favorecer o desenvolvimento da inteligência, da personalidade e a afeição com os colegas enquanto realizam trabalhos em grupos. Também contribuem estimulando a criatividade, ao possibilitar que os alunos construam materiais e/ou metodologias que expliquem suas próprias dúvidas (CUNHA, 1988; MIRANDA, 2001).

Assim sendo, muitos docentes têm se dedicado no desenvolvimento de ferramentas alternativas para elucidar conteúdos teóricos de modo didático e também lúdico, utilizando materiais caseiros de fácil obtenção para produzir jogos pedagógicos, modelos tridimencionais e até mesmo o desenvolvimento de atividades cênicas e musicais com a efetiva participação dos alunos (TEMP *et al*. 2011; WEYH *et al*., 2015; BERTOCCHI *et al*., 2016; TATSCH e SEPEL, 2017).

Dentre os temas trabalhados em genética, as alterações cromossômicas numéricas, constituem um assunto bastante interessante para o desenvolvimento de uma abordagem alternativa de ensino. As alterações cromossômicas numéricas podem ocorrer através da perda ou acréscimo de um ou mais cromossomos (aneuploidias), ou mesmo do complemento cromossômico inteiro (euploidias) (GRIFFITHS, 2006).

As aneuploidias, podem ter sua origem da não disjunção de cromossomos durante as divisões mitótica e/ou meiótica, seja no momento da separação de cromossomos homólogos ou de cromátides irmãs. Estes erros durante a divisão celular têm especial importância àqueles que envolvem a formação dos gametas, onde uma célula diploide é reduzida ao estado haploide, e quando fertilizados resultam em combinações cromossômicas que diferem numericamente no valor diploide padrão para a espécie (GRIFFITHS, 2006).

As aneuploidias são ainda classificadas em: nulissomias, quando ocorre a perda completa de um par de cromossomos homólogos; monossomias, resulta da perda de um cromossomo no par; trissomias, resultam do acréscimo de um cromossomo no par; tetrassomia, ocorre quando existem quatro cópias de um

mesmo cromossomo no cariótipo. Além disso, tais alterações também podem afetar os cromossomos sexuais X e Y (GRIFFITHS, 2006).

4. CAPÍTULO 1:

KARYOTYPE DESCRIPTION AND COMPARATIVE ANALYSIS IN RINGED KINGFISHER AND GREEN KINGFISHER (CORACIIFORMES, ALCEDINIDAE)

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Karyotype description and comparative analysis in Ringed Kingfisher and Green Kingfisher (Coraciiformes, Alcedinidae)

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Abstract

Kingfishers comprise about 115 species of the family Alcedinidae, and are an interesting group for cytogenetic studies, for they are among birds with most heterogeneous karyotypes. However, cytogenetics knowledge in Kingfishers is extremely limited. Thus, the aim of this study was to describe the karyotype structure of the Ringed Kingfisher (*Megaceryle torquata* Linnaeus, 1766) and Green Kingfisher (*Chloroceryle americana* Gmelin, 1788) and also compare them with related species in order to identify chromosomal rearrangements. The Ringed Kingfisher presented 2n = 84 and the Green Kingfisher had 2n = 94. The increase of the chromosome number in the Green Kingfisher possibly originated by centric fissions in macrochromosomes. In addition, karyotype comparisons in Alcedinidae show a heterogeneity in the size and morphology of macrochromosomes, and chromosome numbers ranging from 2n = 76 to 132. Thus, it is possible chromosomal fissions in macrochromosomes resulted in the increase of the diploid number, whereas chromosome fusions have originated the karyotypes with low diploid number.

Keywords

Aves, chromosome, evolution, karyotype

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Introduction

Avian karyotypes are characterized by internal variation in the size of chromosomes, presenting two distinct groups, macrochromosomes and microchromosomes. About eight pairs of macrochromosomes are seen in most of birds, and the remaining are microchromosomes (Rodionov 1996). Diploid number also varies, including species with a low diploid number such as *Burhinus oedicnemus* Linnaeus, 1758 (Charadriiformes) 2n = 40 (Nie et al. 2009), and high 2n = 136-142 in *Corythaixoides concolor* Smith, 1833 (Musophagiformes) (Christidis 1990), but most of the species exhibit karyotypes with 2n = 74–86 (Tegelstrom and Ryttman 1981).

Studies of karyotype structure in birds have given valuable information about evolutionary relationships. Chromosome painting shows that, although relatively conserved, the macrochromosomes evolve through several intra and inter-chromosomal rearrangements (de Oliveira et al. 2010, Kretschmer et al. 2014). While Tandem fusions between microchromosomes and micro- with macrochromosomes have resulted in decrease of diploid number (Nishida et al. 2008, Nie et al. 2009, de Oliveira et al. 2010, 2013). Chromosome fission in recurrent breakpoints has been documented in macrochromosomes, and can result in increase of chromosome number (Skinner and Griffin 2012, Degrandi et al. 2017).

In relation to the sex chromosomes of birds, males have a homogametic ZZ pair and female have a heterogametic ZW (Schartl et al. 2015). The Z chromosome is a highly conserved macrochromosome and it comprises 7% of the haploid genome (Graves and Shetty 2001). In Piciformes, Bucerotiformes, and Coraciiformes the Z chromosome is often the largest chromosome of the complement (de Oliveira et al. 2017). Whereas the W chromosome is highly variable in size, and has been observed from homomorphic to Z in Paleognaths Ratite (Nishida-Umehara et al. 2007) to a small and heterochromatic with variable size in Neognaths birds (Graves and Shetty 2001). This size variation has been attributed to a differential accumulation and degradation of repetitive DNAs (de Oliveira et al. 2017). Also, a multiple sex chromosome system was recently described for the Adelie Penguin (*Pygoscelis adeliae* Hombron et Jacquinot, 1841/ Sphenisciformes) where males have $Z_1Z_1Z_2Z_2$ and females Z_1Z_2W (Gunski et al. 2017).

Kingfishers (Alcedinidae) comprises a diverse family of birds with approximately 115 species distributed worldwide (Gill and Donsker 2017). They are an interesting group for cytogenetic studies since they are among birds with most heterogeneous karyotypes. However, knowledge about cytogenetics in Kingfishers is extremely limited. There are records for *Dacelo novaeguineae* Hermann, 1783, 2n = 76, *Halcyon smyrnensis* Linnaeus, 1758, 2n = 76, *Halcyon pileata* Boddaert, 1783, 2n = 84, *Alcedo atthis* Linnaeus, 1758, 2n = 132, *Ceyx azureus* Latham, 1801, 2n = 122, and *Ceryle rudis* Linnaeus, 1758, 2n = 82 (De Boer and Belterman 1980, Xiaozhuang and Qingwei 1989, Christidis 1990, Youling et al. 1998, Garg and Shrivastava 2013).

The Ringed Kingfisher, *Megaceryle torquata* Linnaeus, 1766 and the Green Kingfisher, *Chloroceryle americana* Gmelin, 1788 belong to subfamily Cerylinae and their

karyotypes are unknown (Moyle 2006). In view of this, the present study aimed to describe the karyotype structure of these species. Secondly, we sought to gather karyotype information from Alcedinidae in order to compare them and to identify the chromosomal rearrangements.

Material and methods

Samples and location

The karyotype of one male and one female of *Megaceryle torquata* (Fig. 1A) collected at the Parque Ecológico El Puma in Argentina, and two males and one female of *Chloroceryle americana* (Fig. 1C) from Santa Maria/Rio Grande do Sul, Brazil were analyzed for this work. Specimens were collected according to license SISBIO 44173-1 and animal research ethics committee (CEUA 018/2014).

Cell culture

Mitotic chromosomes in *M. torquata* specimens were obtained by lymphocyte culture according to Moorhead et al. (1960). In short, blood samples were incubated in medium PBMax (Gibco) for 72 hours at 38 °C. In the last hour of incubation, 0,001 ml of colchicine solution (0.05%) was added. After these procedures, the cells were centrifuged and pellet was incubated in 10 mL of hypotonic solution (0.075 M KCl) for 20 min, followed by fixation in three washes with Methanol: Acetic acid 3:1 solution.

In *C. americana*, mitotic cells were obtained from bone marrow according to Garnero and Gunski (2000). Initially, bone marrow was extracted from femurs and incubated in a 10 ml of RPMI 1640 medium with 0,001 ml of colchicine solution (0.05%) at 39 °C for 1 hour. Finally, cells were incubated in 10 ml of hypotonic solution (0.075 M KCl) for 20 minutes. Then cells were washed three times with Methanol: Acetic acid 3:1 solution.

Chromosomal analyses

The diploid number was determined by analyzing approximately 40 metaphases per specimen, by conventional 0,8% Giemsa staining solution. Karyotypes were organized according to chromosome size and differential staining CBG-banding (Sumner 1972) was applied to identify the W chromosome.

Morphometry of the first 15 autosomal chromosomes pairs and the ZW sex chromosomes, were performed in Alcedinidae species available. Centromeric index (CI) was estimated by ratio of short arm length by total chromosome length. Nomenclature for chromosome morphology were performed according to Guerra (1986) using CI index.

Results

The Ringed Kingfisher presented chromosome number of 2n = 84 (Figure 1B). The chromosome set is composed of ten biarmed pairs, being the submetacentric pairs (1, 3 and 4), metacentric (2, 5, 8 and 13) and acrocentric (6, 7 and 9). The remaining autosomes are telocentric. Z and W are both submetacentric macrochromosomes, with size similar to chromosome 4 and 9, respectively.

The Green Kingfisher had a diploid number of 2n = 94 (Fig.1D), consisting of only four biarmed pairs, where 1, 2 and 3 are submetacentric and 12 is metacentric. All the other chromosome pairs are telocentric. The Z chromosome is submetacentric and is the largest chromosome of the karyotype, while the W chromosome is submetacentric with size between 1 and 2.

C-banding analysis allowed correct identification of the W chromosome, since both species presented a highly heterochromatic pattern for this chromosome (Fig. 2A and B). The Z chromosome was euchromatic in both species. However, in *C. americana* a positive staining was observed near the centromere (Fig. 2 B).

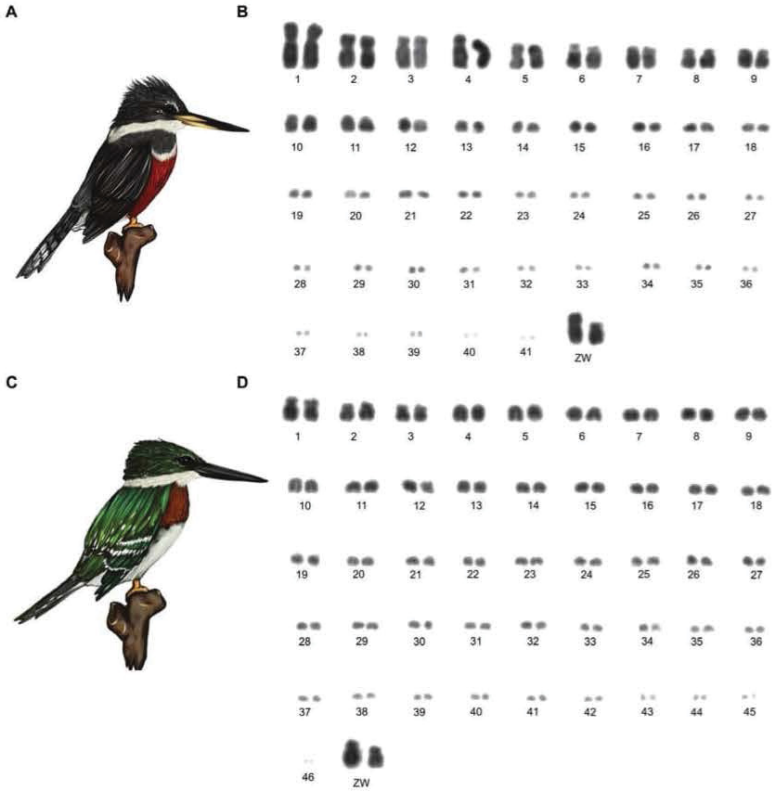

Figure 1. Ringed Kingfisher *Megaceryle torquata* (**A**), and karyotype with 2n = 84 (**B**). Green Kingfisher *Chlorocerylle americana* (**C**), and karyotype with 2n = 94 (**D**).

In the literature, chromosome data were found for *C. rudis*, *H. pileata*, *A. atthis*, *H. smyrnensis*, *D. novaeguineae,* and *C. azureus* (Table 1). Unfortunately, for *H. smyrnensis*, *D. novaeguineae*, *C. azureus* only the diploid number was available. Despite this, some observations can be made: i) diploid number is highly variable; ii) number of biarmed chromosomes (metacentric, submetacentric, and acrocentric) was also variable; iii) the Z chromosome is a conserved submetacentric chromosome; and iv) the W chromosome morphology is variable among species, appearing as metacentric or submetacentric.

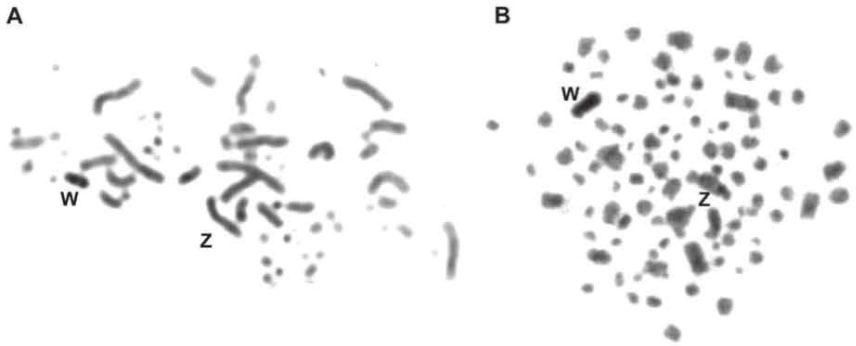

Figure 2. Comparative C-banding analysis of the Ringed Kingfisher *Megaceryle torquata* (**A**) and the Green Kingfisher *Chloroceryle americana* (**B**).

Table 1. Karyotype information's in Alcedinidae species.

Species	2n	Nº biarmed	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Z	W	Reference
Chloroceryle americana	94	4	S	S	S	T	T	T	T	T	T	T	T	M	T	T	T	S	S	Present work
Ceryle rudis	82	13	M	M	M	M	M	M	S	S	A	A	A	A	A	T	T	S	M	Garg and Shrivastava 2013.
Megaceryle torquata	84	10	S	M	S	S	M	A	A	S	A	T	T	T	M	T	T	S	S	Present work
Halcyon pileata	84	12	M	M	S	S	M	M	M	S	T	T	M	T	M	M	S	S	M	Xiaozhuang and Qingwei 1989.
Halcyon smyrnensis	76	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Youling et al. 1998.
Dacelo novaeguineae	76	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	De Boer and Belterman 1980.
Alcedo atthis	132	15	M	M	M	S	M	M	M	M	S	M	S	M	M	M	M	S	M	Xiaozhuang and Qingwei 1989.
Ceyx azureus	122	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Christidis 1990.

2n= diploid number; Nº biarmed= Number of biarmed autosomes; Chromosome morphology: (M=metacentric, S=submetacentric, A=Acrocentric and T=Telocentric); - = Not was possible to obtain the information in original work; Species names in accordance to IOC WORLD BIRD LIST (7.3) http://dx.doi.org/10.14344/IOC.ML.7.

Discussion

Unfortunately, forty years after the publication of the karyotype of *D. novaguineae* (*D. gigas* by De Boer and Beltrman 1980), information about cytogenetics of Alcedinidae species is still limited. Nevertheless, comparisons done in this work (Tab. 1) show that Kingfishers present karyotype plasticity, evidenced by variation in diploid number, number of biarmed chromosomes, and in size and morphology of macrochromosomes.

According to White (1977), chromosome fusions result in the reduction of diploid number and increase of number of biarmed chromosomes, while chromosome fissions increase the diploid number and decrease the number of biarmed chromosomes. These mechanisms appear to be adequate to explain the differences in the karyotypes of Alcedinidae species.

In this work, the increasing of diploid number observed in *M. torquata* (2n = 84) to *C. americana* (2n = 94), (Fig. 1B and D) may have originated by chromosome fissions. Some characteristics support this hypothesis, such as, the number of biarmed chromosomes is reduced from 9 pairs in *M. torquata* for to 4 in *C. americana*, and Z chromosome size is similar to chromosome 4 in *M. torquata*, while in *C. americana*, the Z chromosome is the largest in the karyotype. However, experiments with chromosome painting with specific probes could confirm these hypotheses.

According to Graves and Shetty (2001) Z chromosome size is conserved in most birds. So, Z chromosome size in relation to other macrochromosomes can be considered as a marker for size and evidence of occurrence of chromosome fission or fusions. Chromosome W in *M. torquata* and *C. americana* did not present differences and shows a pattern of heterochromatinization, similar of what has been observed in other Neognaths species. However, when compared to other species of Kingfishers, it is observed that there is a variation in chromosome morphology, ranging from metacentric to submetacentric.

Conclusion

Kingfishers present interesting chromosomal characteristics. These species have a diploid number which is highly variable and probably originated by fusions and/or fissions involving macrochromosomes. Hence rearrangements in macrochromosomes result in size and morphology variations, characterizing an intra-familial karyotypic heterogeneity. Absence of G-banding pattern and chromosome painting data did not allow comparisons. Therefore, we hope that this work may encourage the development of other cytogenetic studies in Kingfishers, and that our hypothesis of fission and chromosomal fusions as mechanisms responsible for karyotypes differentiation in Kingfishers can be confirmed.

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5. CAPÍTULO 2:

CHROMOSOME PAINTING IN TROGON S. SURRUCURA (AVES, TROGONIFORMES) REVEALS A KARYOTYPE DERIVED BY CHROMOSOMAL FISSIONS, FUSIONS, AND INVERSIONS

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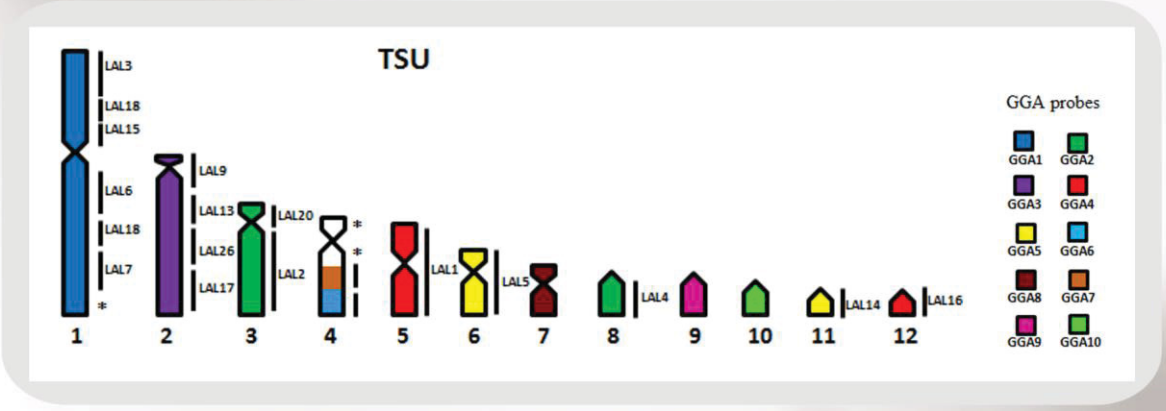

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Chromosome Painting in *Trogon s. surrucura* (Aves, Trogoniformes) Reveals a Karyotype Derived by Chromosomal Fissions, Fusions, and Inversions

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Keywords
Comparative chromosome painting · Cytogenetics · Homology map · Karyotype evolution · Trogon

Abstract
Trogons are forest birds with a wide distribution, being found in Africa, Asia, and America, and are included in the order Trogoniformes, family Trogonidae. Phylogenetic studies using molecular data have not been able to determine the phylogenetic relationship among the different genera of trogons. So far, no cytogenetic data for these birds exist. Hence, the aim of this study was to characterize the karyotype of *Trogon surrucura surrucura* by means of classical and molecular cytogenetics. We found a diploid chromosome number of 2n = 82, similar to most birds, with several derived features compared to chicken and the putative ancestral avian karyotype. *T. s. surrucura* showed 3 pairs of microchromosomes bearing 18S rDNA clusters. The Z and W sex chromosomes were of similar size but could readily be identified by morphological differences. Using chromosome painting with whole chromosome probes from *Gallus gallus* and *Leucopternis albicollis*, we found that the chromosomes homologous to chicken chromosomes 2 and 5 correspond to 2 different pairs in *T. s. surrucura* and *L. albicollis*, due to the occurrence of centric fissions. Paracentric inversions were detected in the segment homologous to chicken chromosome 1q, and we confirmed the recurrence of breakpoints when our results were compared to other species of birds already analyzed by FISH or by in silico genome assembly.

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Trogons are pantropical forest birds occurring in Africa, Asia, and America and are included in the only family of the order Trogoniformes, Trogonidae. There are about 40 different species, of which 10 are found in Brazil: *Pharomachrus pavoninus, Trogon melanurus, T. viridis, T. ramonianus, T. violaceus, T. curucui, T. surrucura, T. rufus, T. collaris*, and *T. personatus* (Avibase, http://avibase.bsc-eoc.org/avibase.jsp?lang=EN).

Phylogenetic studies group trogons to the clade Coraciomorphae, which comprises Coraciiformes, Piciformes, Bucerotiformes, Trogoniformes, Leptosomiformes, and Coliiformes [Jarvis et al., 2014; Prum et al., 2015]. The monophyly of the species included in each genus of the Trogonidae family is clear and strongly supported by molecular data of nuclear and mitochondrial genes. However, the intergeneric phylogeny has been debated in recent years, because some relationships are incompatible with other data, as in the case of the genus *Apalharpactes* from Asia, which was found to be a sister group of *Apaloderma* (Africa) instead of *Harpactes* (Asia) [Hosner et al., 2010].

The species *T. surrucura*, is divided into 2 different subspecies (*T. s. surrucura* and *T. s. aurantis*), based on phenotypical differences such as the feather color in some regions of the body. Of these, *T. s. surrucura* is found in Brazil, from Rio de Janeiro to Rio Grande do Sul States, and also in Paraguay, Uruguay, and Northeast Argentina [Pinto, 1950].

Chromosome features, such as the number, morphology, and symmetry, can be used along with other traits to help in phylogenetic studies. Although birds are characterized by a very constant karyotype formula, the combination of different molecular cytogenetic approaches, such as FISH with whole chromosome probes, repetitive sequences, and BAC mapping, has revealed a high degree of chromosomal rearrangements that proved useful in clarifying and corroborating some phylogenetic proposals [Nanda et al., 2007; Kretschmer et al., 2014, 2015; Rodrigues et al., 2014; de Oliveira Furo et al., 2015; dos Santos et al., 2015].

Despite the uncertain phylogenetic position of trogons, there are no chromosomal studies for any of the species of this order. Hence, the main aim of this study was to analyze the karyotype of a species of trogon, *T. s. surrucura*, found in Brazil, by means of classical and molecular cytogenetics, in order to generate a homology map with *Gallus gallus* and *Leucopternis albicollis*, 2 species commonly used in avian chromosome evolution studies. The results were compared to other bird species, in order to reveal derived chromosomal characters that could contribute to a better understanding of the phylogenetic position of these birds.

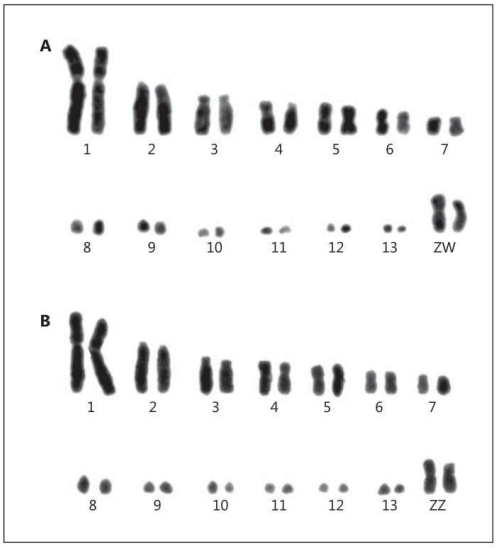

Fig. 1. Partial Giemsa-stained female (**A**) and male (**B**) karyotypes of *Trogon s. surrucura*, 2n = 82.

Materials and Methods

We analyzed the karyotypes of 4 individuals (3 males, 1 female) of *T. s. surrucura* (Trogoniformes, Trogonidae) caught in 2 different cities in Rio Grande do Sul State, Brazil: Santa Maria and Porto Vera Cruz.

Mitotic chromosomes were obtained by direct culture of bone marrow, according to Garnero and Gunski [2000]. Initially, bone marrow was extracted from femurs and resuspended in a centrifuge tube containing 10 mL of RPMI 1640 medium and 0.001 mL of colchicine solution (0.05%). The tubes were maintained in a water bath at 39°C for 1 h. Finally, the cells were treated with 10 mL 0.075 M KCl hypotonic solution for 20 min, washed, and fixed in fixative (methanol:acetic acid, 3:1).

Chromosome Analyses

The diploid number was determined by analyzing approximately 40 conventionally Giemsa-stained metaphase plates per individual. Karyotypes were arranged according to chromosome size and centromere position. C-banding [Sumner, 1972] was performed in order to define the amount and distribution of constitutive heterochromatin blocks.

18S rDNA and AgNORs

Clusters of 18S rDNA were visualized by FISH. First, 18S rDNA probes were synthesized according to Cioffi et al. [2009], using the genomic DNA of *Hoplias malabaricus* and primers 18SF (5′-CCGAGGACCTCACTAAACCA-3′) and 18SR (5′-CCGCTTTGGT-

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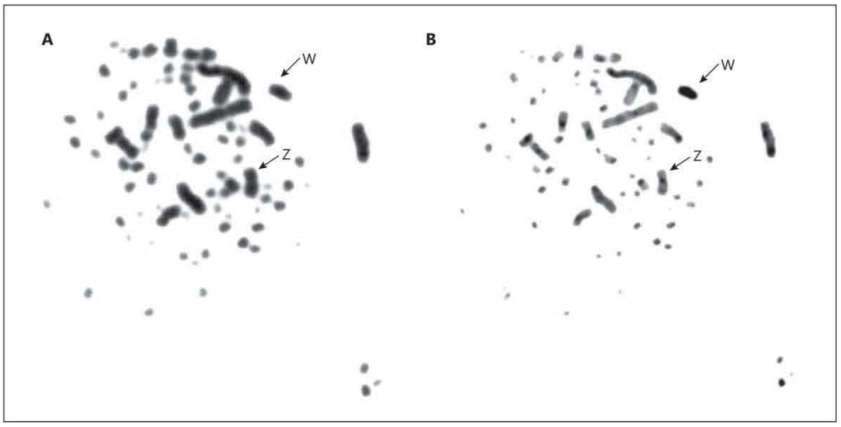

Fig. 2. Metaphase of the female *Trogon s. surrucura* after Giemsa staining (**A**) and subsequent C-banding (**B**). The arrows point to the Z and W sex chromosomes.

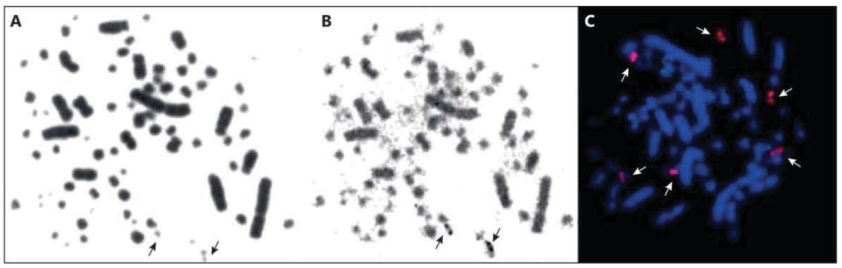

Fig. 3. Metaphase of *Trogon s. surrucura* after Giemsa staining (**A**) and subsequent AgNOR banding (**B**). **C** FISH with 18S rDNA. Two chromosomes carry active NORs after AgNOR staining (indicated by arrows in **A** and **B**), but 6 chromosomes hybridize with the 18S rDNA probe (**C**).

GACTCTTGAT-3′). The fragments generated, approximately 1,400 bp in size, were then labeled with biotin-16-dUTP using the nick translation mix (Roche) following the manufacturer's instructions.

FISH experiments were performed according to Daniels and Delany [2003]. Briefly, the slides were treated with RNAse A (10 μg/mL) for 20 min and then denatured in 70% formamide at 70°C for 1 min 20 s. Afterwards, 100 ng of the 18S probe in hybridization mix was applied to the slides, sealed with a cover slip and incubated at 37°C overnight. Finally, the slides were washed, and signals were detected with Cy3-streptavidin. Chromosomes were counterstained with DAPI. Hybridization results were analyzed using a Zeiss Axioplan2 fluorescence microscope.

In addition, we applied the AgNOR technique [Howell and Black, 1980] to detect the transcriptional activity of these genes.

Chromosome Painting

Two sets of whole chromosome probes (WCPs) were used in FISH experiments: (1) *G. gallus* WCPs corresponding to chicken chromosomes 1–10 (GGA1–10) [Griffin et al., 1999], and (2) *L. albicollis* WCPs, LAL1–7, LAL9, LAL13–18, LAL20, and LAL26, homologous to segments covering GGA1 to GGA6 [de Oliveira et al., 2010]. Probes were labeled by DOP-PCR using biotin-dUTP, and FISH experiments followed standard protocols according to de Oliveira et al. [2010].

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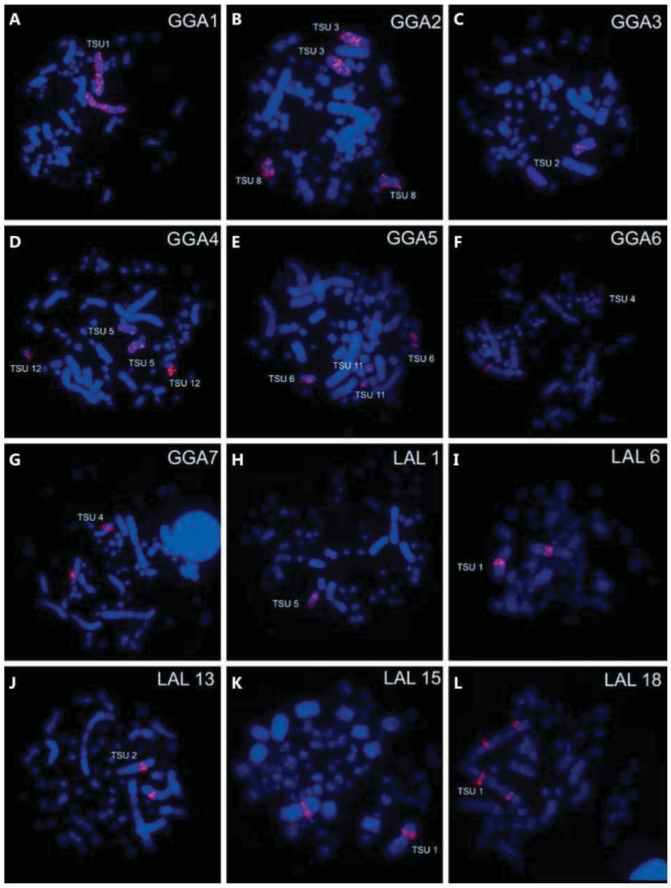

Fig. 4. A–L FISH experiments in *Trogon s. surrucura* (TSU) using chromosome probes from *Gallus gallus* (GGA) and *Leucopternis albicollis* (LAL) as indicated in the upper right corner of each panel.

Results

Karyotype Description

We found a diploid chromosome number of 82 in *T. s. surrucura*. Nine autosome pairs and the sex chromosomes were classified as macrochromosomes, the remainder as microchromosomes (Fig. 1). Pairs 1, 6, and 7 were submetacentric, pairs 2, 3, and 4 were acrocentric, and pair 5 metacentric. The sex chromosomes were of similar size, but the Z was metacentric, while the W was submetacentric.

Constitutive heterochromatin was observed in the centromeric regions of the macrochromosomes and in some microchromosomes (Fig. 2B). The W chromosome showed a high content of constitutive heterochromatin, while the Z only displayed a positive C-band in the centromeric region.

Although silver staining showed only 2 microchromosomes with transcriptionally active NORs (Fig. 3B), FISH experiments revealed that 18S rDNA clusters are distributed in 3 pairs of microchromosomes in *T. s. surrucura* (Fig. 3C).

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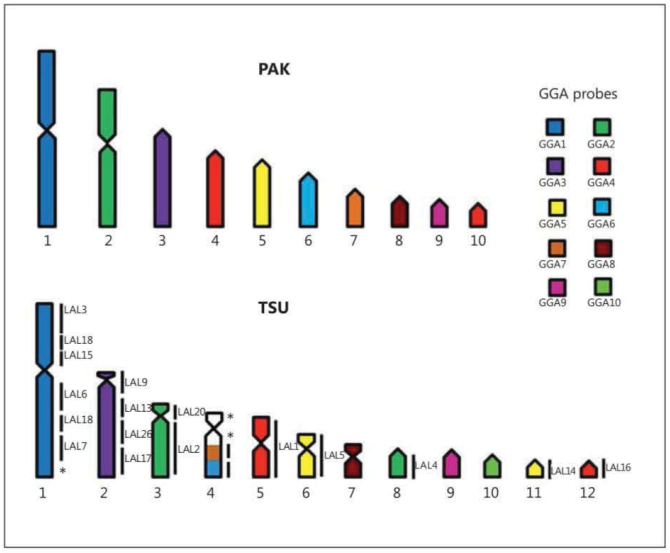

Fig. 5. Map of chromosome homology between *Trogon s. surrucura* (TSU) and the putative ancestral avian karyotype (PAK) according to Griffin et al. [2007]. Chromosome probes from *Gallus gallus* (GGA) are shown in color and *Leucopternis albicollis* (LAL) homologies are indicated on the right of the TSU chromosomes. Segments not hybridized are indicated with an asterisk.

Chromosome Painting

G. gallus WCPs showed that some chicken chromosomes are conserved entirely in *T. s. surrucura* (TSU). Hence, GGA1 (Fig. 4A), GGA3 (Fig. 4C), GGA8, GGA9, and GGA10 are homologous to TSU1, TSU2, TSU7, TSU9, and TSU10, respectively. On the other hand, GGA2, GGA4, and GGA5 hybridized onto 2 different pairs each (TSU3/TSU8, TSU5/TSU12, and TSU6/TSU11, respectively) (Fig. 4B, D, E). Additionally, both GAA6 and GGA7 hybridized in TSU4q (Fig. 4F, G).

The *L. albicollis* WCPs allowed the identification of intrachromosomal rearrangements and breakpoints of syntenic groups corresponding to GGA2 and GGA5. For instance, LAL18 hybridized in both the short and the long arm of TSU1, suggesting the occurrence of pericentric inversions (Fig. 4L). LAL4 and LAL20, which correspond to segments homologous to GGA2p and GGA2q_{prox}, respectively, revealed that the fission observed in GGA2 was centromeric (Fig. 4B). A similar situation was found with LAL5 and LAL14 (GGA5p and GGA5q), which confirmed a centric fission in GGA5 (Fig. 4E).

The homology map based on the FISH results and indicating homologies to syntenic groups of the putative ancestral avian karyotype is shown in Figure 5.

Discussion

We found 2n = 82 in *T. s. surrucura*, the first karyotype described for a species belonging to the order Trogoniformes. This value is within the range reported for more than 60% of the bird karyotypes described so far, which have diploid numbers between 2n = 74 and 86 [Christidis, 1990].

Some interesting features were observed in this karyotype, for example the large size of the W chromosome. The W is very similar to the Z except for its centromere position, which allows a clear distinction between them. Additionally, C-banding revealed that most of the W is constitutive heterochromatin. Similar findings have been reported by Nieto et al. [2012] in *Nyctibius griseus* (Caprimulgiformes). This species shows homomorphic sex chromosomes, which can be differentiated only by C-banding, because the W chromosome is completely heterochromatic. According to Schartl et al. [2016], large and heterochromatic W chromosomes in different lineages of Neoaves arose by the accumulation of repetitive sequences and their retention due to low degeneration rates of the W chromosome. In fact, a case of homomorphic sex chromosomes has recently been found in *Myiopsitta monachus* (Psittaciformes, Psittacidae), and microsatellite probes revealed an accumulation of 3 different

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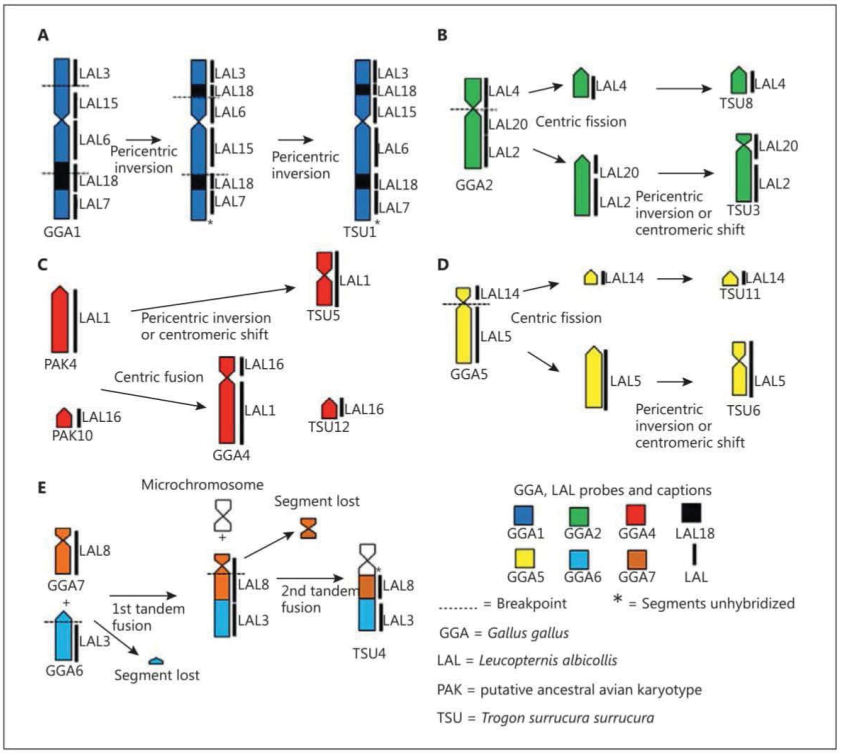

Fig. 6. A–E Schematic representation of the rearrangements detected in *Trogon s. surrucura* (TSU) based on the results of comparative FISH experiments. The karyotype of TSU was compared to *Gallus gallus* (GGA) and to the putative ancestral avian karyotype (PAK), proposed by Griffin et al. [2007]. Segments which did not hybridize with the available probes are marked with an asterisk.

microsatellite sequences in the large W chromosome [de Oliveira Furo et al., 2017].

Most studies concerning rDNA in birds describe only the number of clusters by FISH or their activity by the AgNOR technique. From what is known so far, most birds show only 1 chromosome pair bearing these clusters, as in *G. gallus,* ratites, and some Accipitriformes [Nishida-Umehara et al., 2007; Tagliarini et al., 2009]. However, some species have more than 1 pair of chromosomes carrying 18S rDNA, and this feature is considered to be a derived state. The highest number recorded to date is in *Falco peregrinus* (Falconiformes), which has 7 pairs of chromosomes with 18S rDNA clusters [Nishida et al., 2008]. We found that *T. s. surrucura* has 18S rDNA clusters in 3 pairs of microchromosomes, although AgNOR staining revealed that only 1 pair of NORs is transcriptionally active.

Comparisons with *G. gallus* and *L. albicollis* indicate the conservation of syntenic groups corresponding to GGA1, 3, 8, 9, and 10, confirming their homology to TSU1, 2, 7, 9, and 10, respectively. Similar to most bird species, 2 different pairs of chromosomes are homologous to GGA4, i.e., TSU5 (GGA4q) and TSU12 (GGA4p). This configuration is considered a plesiomorphic state, found in the putative ancestral avian karyotype [Griffin et al., 2007].

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TSU4 is homologous to GGA6 and GGA7, suggesting the occurrence of an in-tandem fusion. In addition, a segment comprising TSU4p and TSU4q_{prox}, did not hybridize with any of the probes used, indicating fusion with a third element, possibly a microchromosome (Fig. 6E). Interestingly, the association GGA6/GGA7/microchromosome has also been reported in some species of Psittaciformes (*Agapornis roseicollis*, *Ara macao*, *Psittacus erithacus*, *Ara chloropterus,* and *Anodorhynchus hyacinthinus*) [Nanda et al., 2007; Seabury et al., 2013; de Oliveira Furo et al., 2015; Seibold-Torres et al., 2015]. However, in this group, the order in the chromosome is inverted compared to TSU4, with GGA7 being terminal and GGA6 interstitial. This indicates that these fusions occurred independently in these lineages, which is consistent with recent phylogenetic proposals, placing Trogoniformes and Psittaciformes in different clades [Jarvis et al., 2014; Prum et al., 2015]. Such reoccurrences of chromosomal rearrangements are well-documented in birds. Another example is the centric fission of the syntenic group corresponding to GGA5, observed in *T. s. surrucura*, which is also found in Accipitriformes, such as *L. albicollis* (Falconiformes) [de Oliveira et al., 2010] and *Nisaetus nipalensis orientalis* (Accipitriformes) [Nishida et al., 2013].

In recent years, LAL paint probes have been essential tools in revealing intrachromosomal rearrangements in the large macrochromosomes of birds, which were undetected by GGA probes [Kretschmer et al., 2014; dos Santos et al., 2015]. These findings are in concordance with the results obtained by in silico genome assemblies of some birds [Jarvis et al., 2014; Prum et al., 2015]. LAL probes are very important in determining the breakpoints of rearrangements. For instance, the results of LAL probes in *T. s. surrucura* confirm the findings obtained with GGA probes and also reveal some intrachromosomal rearrangements, such as the inversion in the segment homologous to LAL18 (Fig. 6A). In addition, it was confirmed that the GGA2 homologue underwent centric fission, corresponding to TSU3 and TSU8 (Fig. 6B), and another example is the centric fission that occurred in the GGA5 homologues (TSU6 and TSU11) (Fig. 6D). These findings show that *T. s. surrucura* and *L. albicollis* share common breakpoints, despite their phylogenetic distance, reinforcing the fact that recurrent breakpoints are an important aspect of avian karyotype evolution, as proposed by Skinner and Griffin [2012] based on in silico data.

Furthermore, it is important to highlight that some conserved syntenic groups show morphological differences when compared to the putative ancestral avian karyotype (and chicken), such as TSU3 (GGA2), TSU5 (GGA4q), and TSU6 (GGA5q) (Fig. 6B, C, D). These differences indicate the occurrence of pericentric inversions or centromeric shifts, which could not be confirmed with the probes used in this study.

In summary, we present the first cytogenetic description of a species belonging to the order Trogoniformes. Our findings show that *T. s. surrucura* has a diploid number of 2n = 82, similar to most species of birds. However, its karyotype shows some derived features, including fusions, fissions, and chromosomal inversions, when compared to *G. gallus* and to the putative ancestral avian karyotype. Finally, despite the occurrence of many chromosomal rearrangements, the homology map confirms that breakpoints in *T. s. surrucura* are shared by other species of birds, confirming that avian karyotype evolution is characterized by recurrent breakpoints, possibly due to the small amounts of repetitive sequences within their genomes.

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Statement of Ethics

Samples were collected according to license SISBIO 44173-1 and the animal research ethics committee (CEUA 018/2014).

Disclosure Statement

The authors have no conflicts of interest to declare.

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6. CAPÍTULO 3:

THE DISTRIBUTION OF 45S RDNA SITES IN BIRD CHROMOSOMES SUGGESTS MULTIPLE EVOLUTIONARY HISTORIES

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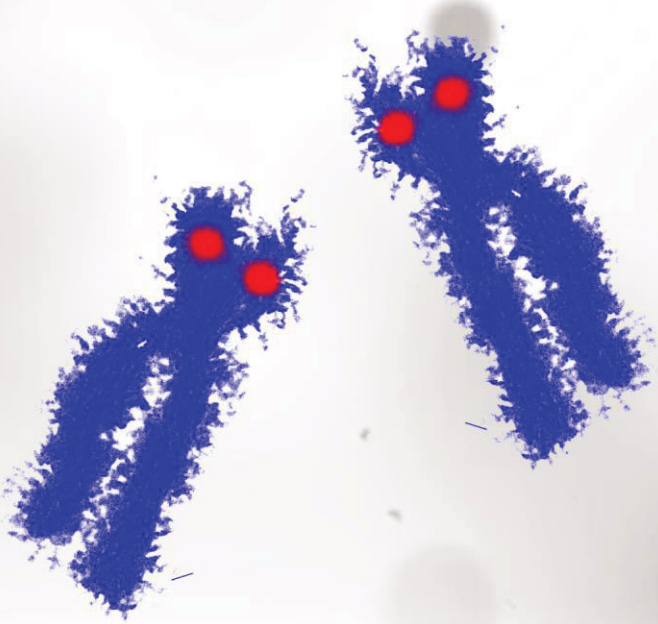

The distribution of 45S rDNA sites in bird chromosomes suggests multiple evolutionary histories

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Abstract

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

Keywords: FISH, chromosome, chromosome evolution, cytogenetics

Introduction

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

Identification of chromosomes that bearer 45S rDNA can be performed by the silver nitrate impregnation technique (Ag-NORs) (Howell and Black, 1980). However, this procedure only identifies the chromosomes with 45S rDNA sites in transitional activity, exhibiting intercellular and interindividual variations (Zutita et al. 1997). In this way, the Fluorescence in situ Hybridization (FISH) experiments are more appropriate for this type of study, since they allow the precise identification of the bearing chromosomes when using probes for the genes that make up the rDNA cluster even when they are not active (O'Connor, 2008).

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

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

in the chromosomal distribution of the rDNA sites in closely related species (Datson and Murray, 2006; Degrandi et al. 2014).

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

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

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

Materials and Methods

In this work, we analyzed the basic karyotype structure and distribution of the 45S rDNA sites in bird karyotypes. The following data were considered in each species: diploid number, number of 45S rDNA-bearing chromosomes, their type (macro- or microchromosome), and position of the clusters on the chromosome arm. First, the data were obtained from literature, considering only the species in which the 45S rDNA clusters were identified by FISH-rDNA. Ag-NORs data were

disregarded due to the intercellular and individual variations or possible false positive results, already reported in the literature.

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

Chromosome preparation

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

FISH 18S rDNA

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

The PCRs were run in a final volume of 25 µl containing: 2 ng of genomic DNA from *H. malabaricus*, 0.2 µM of each primer (18SF and 18SR), 0.2 mM of dNTP, 10X buffer (1x), 50 mM of MgCl2 (2 µM), 1 mM of Fluorescein-12-dUTP solution, 1 U/µl of Taq polymerase, and sterile H_2O to complete final volume. The thermal cycling

parameters were 94ºC/1 min, 30 cycles of 94ºC/1 min, 60ºC/1 min, 72ºC/1:30 min, followed by a cycle of 5 min at 72ºC (Cioffi et al. 2009).

For the FISH procedures, slides with metaphases were treated with RNAse A (10 µg/mL) for 20 minutes and then denatured in 70% Formamide at 70°C for 1 min 20s. Subsequently, 300 ng of the 18S probe were added to each slid, which was then sealed with a cover slip and incubated overnight at 37°C (Daniels and Delany, 2003). The slides were then washed in 50% Formamide at 42 ° C for 1 min (x2), 2xSSC at 40 ° C for 2.30 min (x2) and once in 4xSSC Tween (1X) at room temperature. The chromosomes were counterstained with DAPI. Hybridization results were analyzed using a Zeiss Axioplan2 fluorescence microscope.

Chromosomal analyses

The diploid number of each specimen was determined from the analysis of approximately 30 mitotic cells stained with Giemsa and observed under an optical microscope. The variation in the number of rDNA clusters was evaluated based on the number of chromosomes that presented a fluorescent signal. The rDNA cluster-bearing chromosomes were classified as either macrochromosomes or microchromosomes, according to their length (Rodionov, 1996). The position of the 45S rDNA cluster was classified as: (i) pericentromeric (adjacent to the centromere), (ii) subtelomeric (adjacent to the telomere), and (iii) interstitial (between the centromere and the telomere) (Cazaux et al. 2011). Ideograms were created using these characteristics to represent the rDNA-bearing chromosomes in each species.

Phylogenetic Comparison

The species were compared using the phylogenetic relationships proposed by Jarvis et al. (2014) and Prum et al. (2015). In this step, the chromosomal location characteristics of the 45S rDNA clusters were plotted in a modified phylogenetic tree of Jarvis et al. (2014). In this tree, we used the Mesquite software to exclude groups of birds for which rDNA location data were not available. We also considered the presence of 45S rDNA in a single pair of microchromosomes as an ancestral condition for birds, according to the Nishida-Umehara hypothesis (2007). Based on this hypothesis, we analyzed these evolutionary relationships and the probable chromosomal rearrangements that would explain the variations observed in chromosomes carrying 45S rDNA.

Results

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

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

Variation in the diploid number in birds

Considering only the bird species for which the location of 45S rDNA sites is available (73), diploid numbers ranged from 2n= 40 to 2n= 112 (Table 1). Despite this ample variation, most (38) of the species had diploid numbers between 78 and 82, and 21 are 2n= 80 (Figure 2A). While the Paleognathae species were relatively conserved, with most species having around 80 chromosomes, higher variability in 2n was observed in Neognathae (Table1).

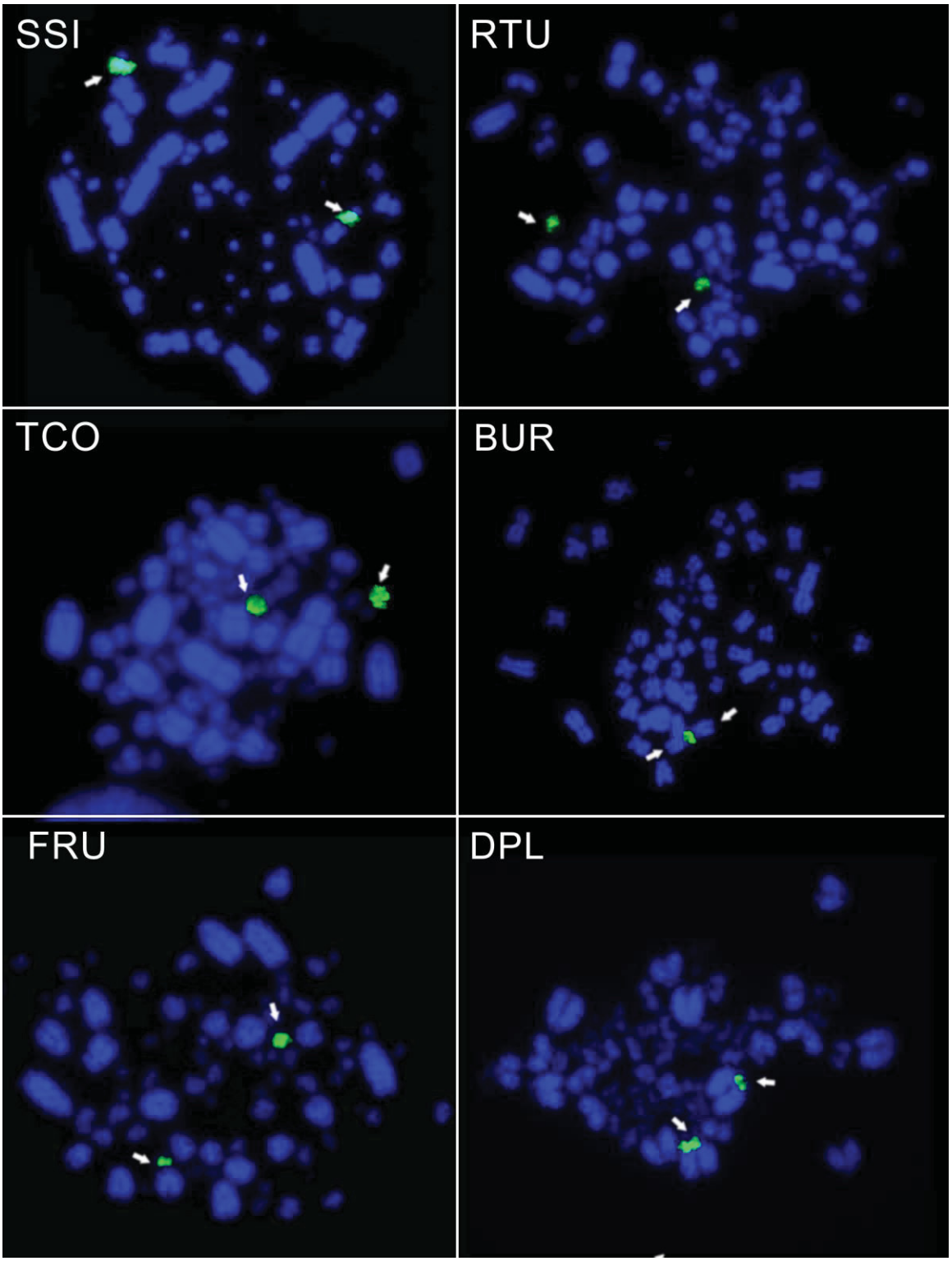

Figure. 1. Examples of the metaphases analyzed in the present study using the 18S rDNA probe (green) to identify the chromosomes (blue) carrying 45S rDNA sites (arrows). The acronym shown in the upper right corner of each metaphase indicates the species: *Syrigma sibilatrix* (SSI), *Ramphastos tucanus*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

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

				45S rDNA			
Infraclass/ order	**Family**	**Species**	**2n**	**Nº**	**Type of chromosome**	**Position**	**Reference**
Neognathae							
Passeriformes Oscines	Turdidae	*Turdus rufiventris*	78	6	Micro	NA	Kretschmer et al. (2014)
		Turdus albicollis	78	4	Micro	NA	Kretschmer et al. (2014)
	Thraupidae	*Saltator similis*	80	2	Micro	NA	dos Santos et al. (2015)
		Saltator aurantiirostris	80	2	Micro	NA	dos Santos et al. (2015)
		*Tachyphonus coronatus**	80	2	Micro	NA	Present study
		Coryphospingus cucullatus	80	2	Micro	NA	Present study
	Icteridae	*Agelaioides badius*	80	4	Micro	NA	Present study
		Molothrus bonariensis	80	2	Micro	NA	Present study
	Fringillidae	*Serinus canaria*	80	4	Micro	NA	dos Santos et al. (2017)
	Parulidae	*Basileuterus culicivorus*	80	2	Micro	NA	Present study
	Estrildidae	*Taeniopygia guttata*	80	2	Micro	NA	dos Santos et al. (2017)
		Elaenia spectabilis	80	4	Micro	NA	Kretschmer et al. (2015)
Passeriformes Suboscines	Tyrannidae	*Pitangus sulphuratus*	78	2	Micro	NA	Present study
		Myiarchus ferox	76	2	Micro	NA	Present study
	Tityridae	*Schiffornis virescens*	82	2	Micro	NA	Present study
	Furnariidae	*Dendrocolaptes platyrostris**	82	2	Macro, 1th	P	Present study
		Anumbius annumbi	82	2	Micro	NA	Present study
		Synallaxis albescens	82	2	Micro	NA	Present study
		*Furnarius rufus**	82	2	Micro	NA	Present study
		Cranioleuca obsoleta	82	2	Micro	NA	Present study

		Syndactila rufosuperciliata	82	2	Micro	NA	Present study
Psittaciformes		*Psittacus erithacus*	62-64	8	Micro	NA	Seibold-Torres et al. (2015)
Falconiformes	Falconidae	*Falco tinnunculus*	52	4	Micro	NA	Nishida et al. (2008)
		Falco peregrinus	50	12 or 14	Micro	NA	Nishida et al. (2008)
		Falco columbarius	40	9	Micro	NA	Nishida et al. (2008)
Coraciiformes	Alcedinidae	*Chloroceryle americana*	94	2	Micro	NA	Present study
Piciformes	Picidae	*Colaptes campestres*	84	2	Macro, 13th	I	de Oliveira et al. (2017)
		Colaptes melanochloros	84	2	Macro, 13th	I	de Oliveira et al. (2017)
		Melanerpes candidus	64	2	Micro	NA	de Oliveira et al. (2017)
	Ramphastidae	*Ramphastos tucanus**	112	2	Micro	NA	Present study
Trogoniformes	Trogonidae	*Trogon s. surrucura*	82	6	Micro	NA	Degrandi et al. (2017)
Accipitriformes Eagles	Pandionidae	*Pandion haliaetus*	74	2	Macro, 2th	P, q	Nishida et al. (2014)
	Accipitridae	*Pseudastur albicollis*	66	2	Macro, 8th	P, q	Present study
		*Buteogallus urubitinga**	68	2	Macro, 8th	P, q	Present study
		Buteo nitidus	68	2	Macro, 8th	P, q	de Oliveira et al. (2013)
		Rupornis magnirostris	68	2	Macro, 8th	P, q	de Oliveira et al. (2013)
		Buteogallus meridionalis	68	2	Macro, 8th	P, q	de Oliveira et al. (2013)
		Harpia harpyja	58	4	Macro, 6th and Micro, 25th	S	Tagliarini (2013)
		Morphnus guianensis	82	2	Macro, 1th	S	Tagliarini (2013)
		Nisaetus n. orientalis	66	2	Micro, 29th	NA	Nishida et al. (2013)
Accipitriformes Vultures	Cathartidae	*Sarcoramphus papa*	80	2	Micro	NA	Tagliarini et al. (2009)
		Cathartes burrovianus	80	2	Micro	NA	Tagliarini et al. (2009)

		Cathartes aura	80	2	Micro	NA	Tagliarini et al. (2009)
		Gymnogyps californianus	80	2	Micro	NA	Raudsepp et al. (2002)
Pelecaniformes	Ardeidae	*Syrigma sibilatrix**	62	2	Micro	NA	Present study
Charadriiformes	Stercorariidae	*Stercorarius antarcticus*	84	2	Micro	NA	Present study
	Burhinidae	*Burhinus oedicnemus*	42	2	Macro, 13th	I	Nie et al. (2009)
Caprimulgiformes Hummingbirds	Trochilidae	*Amazilia versicolor*	82	2	Micro	NA	Present study
Caprimulgiformes Nigthjars	Nyctibiidae	*Nyctibius griseus*	*86*	2	Micro	NA	Present study
	Caprimulgidae	*Hydropsalis torquata*	74	2	Micro	NA	Present study
Cuculiformes	Cuculidae	*Coccyzus melacoryphus*	-	2	Micro	NA	Present study
		Piaya cayana	88	2	Macro 7th	P, p	Present study
		Guira guira	76	2	Macro, 6th	P, q	Present study
Columbiformes	Columbidae	*Columbina talpacoti*	76	2	Micro	NA	Present study, Kretschemer et al. (2018b)
		Zenaida auriculata	76	2	Micro	NA	Kretschemer et al. (2018b)
		Geotrygon montana	86	2	Micro	NA	Kretschemer et al (2018b)
		Geotrygon violacea	86	2	Micro	NA	Kretschemer et al. (2018b)
		Leptotila verreauxi	78	2	Micro	NA	Kretschemer et al. (2018b)
		Patagioenas cayennensis	76	2	Micro	NA	Kretschemer et al. (2018b)
		Columba livia	80	2	Micro	NA	Kretschemer et al. (2018b)
		Columbina passerina	76	2	Micro	NA	Kretschemer et al. (2018b)
		Columbina picui	76	6	Micro	NA	Kretschemer et al. (2018b)
Galliformes	Phasianidae	*Coturnix japonica*	78	6	Micro	NA	McPherson et al. (2014)

		Meleagris gallopavo	80	2	Micro, 18th	NA	McPherson et al. (2014)
		Gallus gallus	78	2	Micro, 16th	NA	Dyomin et al. (2016)
Paleognathae							
Tinamiformes	Tinamidae	*Nothura maculosa*	78	4	Micro	NA	Present study
		Eudromia elegans	80	4	Micro	NA	Nishida-Umehara et al. (2007)
		Rhynchotus rufescens	78	2	Micro	NA	Present study
Casuariiformes	Dromaiidae	*Dromaius novaehollandiae*	80	2	Micro	NA	Nishida-Umehara et al. (2007)
	Casuariidae	*Casuarius casuarius*	92	2	Micro	NA	Nishida-Umehara et al. (2007)
Struthioniformes	Struthionidae	*Struthio camelus*	80	2	Micro	NA	Nishida-Umehara et al. (2007)
Rheiformes	Rheidae	*Rhea pennata*	80	2	Micro	NA	Nishida-Umehara et al. (2007)
		Rhea americana	80	2	Micro	NA	Nishida-Umehara et al. (2007)

2n = diploid number;
Nº = number of 45S rDNA-bearing chromosomes;
Type of chromosome: Macro = macrochromosome; Micro = microchromosome;
Nomenclature for the position in the chromosome: I= interstitial; S = subtelomeric; P = pericentromeric;
Arm location: Short arm = p; Long arm = q;
NA = Not applicable;
*Shown in the fig. 1;
Species names are in accordance with Gill and Donsker (2018).

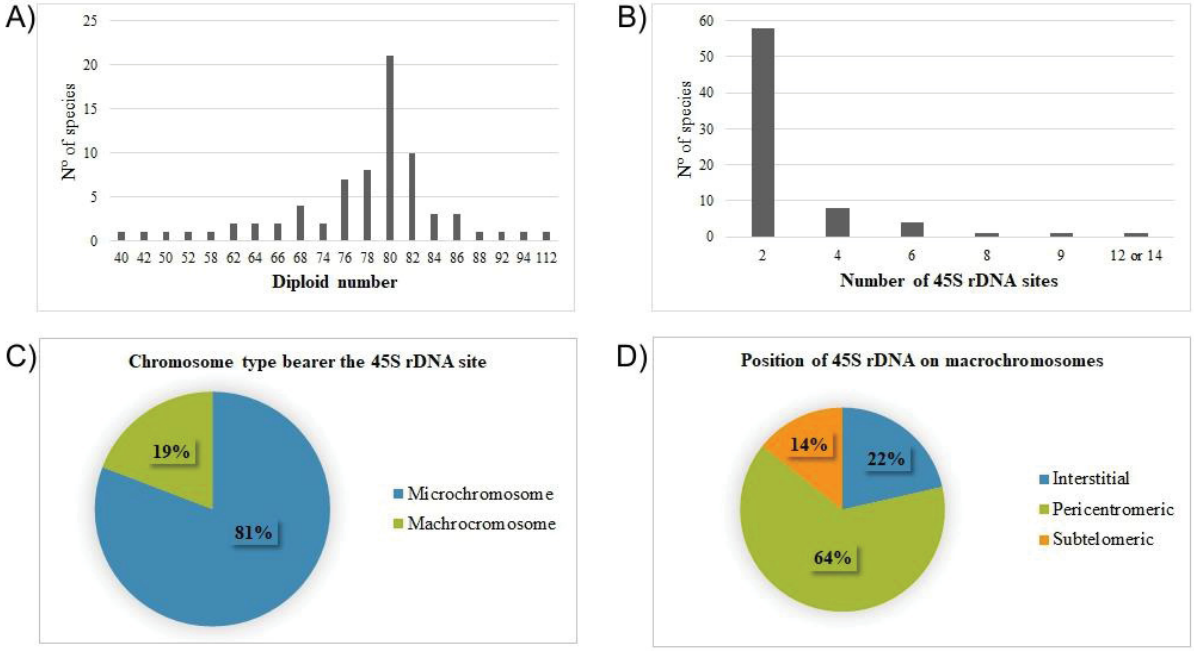

Figure. 2. Chromosomal location of the 45S rDNA sites in all 73 bird species analyzed in the present study. A: variation in the diploid number; B: variation in the number of 45S rDNA bearer chromosomes; C: the proportion of the species with 45S rDNA located in macrochromosomes or microchromosome, and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

Number of 45S rDNA sites

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

Types of rDNA-bearing chromosomes

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

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

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

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

* Homologies established by chromosome painting; Micro= microchromosome;

The position of the 45S rDNA site in the chromosomes

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

The 45S rDNA was observed in a pericentromeric position in most (64%) cases, that is, in *P. haliaetus*, *P. albicollis*, *B. urubitinga*, *B. nitidus*, *R. magnirostris*, *B. meridionalis* (Accipitriformes), *G. guira, P. cayana* (Cuculiformes), and *D.*

platirostris (Passeriformes). The interstitial position was the second most frequent, being observed in 22% of the species, *B*. *oedicnemus* (Charadriiformes), *C*. *campestres*, and *C*. *melanochloros* (Piciformes). Finally, a subtelomeric position was recorded in two (14%) species, *M*. *guianensis*, *H*. *harpyja* (Accipitriformes) (Figure 2D, Table 1).

Phylogenetic comparisons

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

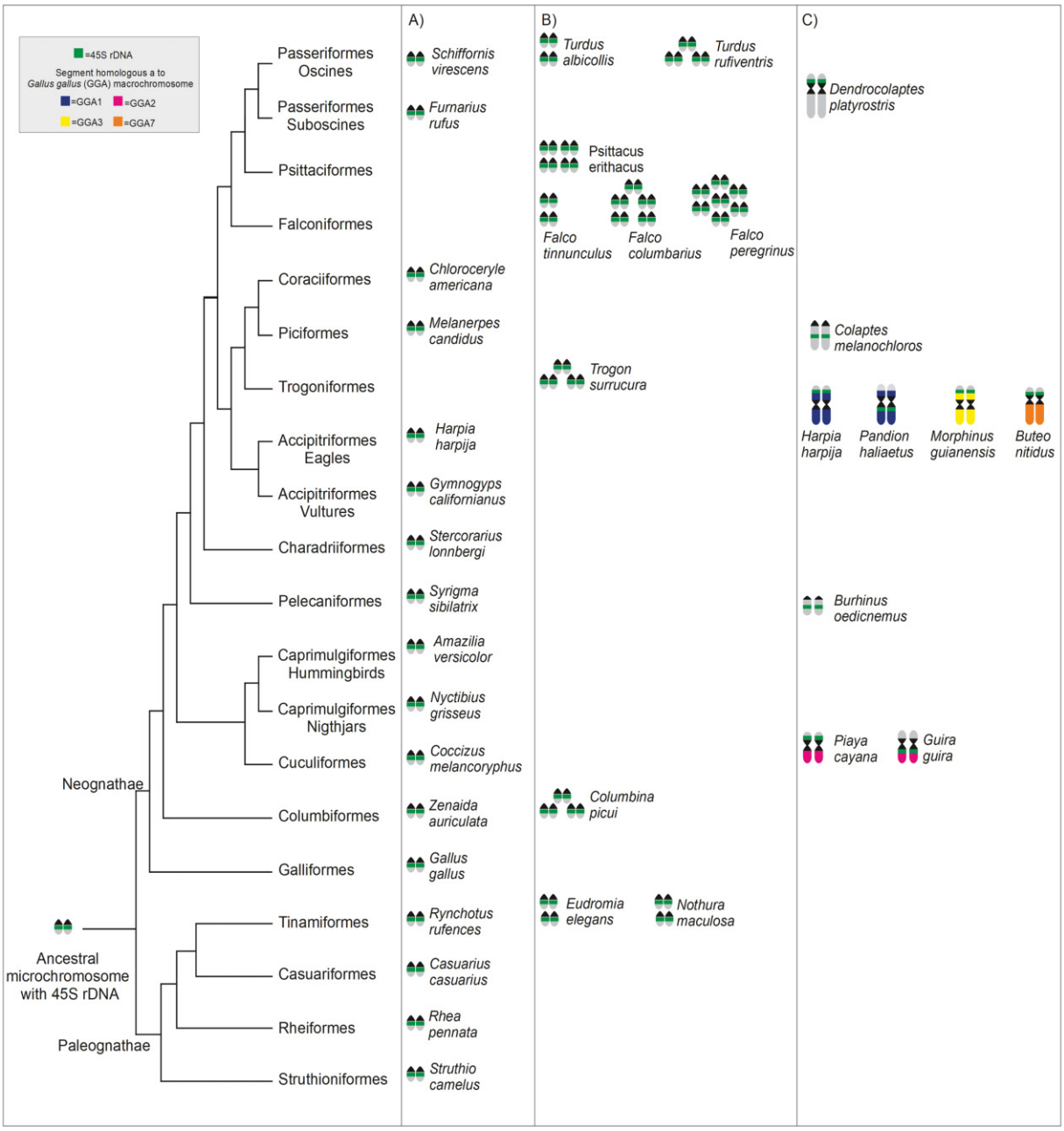

Figure. 3. Phylogenetic relationships among the birds modified from Jarvis et al. 2014. The data of chromosomal location of the 45S rDNA from species analyzed in the present study were plotted in the tree. In A shows the species with rDNA located only in a microchromosome pair; in B: species with rDNA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

Discussion

Here we have presented for the first time a broad analysis of the distribution of 45S rDNA in avian karyotype. Although an impressive variation was observed in the

chromosomes carrying the 45S rDNA cluster, we recorded that in most species it is located in a single pair of microchromosomes. Interestingly, it was also observed that most these species have a karyotype with 2n = 80 chromosomes (Figure 2A).

A study with rodents indicated that there is no relationship between the 2n and the number of 45S rDNA cluster bearing chromosomes (Cazaux et al. 2011). Nevertheless, birds with 2n=80 chromosomes which carry just a single pair of 45S rDNA microchromosomes seem to reflect the karyotype conservation status of these species in relation to the ancestral karyotype of birds (PAK), as proposed by Griffin et al. (2007). This karyotype uniformity of birds has been observed in species from Paleognathae and Neognathae using the GGA whole chromosome paint (Kretschmer et al. 2018a).

The presence of a single pair of microchromosomes with 45S rDNA conserved among the species of Paleognaths (*Dromaius novaehollandiae*, *Casuarius casuarius*, *Struthio camelus*, *Rhea pennata* and *Rhea americana*) suggests that this would be an ancestral condition of rDNA (Nishida-Umehara et al. 2007). Using the phylogenetic relationships proposed by Jarvis et al. (2014) and Prum et al. (2015), we have compared these data and also identified that several species of the Neognaths infraclass, preserve the 45S rDNA in a pair of microchromosome (Figure 3), a fact that reinforces the hypothesis of PAK ancestral condition (Griffin et al. 2007).

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

45S rDNA in multiple microchromosomes

Multiple microchromosomes carrying 45S rDNA can be found in some species of the orders: Tinamiformes, Columbiformes, Trogoniformes, Falconiformes. It is

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

45S rDNA macrochromosomes distribution

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

In Accipitriformes, for example, multiple associations were recorded, including GGA1, GGA3 and GGA7. In *B. nitidus*, *R. magnirostris and B. meridionallis*, an association with the homologous GGA7 segment was found, although the short arm of the chromosome pair containing the rDNA of these species was not hybridized by any of the GGA probes used (de Oliveira et al. 2013). This unhybridized region probably corresponds to the homologous of the ancestral microchromosome containing the rDNA, reinforcing the fusion hypothesis. Similarly, in *P. haliaetus*, the rDNA located on the q-arm of chromosome 2 was associated with the homologous GGA1 segment (Nishida et al. 2014). In this species, the short arm did not hybridize by any GGA probes. However, *P. haliaetus* shows rDNA in the long arm, suggesting that a pericentric inversion should have occurred after fusion with the 45S rDNA microchromosome, shifting the cluster position to the long arm.

45S rDNA related to intrachromosomal rearrangements

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

Conclusion

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

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7. CAPÍTULO 4:

"BIRD CHROMOSOME DATABASE" AN OVERVIEW OF CYTOGENETIC KNOWLEDGE ON BIRDS

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"Bird Chromosome database" an overview of cytogenetic knowledge on birds

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Abstract

Background: The cytogenetics is historically utilized in birds, initially to identify differences in the diploid number and karyotype structure of species. More recently, the comparative whole chromosome paint it has shown some chromosomal rearrangements important to infer evolutionary relationships. However, these data are found represent only some birds orders which makes it difficult for researchers to have an overview and to reconstruct the evolutionary history of birds. It is known that the online databases include a valuable compilation of research data and permitting to researchers easily identify this information, the gaps as well as the need for new approaches and methodologies to be used. Thus, the present study aimed to compile the current cytogenetic data for birds, gathering data from the chromosome number (2n) and whole chromosome painting (WCP) with probes from the of the *Gallus gallus* to create a database.

Results: In the first version the "Bird Chromosome database" (https://sites.unipampa.edu.br/birdchromosomedatabase/) we compile the data of the 2n for 1032 and of WCP for 83 bird species. In the analyses, we observed that the diploid number in birds ranging from 2n = 40 to 142, however, the most frequent karyotype is 2n= 80. The orders with more species karyotyped were Acciptriformes (25%), Falconiformes (19%), Psittaciformes (21%) and Passeriformes (7%). While Phaethontiformes, Mesitornithiformes, Pterocliformes, Leptosomiformes, did not have any record. The WCP has been used in less than 1% of all birds species.

Conclusions: The data available here will allow the researchers to identify the main gaps in the cytogenetic knowledge of birds, as well as to identify groups of with little studied, and to make inferences about the chromosomal homologies for phylogenetic tests.

Key words: Aves chromosomes, diploid number, evolution chromosomes, karyotypes

Background

The Aves class is an important biological group that is globally distributed and highly diverse, including more 10 thousand species (Gill and Donsker 2018). In the last decade, there was a great effort to reconstruct the phylogeny of birds and more than 50 species had their genome completely sequenced (Jarvis et al. 2015). These genome analyses allowed reviewing the evolutionary relationships between the orders of the group and supporting an initial divergence of birds in Paleognathae and Neognathae infraclass (Jarvis et al. 2014; PRUM et al. 2015). Despite the great advances obtained in the genomic studies, this knowledge is available for a smal fraction of birds species (less of 2%), so it is important the use of other approaches e.g the cytogenetics (Kretschmer et al. 2018).

In cytogenetic, researchers analyze the chromosomes, initially to determine the number (n or 2n), the morphology and karyotype of the species (Guerra 1988). In addition, others markers chromosomic cam be determined such as the structural characteristics such as chromosomic regions rich in GC and AT (CBG-band) (Seabright 1971), constitutive heterochromatin (CBG- band) (Sumner, 1972), the localization of regions organizers nucleoli (ROM) (Howell and BLACK 1980). Most recently the homology between chromosomes that is determined by whole chromosome paint in the fluorescence hybridization in situ (FISH) experiments (Pinkel et al. 1986). These characteristics can be different applications e.g in clinical diagnostic, differentiation of males and females, or for predictions of evolutionary relationships (Dobigny et al. 2004).

Birds chromosomes are investigated a most one century (Guyer 1902). In general, they have karyotypes quite conserved among the species and are characterized by the have two groups of chromosomes that are distinguished by size, in macrochromosomes (6µm- 2.5µm) and microchromosomes (less than 2.5µm) (Rodionov 1996). The diploid number is considered high, around 80 chromosomes, where the microchromosomes are the most numerous elements of the karyotype (Griffin et al. 2007). The females have sex heterogametic with chromosomes ZW and males are homogametic ZZ (Graves and Shetty 2001; Wang et al. 2014).

From FISH technique and chromosome painting with whole chromosome probes, it was possible to identify several important information regarding to avian chromosome complement (Kretschmer et al. 2018). The domestic fowl, *Gallus gallus* (GGA), 2n = 78, was the first bird species used to isolate whole chromosomes

probes (Griffin et al. 1999). Through the use of GGA probes, it was possible to determine the chromosomal homology between distantly related species such as Ostrich (*Struthio camelus*), Emu (Rhea americana), Canary (*Serinus canaria*) and Zebra Finch (*Taeniopygia guttata*) (Nishida-Umehara et al. 2007; dos Santos et al. 2017). E.g in eagles (Accipitridae) was observed that macrochromosomes evolved through interchromosomal rearrangements such as fusions, fission and chromosome translocations, which resulted in the reduction of the diploid number (2n=58- 68) observed in species this group (de Oliveira et al. 2005).

Despite the importance of cytogenetic knowledge for the birds, is observed the absence of a periodical specialized in this theme. Thereby the most of publications found are disperse in unspecialized journals and difficult for researchers to find the data and identify gaps in this type of knowledge. It is known that, online databases include a valuable compilation of research data on various topics that ranging from complete lists of species, about the cytogenetic knowledge of some groups, the chromosomal distribution of rDNA to the complete sequence of genomes (Peruzzi and Bedini 2014; Jarvis et al. 2015; Paresque et al. 2017; Cardoso et al. 2018; Gill and Donsker 2018; Socorova et al. 2018). They also provide a overview of this knowledge and contributed to researchers easily identify the information, the gaps in knowledge as well as the need for new approaches and new methodologies to be used.

Thus, the present study aimed to compile the current cytogenetic data of birds, gathering data from the diploid number and chromosome painting to create a database. The information provided will allow the researchers to identify priority groups of birds for new studies, as well as to identify patterns of chromosomal homology and processes involved in the karyotype structure and evolution of birds.

Methods

In this study, we analyzed the knowledge about chromosome number (2n) and chromosomal homology of several birds species with the G. gallus karyotype, available in the literature until the year 2018. The compilation of these data was used for the creation of the “Bird Chromosome database”.

Initially, we examined the data available in review studies such Bloom (1969), Ray-Chaudhuri (1973), Shields (1982), De Boer (1984), Capanna et al. (1987), Santos and Gunski (2006), Cuervo et al. (2011), and Kretschmer et al. (2018)

verifying each citation of these authors. In addition, Web of Science and Google Scholar was used to find more studies. In this search, were used the keywords "birds", "chromosome", "diploid number", "description", "karyotype", "chromosome painting" and "FISH".

In all were analyzed 300 publications, which included abstracts in congresses, theses, dissertations and research articles. The division was made according to the type of data available in each study, i) studies containing data of the description of the diploid number and karyotype; ii) studies involving chromosome painting with *Gallus gallus* probes. The data were organized into a Table that includes the list of species and the bibliographic references. To verify the scientific name of each species and the existence of synonymous we use the World Bird List v8.2 from the International Community of Ornithologists (Gill and Donsker 2018). The synonyms were kept in a separate column, to easy search by users.

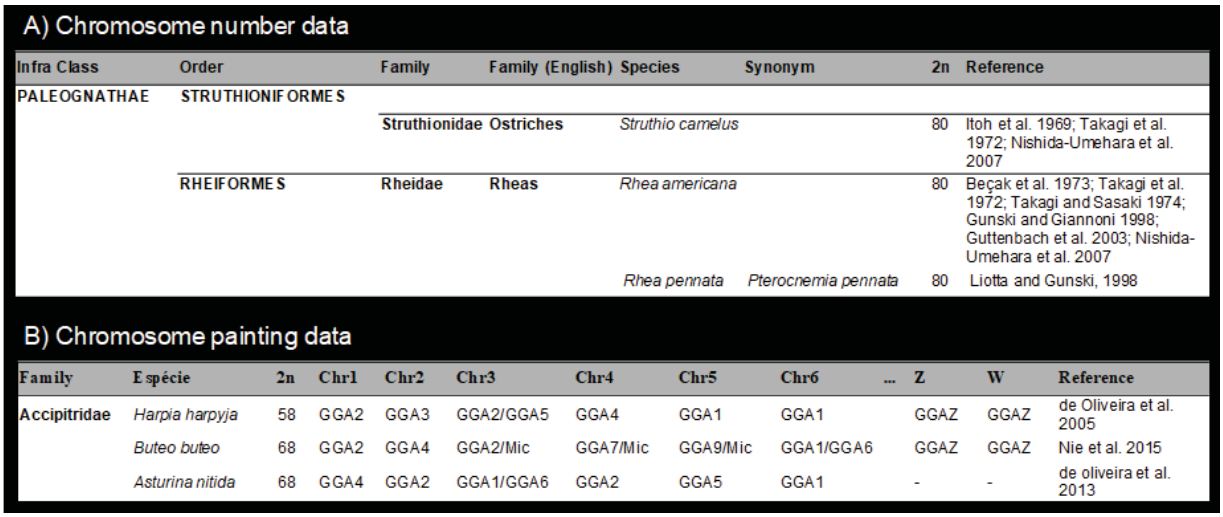
To obtain an overview of the current cytogenetic data of birds available on the database, we performed basic statistical analyses such as total percentage of karyotyped species, frequency of diploid number (2n), 2n variation, using Libreoffice tools.

Results

Website designing

The Bird Chromosomes database (BCD) is available in https://sites.unipampa.edu.br/birdchromosomedatabase. In this first version, the BCD included data from the chromosome number of 1032 species and the data of chromosome homology with *Gallus gallus* karyotype from 83 species. To access this data, the users who must download the files in XLS format that are available on the web page in the guide "Chromosome Number Data" and "Chromosome Painting Data". A sample of the information that can be found in the database is shown in Figure 1.

All users are encouraged to send new contributions and suggestions to the database through the e-mail (birdschromosome@gmail.com). We will annually update on the species list and bird cytogenetic studies.



A) Chromosome number data

Infra Class	Order	Family	Family (English)	Species	Synonym	2n	Reference
PALEOGNATHAE	STRUTHIONIFORMES						
		Struthionidae	Ostriches	*Struthio camelus*		80	Itoh et al. 1969; Takagi et al. 1972; Nishida-Umehara et al. 2007
	RHEIFORMES	Rheidae	Rheas	*Rhea americana*		80	Beçak et al. 1973; Takagi et al. 1972; Takagi and Sasaki 1974; Gunski and Giannoni 1998; Guttenbach et al. 2003; Nishida-Umehara et al. 2007
				Rhea pennata	*Pterocnemia pennata*	80	Liotta and Gunski, 1998

B) Chromosome painting data

Family	Espécie	2n	Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	...	Z	W	Reference
Accipitridae	*Harpia harpyja*	58	GGA2	GGA3	GGA2/GGA5	GGA4	GGA1	GGA1		GGAZ	GGAZ	de Oliveira et al. 2005
	Buteo buteo	68	GGA2	GGA4	GGA2/Mic	GGA7/Mic	GGA9/Mic	GGA1/GGA6		GGAZ	GGAZ	Nie et al. 2015
	Asturina nitida	68	GGA4	GGA2	GGA1/GGA6	GGA2	GGA5	GGA1		-	-	de oliveira et al. 2013

Figure 1: Sample of cytogenetic data available on Bird Chromosome database. In A is shown data from the diploid number (2n) e.g in Struthioniformes and Rheiformes) and B is show the homology of each chromosome pair (Chr1,…) with *Gallus gallus* (GGA) karyotype. The associations between macrochromosomes are represented by / e.g. GGA2 / GGA5 in *Harpia harpyja* (Accipitridae). In both tables is present the currently zoological classification of each species and list of reference from which the information was found.

Chromosome number data

The diploid number data for 1032 species are available on the database website, and corresponds to 9.5% of the total bird species in the world. This contingent of species represents 36 of 40 current avian orders. We analyzed the percentage of species with known karyotypes for each order that is shown in table 1. The orders Rheiformes, Cariamiformes, Opisthocomiformes have 100% of their species karyotyped, while Phaethontiformes, Mesitornithiformes, Pterocliformes, Leptosomiformes, did not have any record. Acciptriformes (25%), Falconiformes (19%), Psittaciformes (21%) and Passeriformes (7%) are the most studied groups (Table 1).

Table 1: Number of bird species karyotyped and analyzed by chromosome painting with *Gallus gallus* whole chromosome probes available on database

Order	Total number species*	Number of species karyotyped	% karyotyped	Number species with chromosome painting	% species with chromosome painting
Struthioniformes	2	1	50%	1	50%
Rheiformes	2	2	100%	2	100%
Apterygiformes	5	1	20%	-	-
Casuariiformes	4	2	50%	2	50%
Tinamiformes	47	6	13%	1	2,1%
Anseriformes	177	45	25%	6	3,3%
Galliformes	300	50	17%	9	3%
Gaviiformes	5	1	20%	-	-
Sphenisciformes	18	8	44%	-	-
Eurypygiformes	2	1	50%	1	50%
Procellariiformes	147	5	3%	-	-
Podicipediformes	23	4	17%	-	-
Phoenicopteriformes	6	3	50%	-	-
Phaethontiformes	3	-	0%	-	-
Ciconiiformes	19	14	74%	-	-
Pelecaniformes	118	31	26%	-	-
Suliformes	61	5	8%	-	-
Accipitriformes	266	66	25%	11	4,1%
Otidiformes	26	1	4%	-	-
Mesitornithiformes	3	-	0%	-	-
Cariamiformes	2	2	100%	-	-
Gruiformes	189	26	14%	2	1,0%
Charadriiformes	383	62	16%	3	0,78%
Pteroclidiformes	16	-	0%	-	-
Opisthocomiformes	1	1	100%	1	100%
Columbiformes	344	30	9%	5	1,4%
Opisthocomiformes	1	-	0%	-	-
Musophagiformes	23	3	13%	-	-
Cuculiformes	149	10	7%	-	-
Strigiformes	243	32	13%	3	1,2%
Caprimulgiformes	122	10	8%	-	-
Apodiformes	481	7	1%	-	-
Coliiformes	6	1	17%	-	-
Trogoniformes	43	2	5%	1	2,3%
Leptosomiformes	1	-	0%	-	-
Coraciiformes	177	13	7%	-	-
Bucerotiformes	74	6	8%	-	-
Piciformes	445	31	7%	-	-
Falconiformes	67	13	19%	4	5,9%
Psittaciformes	398	82	21%	9	2,2%
Passeriformes	6459	455	7%	22	0,34%
Total entries	**10857**	**1032**	**9,5%**	**83**	**0,92%**

* According Gill and Donsker (2018); - =No records found.

We also analyzed the diploid number variation, and observe this range was of 2n= 40 in *Falco columbarius* (Falconiformes) and *Bycanistes bucinator* (Coraciiformes) to 2n= 142 chromosomes in *Corythaixoides concolor* (Musophagiformes). Despite of wide variation, the most birds species have diploid

number range 2n = 78-82, which represent 50% of the species and a karyotype with 2n= 80 is most 22,3 % of the records (Figure 2).

Figure 3 shows the 2n variation stratified by orders. It is remarkable that Struthioniformes, Rheiformes, Apterygiformes species have few variation in chromosome number, while Coraciiformes, Piciformes, present a large 2n variation among the analyzed species so far.

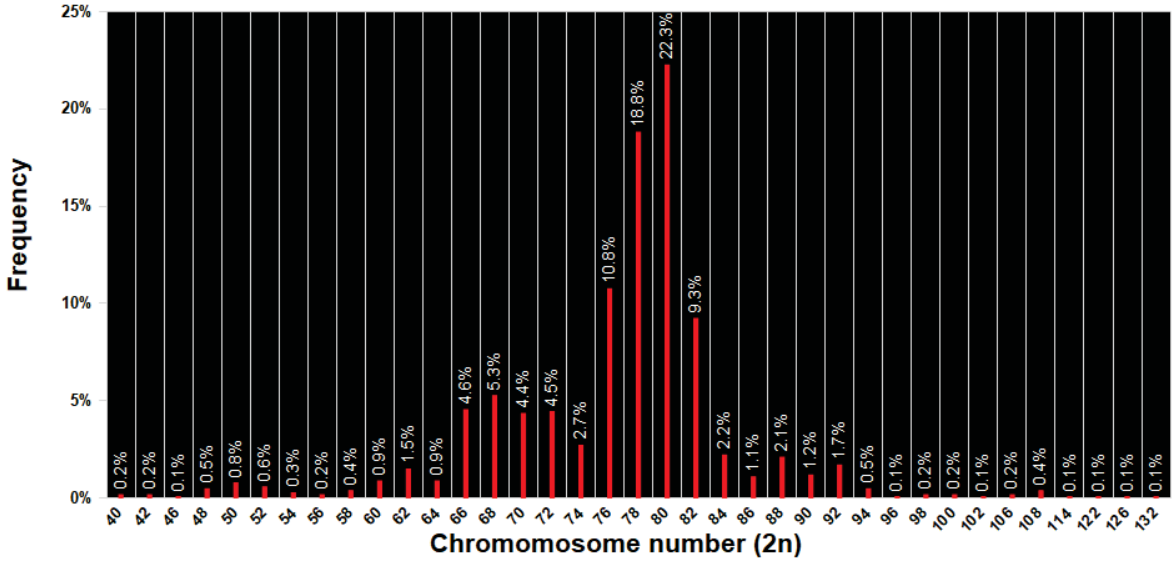


Figure 2: Diploid number (2n) frequency in birds.

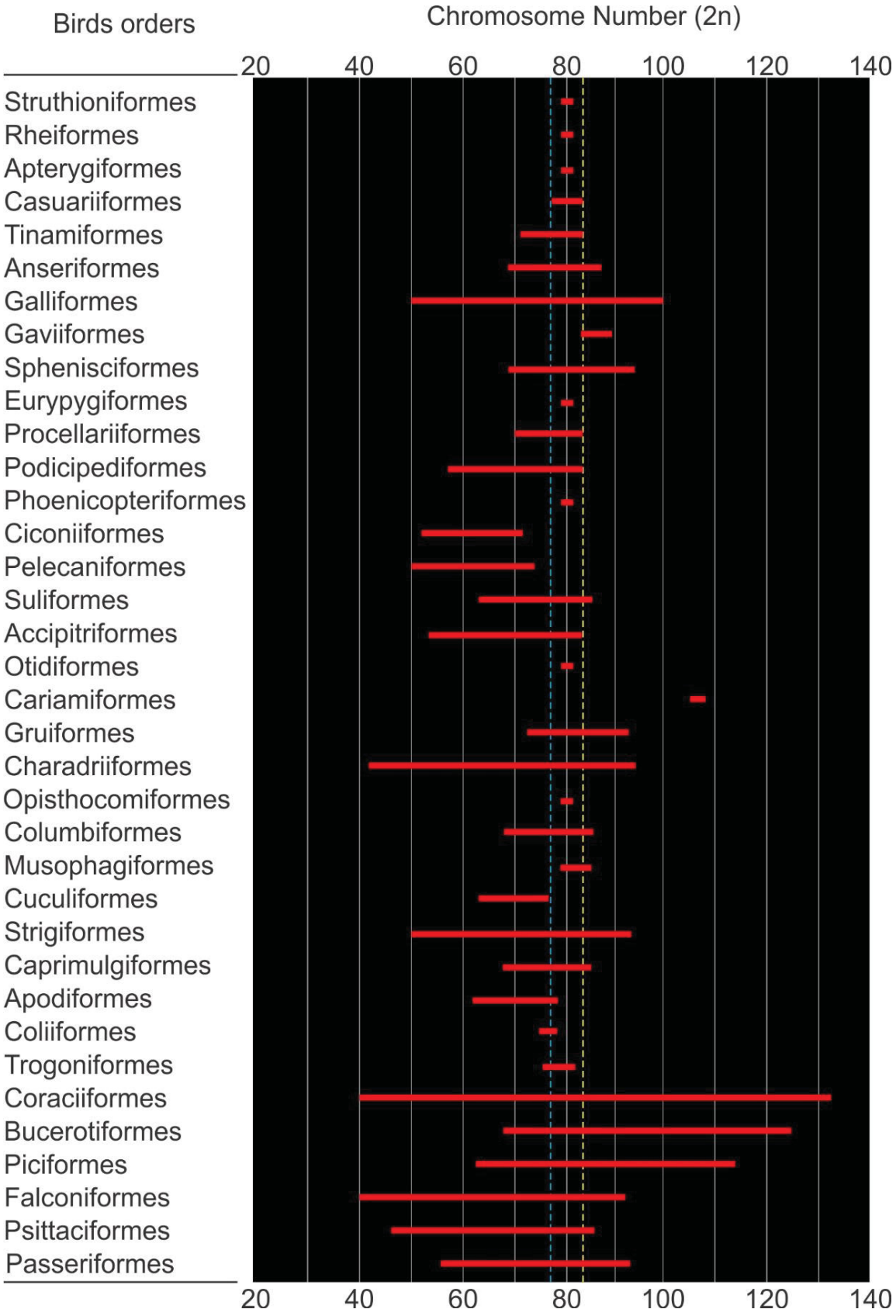


Figure 3: Diploid number variation observed in birds. About 50% of species have a diploid number ranging from 78 to 82 (vertical lines in blue and yelow, respectively). This figure is a modified version from the review study by Shields (1982).

Chromosome painting data

The database provides information about 83 bird species which were studied by chromosome painting with *G. gallus* (GGA) whole chromosome probes. These species are distributed into 17 orders and 33 families that represents less than 1% of the total bird species (Table 1). Among the most studied orders find the Falconiformes, Accipitriformes, Galliformes and Passeriformes (Table 1).

Discussion

Over the years several bird cytogenetic review studies have been published e.g Bloom (1969), Ray-Chaudhuri (1973), Shields (1982), De Boer (1984), Capanna et al. (1987), Santos and Gunski (2006), Cuervo et al. (2011), Kretschmer et al. (2018). In De Boer (1984) was listed the karyotype of 587 species and Capanna et al 1987 with 484 species. In this first edition, the website "Bird Chromosome database" compiled an important number of data including the diploid number of 1032 species. Despite this increase in the contingent of karyotyped species, is observed the birds are a group very little studied from the cytogenetic point of view, karyotype numbers are known in less than 10% of the species.

The chromosome painting is still a technique little used in birds, the database showed that less than 1% of the bird species had their karyotype analyzed with the probes of chromosomes of G. gallus. E.g in Acciptriformes, the accumulated knowledge allowed to understand the evolutionary chromosomal processes involved in the reduction of the diploid number 2n = 66 that is observed in the group. In this sense, the compilation of the diploid number presented in the database also served to identify the gaps and important questions that can be approached through chromosome painting as the wide numerical chromosome variation observed in the species of the Piciformes and Coraciformes orders (Figure 2).

G. gallus has been an excellent model organism, its karyotype allows to standardize all comparisons between birds of different orders (Guttenbach et al., 2003; Nishida-Umehara et al., 2007). These data are extremely useful not only for cytogeneticists but also for genome sequencing studies, which can identify chromosomal homologies to assemble the genomes.

Conclusions

We hope that, with the contribution of the scientific community, the information available in the database will increase and also provide an overview of the cytogenetic studies of birds. In future updates, we intend to make available the data on the sex chromosomes, C, G, and NOR band patterns, as well as the known data of chromosome painting with the probes of Leucopternis albicollis (Accipitriformes). All contributions and suggestions from the community are encouraged and should be sent to birdschromosome@gmail.com.

Acknowledgements

We thank the Universidade Federal do Pampa (UNIPAMPA), for technical support for in the website creation. The Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) by scholarship to TMD (finance Code 001).

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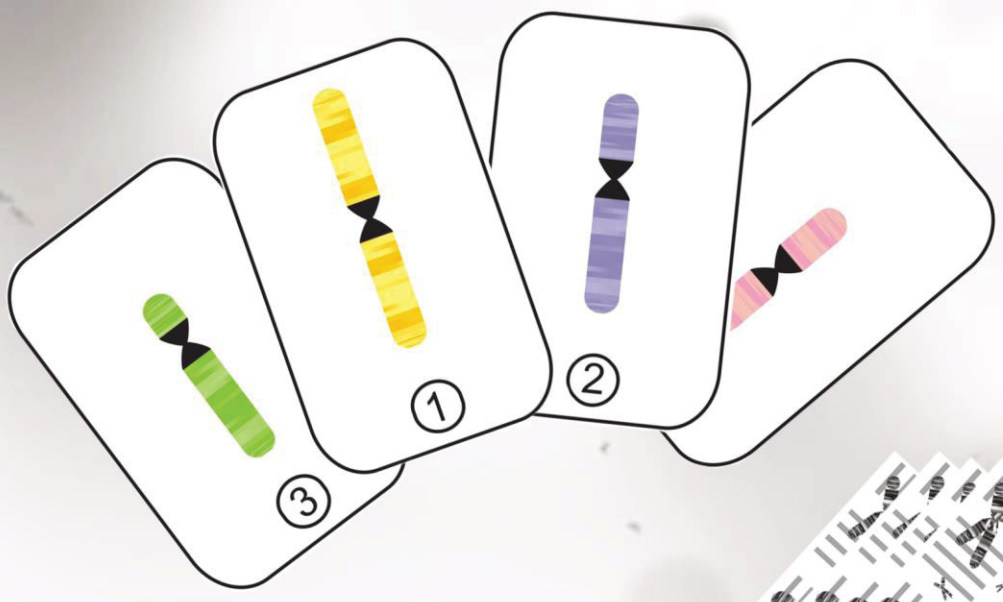
8. CAPÍTULO 5:

BARALHO MUTANTE" PARA O ENSINO DAS ALTERAÇÕES CROMOSSÔMICAS NUMÉRICAS ANEUPLOIDIAS

Artigo publicado no periódico Genética na Escola (ISSN 1980-3540), v.13(2), 2018.



"Baralho mutante" para o ensino das alterações cromossômicas numéricas Aneuploidias

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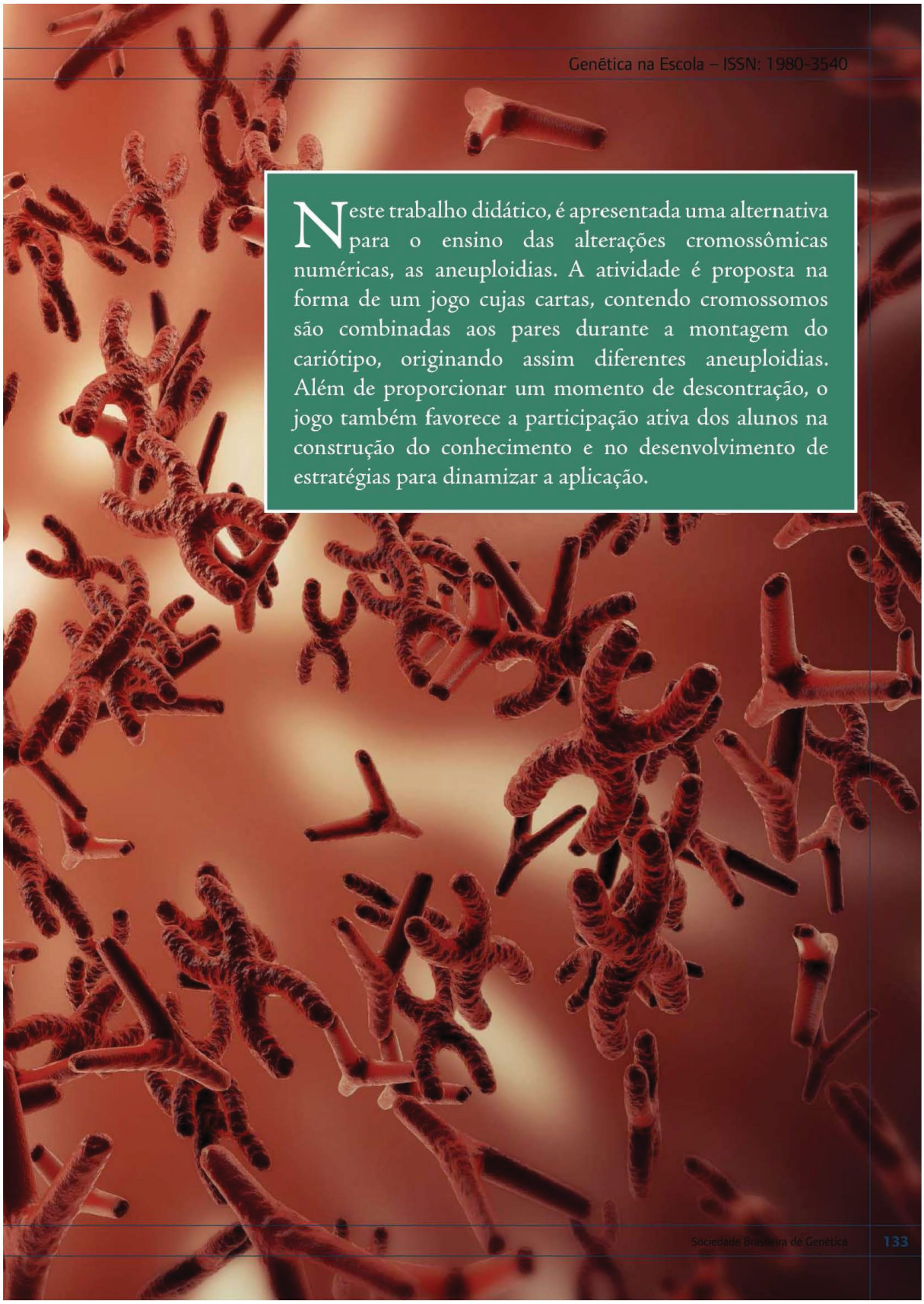

Genética na Escola – ISSN: 1980-3540

Neste trabalho didático, é apresentada uma alternativa para o ensino das alterações cromossômicas numéricas, as aneuploidias. A atividade é proposta na forma de um jogo cujas cartas, contendo cromossomos são combinadas aos pares durante a montagem do cariótipo, originando assim diferentes aneuploidias. Além de proporcionar um momento de descontração, o jogo também favorece a participação ativa dos alunos na construção do conhecimento e no desenvolvimento de estratégias para dinamizar a aplicação.

APRESENTAÇÃO DO JOGO

Para este estudo foi elaborado um jogo de cartas com o objetivo de elucidar o tema das alterações cromossômicas numéricas do tipo Aneuploidias (Anexo). O jogo foi nomeado "Baralho Mutante" e pode ser aplicado em sala de aula para estudantes de ensino médio e superior.

A finalidade do jogo é a montagem de um cariótipo hipotético formado por dez pares de cromossomos (2n=20), que inclui nove pares de cromossomos autossômicos e um par de cromossomos que determinam o sexo (Figura 1).

As cartas do jogo foram elaboradas contendo o número correspondente à posição do cromossomo no cariótipo; assim, do 1 ao 9 posicionam-se os cromossomos autossômicos. As letras X e Y foram utilizadas para definir os cromossomos que determinam o sexo, sendo XX para o cariótipo da fêmea, ou XY, para o macho.

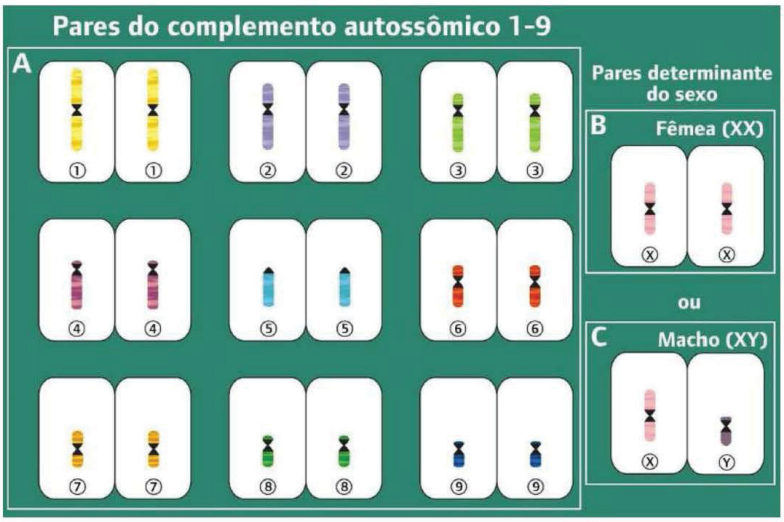

FIGURA 1.
Representação do cariótipo normal com 2n= 20 que pode ser montado com o jogo "Baralho Mutante". No box A estão agrupados os cromossomos do complemento autossômico. Em B e C estão os cromossomos que determinam o sexo, que complementam a representação do cariótipo feminino XX e masculino XY, respectivamente.

Para que ocorra a formação das aneuploidias durante o jogo, as cartas podem conter um, dois e também não conter o idiograma do cromossomo. A combinação destas cartas em pares no cariótipo é que dará origem às diferentes aneuploidias. A nomenclatura para as possíveis aneuploidias do jogo pode ser vista na figura 2.

A fim de promover maior interação entre os jogadores, o jogo contém cartas com instruções que conferem tanto vantagens quanto desvantagens ao jogador, como pode ser visto na figura 5. Ganha o jogo, o jogador que concluir um cariótipo primeiro, independente de conter ou não mutações cromossômicas.

O baralho completo é formado por 110 cartas e com ele o professor pode aplicar a prática para grupos de quatro alunos. Para a reprodução do material, o professor deve imprimir as figuras conforme as instruções apresentadas na Tabela 1.

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Tabela 1.
Instruções para impressão das cartas para construir o baralho.

Passos	Imprimir páginas, selecionar:	Nº de cópias para cada quatro jogadores
1º	Página da figura 3; página da figura 6	9 cópias
2º	Página da figura 4; página da figura 6	1 cópia
3º	Página da figura 5; página da figura 6	1 cópia

*Recomendamos a impressão em papel A4 250 g, para dar mais resistência às cartas. Solicitar aos alunos para que levem para a aula uma tesoura com ponta redonda para recortar as cartas.

COMO PREPARAR O MATERIAL

Para confeccionar o jogo, utilizar o material de apoio: figuras 3, 4, 5 e 6. No momento da impressão, escolher a opção frente e verso. Selecionar imprimir páginas e, no espaço indicado, digitar o número da página em que se encontra a figura e a página da figura no verso das cartas, conforme instruções da Tabela 1.

COMO JOGAR

1. Inicialmente os jogadores devem embaralhar as cartas; distribuir 12 cartas para cada um dos jogadores; o restante das cartas deve ficar sobre a mesa, em um monte que será utilizado para compra;

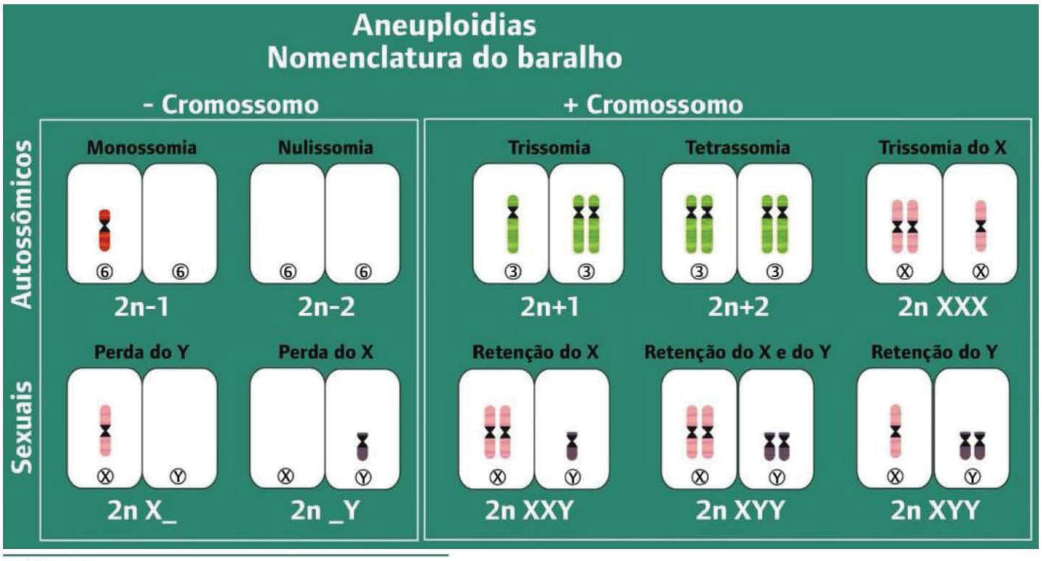

FIGURA 2.
Simbologia e nomenclatura das alterações cromossômicas numéricas, Aneuploidias do Jogo "Baralho mutante". Em cada carta está representado o número correspondente ao par cromossômico no cariótipo e o idiograma do cromossomo pode estar presente, ausente ou em mais de uma cópia.

2. O jogador deve observar, dentre as cartas recebidas, quais pares podem ser montados. Para isso, cada jogador deve guiar-se pelo número ou letra que a carta contém, identificando os pares de cromossomos;

3. Apenas os pares completos no primeiro instante devem ser baixados e organizados em ordem sobre a mesa, dando início à montagem do cariótipo. Ver exemplo do cariótipo na figura 1;

4. Os jogadores devem iniciar a primeira rodada de compra decidindo entre si quem será o primeiro jogador a pegar uma carta do monte, mantendo a mesma ordem de compra nas próximas rodadas;
5. A cada rodada de "compra" o jogador deve observar se pode ser formado um par de cromossomos com a carta que foi comprada. Caso seja formado um par, este deve ser inserido no cariótipo, que está sobre a mesa. Caso não forme um par, o jogador deve escolher uma, dentre suas cartas, para descartar no monte de descarte;
6. A carta que o jogador anterior descartou, poderá ser utilizada apenas pelo próximo jogador, se desejar. Caso a carta descartada não seja útil ao jogador subsequente ele deverá comprar uma carta e seguir a instrução nº5.
    a. Os jogadores não podem utilizar as cartas para substituir/ou corrigir pares que já compõem o cariótipo;
7. Sempre que algum jogador ficar sem cartas na mão, terá o direito de comprar três cartas em uma mesma rodada de compra. Em seguida, ele deve escolher uma delas para descartar, permanecendo com duas cartas na mão;
8. Sempre que terminar o monte de compras, o monte de descarte deve ser reciclado tornando-se o monte de compra. É recomendado embaralhar o mesmo nessa etapa;
9. Cartas com instruções aparecerão durante o jogo e fornecerão uma maior interação entre os jogadores; os jogadores que tiverem em mãos estas cartas podem escolher o momento certo para utilizá-las, sempre que a instrução for cabível. Além disso, não é permitido descartar as cartas de instruções sem utilizá-las. No entanto, após utilizá-las, estas não devem voltar ao baralho de compra ou descarte;
10. O fim do jogo é sinalizado quando 3 jogadores concluírem o cariótipo. Mas recomendamos que seja permitido que o quarto jogador também possa concluir, buscando entre os montes as cartas que lhe faltam. Assim, este poderá participar da discussão com os colegas.
11. Ao final o professor deve auxiliar os grupos na descrição dos cariótipos que foram concluídos. Recomenda-se utilizar o guia de nomenclatura (Figura 2), e os conceitos de cada alteração.

QUESTÕES PARA O PROFESSOR ABORDAR APÓS O JOGO

O professor deverá solicitar aos jogadores que façam uma análise e descrição do cariótipo montado (quadro a seguir). Deve-se observar se ocorreu a formação de uma ou mais alterações cromossômicas numéricas e obedecer à nomenclatura apresentada na figura 2.

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Análise e descrição do cariótipo	
2n =	Número de cromossomos autossomos =
Número de cromossomos determinantes do gênero =	
X =	
Y =	
Foram observadas alterações cromossômicas numéricas	
SIM ( )	NÃO ( )
Descreva	

Quadro.

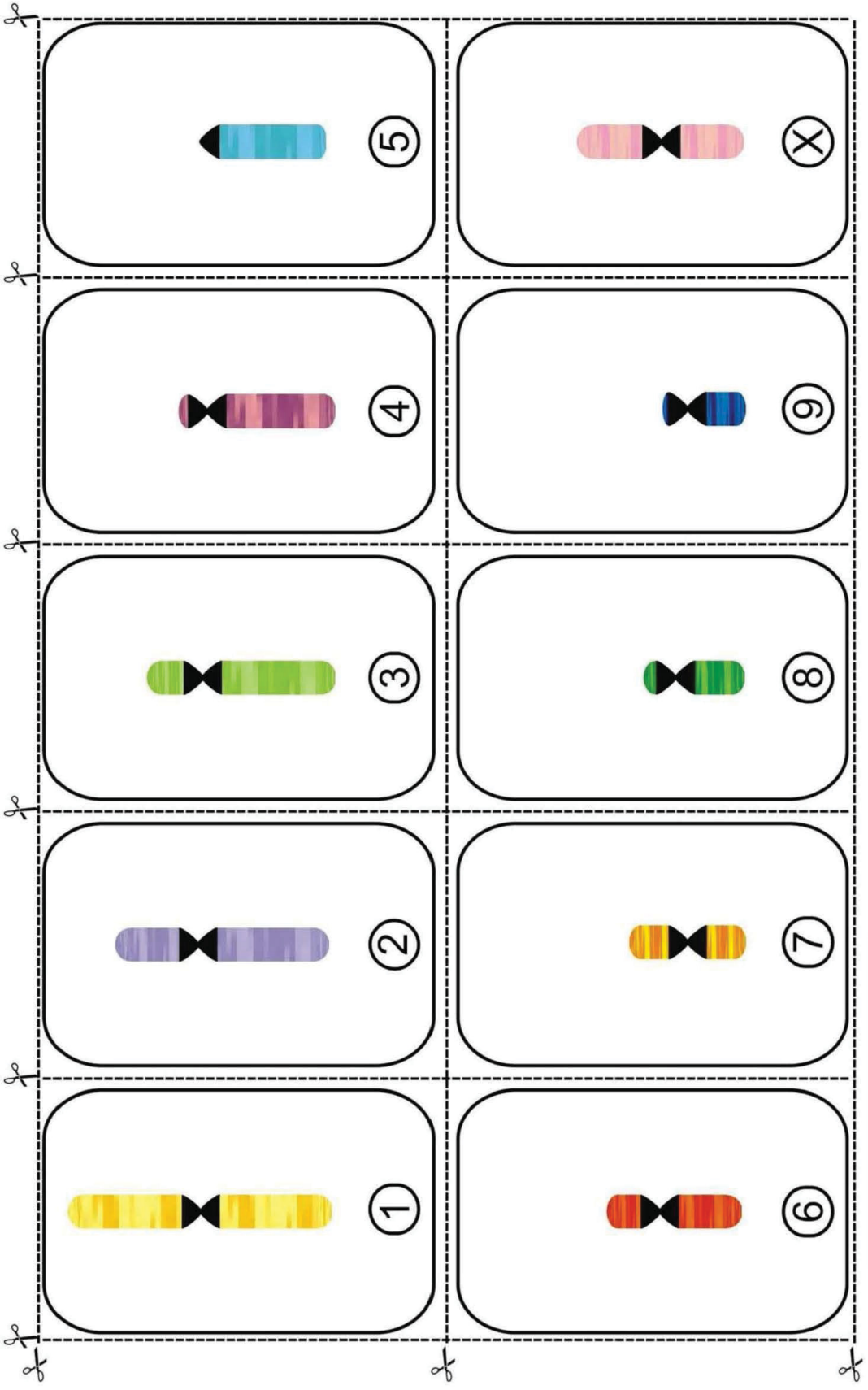

Figura 3.
Cartas para impressão e confecção do jogo, compreendendo os pares autossômicos 1 a 9 e o cromossomo X.

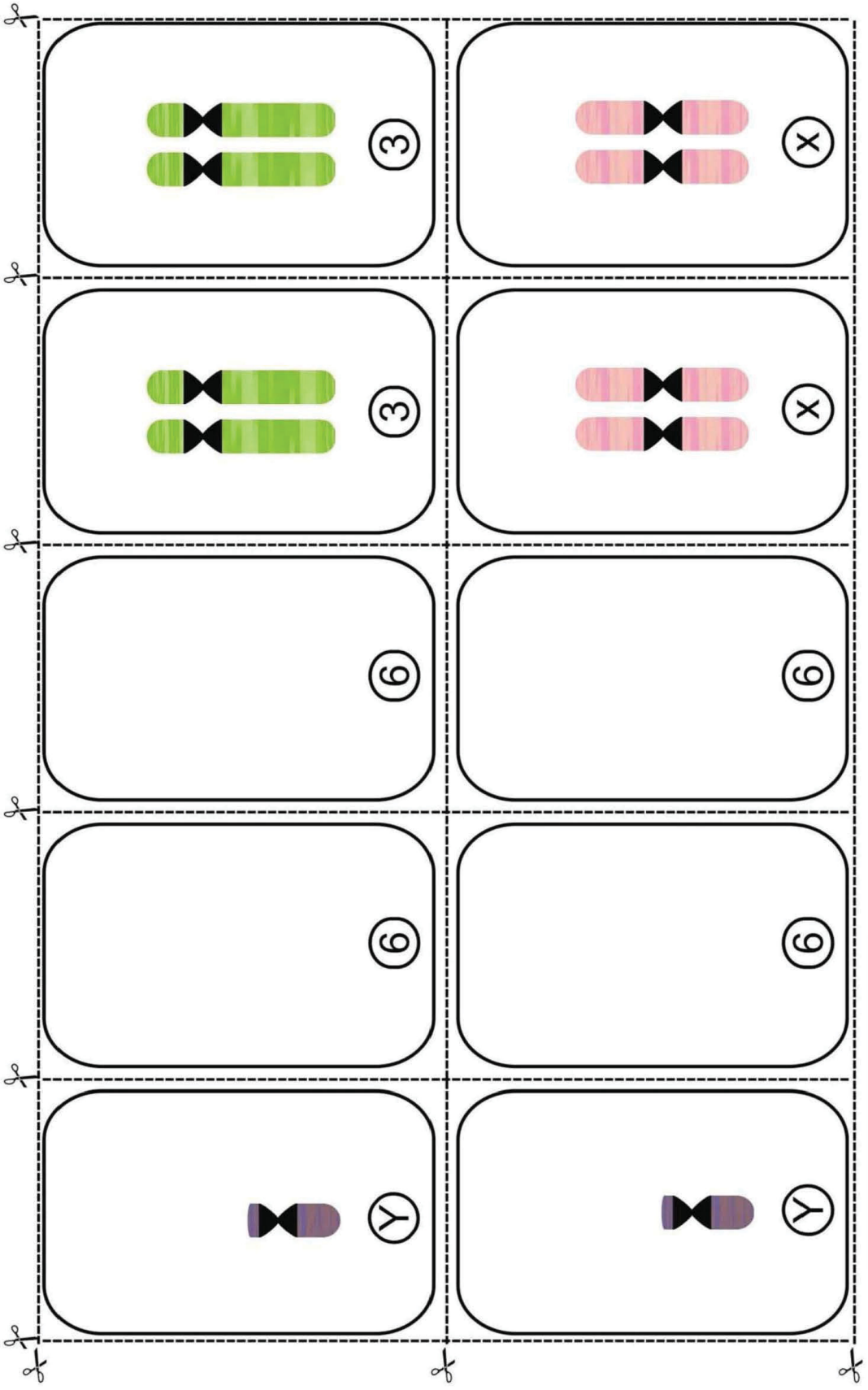

Figura 4.
Cartas para impressão e confecção do jogo, compreendendo o cromossomo Y e cartas com alterações cromossômicas.

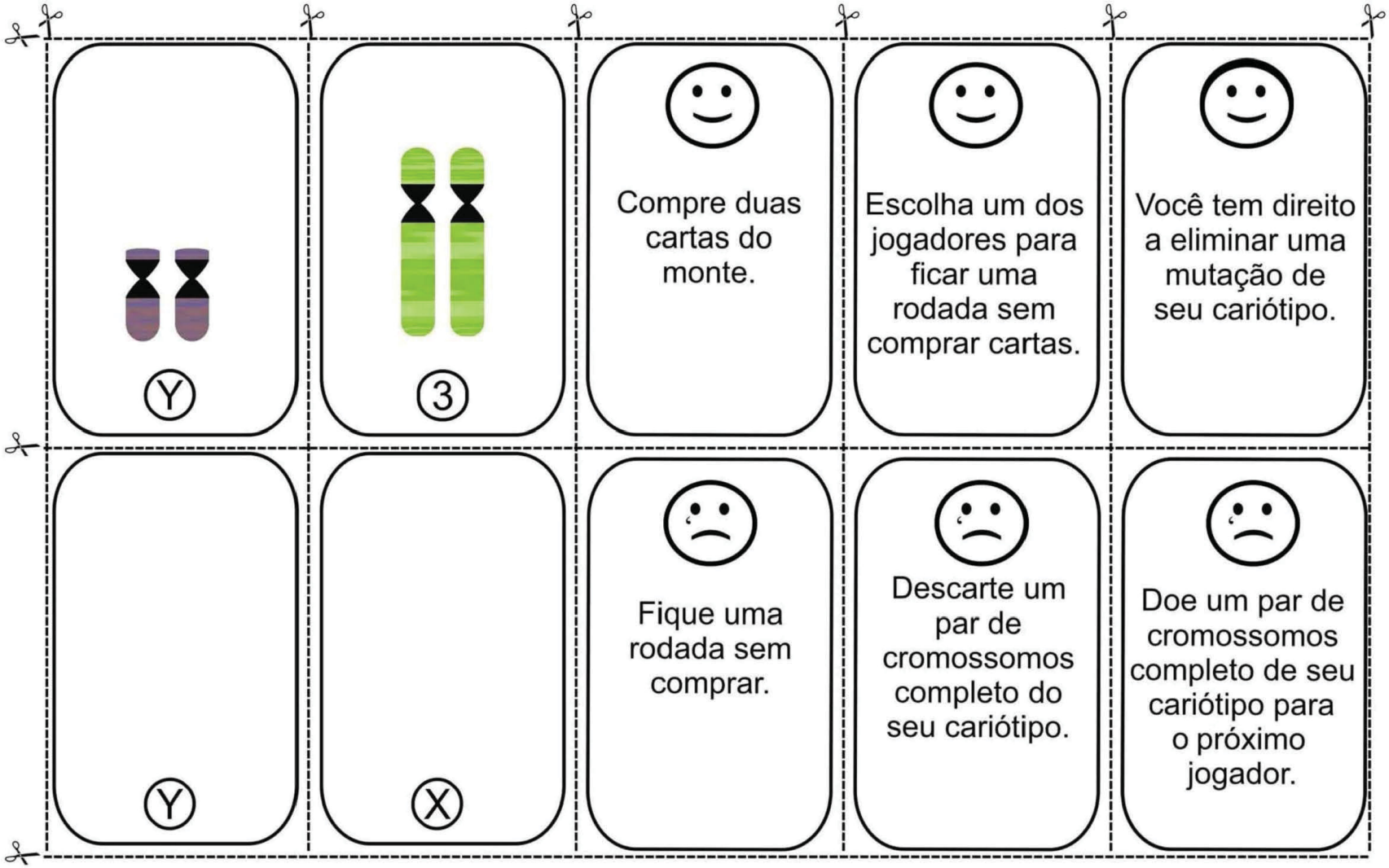

Figura 5.
Cartas para impressão e confecção do jogo, compreendendo cartas com alterações cromossômicas e cartas com instruções para promover a interação entre os jogadores.

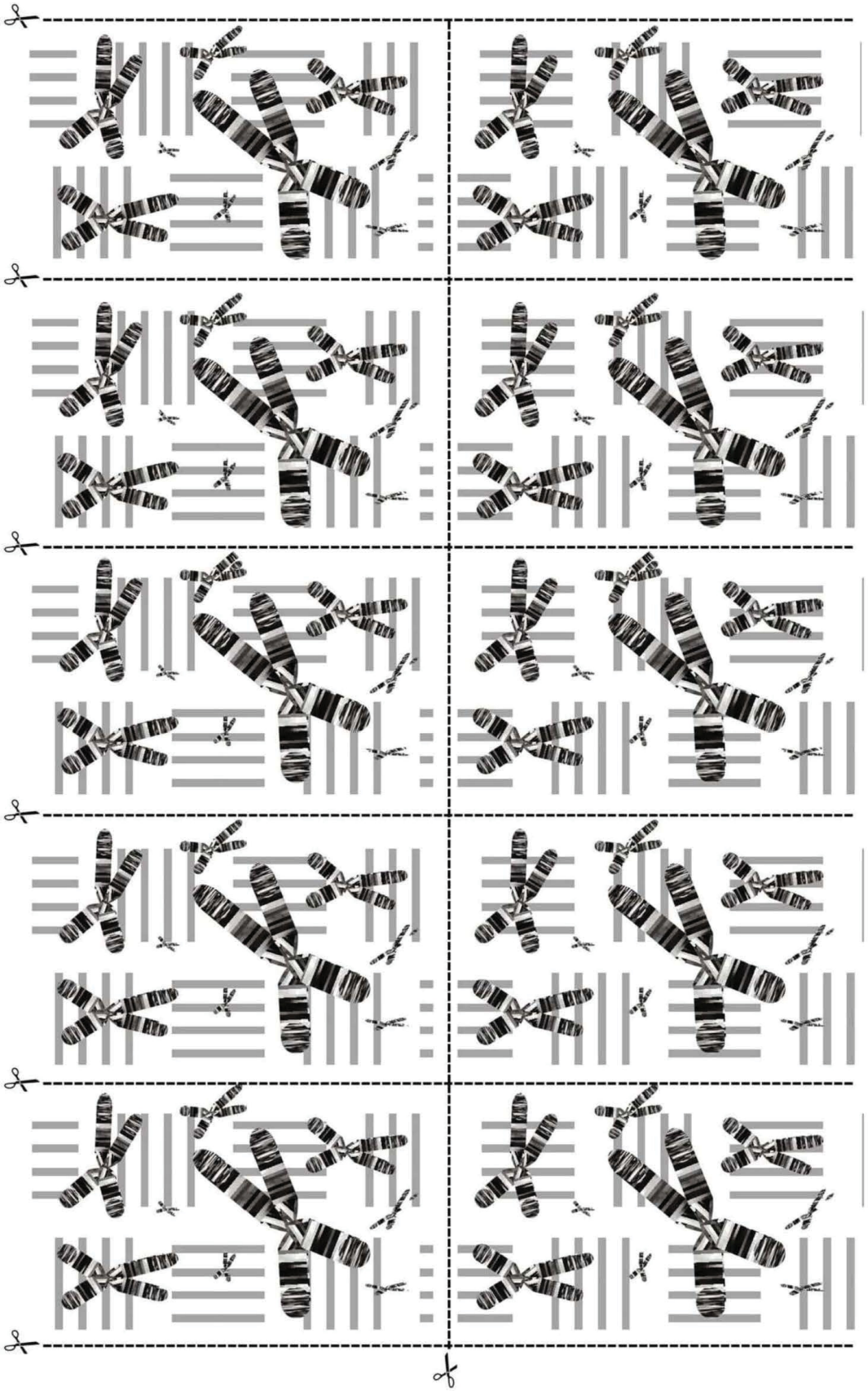

Figura 6.
Cartas para impressão e confecção do jogo, em frente e verso, sendo estas figuras a parte do verso para todas as cartas.

OUTRAS FORMAS DE UTILIZAR O BARALHO

O baralho também pode ser utilizado para abordar temas básicos como montagem de cariótipos, morfologia dos cromossomos, meiose e gametogênese.

1. Montagem do Cariótipo

Para uma abordagem mais simples de montagem do cariótipo, o professor deve retirar as cartas que contêm alterações cromossômicas e também as cartas com instruções, apresentadas nas figuras 4 e 5. Os jogadores podem seguir as mesmas instruções do jogo com as mutações. Devem ser identificados os cromossomos homólogos, seguindo para a ordenação dos cromossomos de acordo com o tamanho (do maior para o menor) e a identificação dos cromossomos que determinam o sexo. Ver exemplo do cariótipo na figura 1.

2. Morfologia dos cromossomos

O baralho também pode ser utilizado para abordar as diferentes morfologias dos cromossomos. Para isso os alunos devem observar o quadro abaixo e classificar as morfologias de acordo com a posição do centrômero (metacêntrico, submetacêntrico, acrocêntrico e telocêntrico) conforme demonstrado na figura 7. O professor deve explicar as diferenças entre os braços cromossômicos (longo e curto) e como é identificado o centrômero.

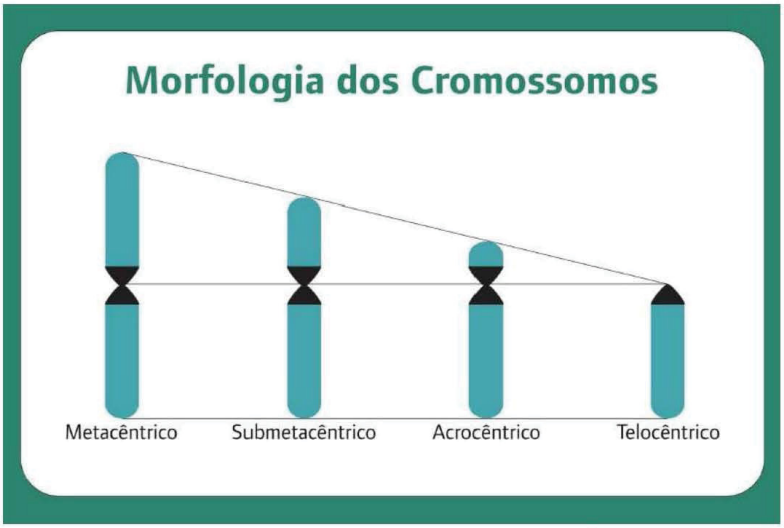

FIGURA 7.
Guia para classificação das morfologias cromossômicas de acordo com a posição do centrômero.

3. Meiose e gametogênese

Os cariótipos montados durante os jogos (tanto os normais ou com Aneuploidias) podem ser utilizados para abordar a meiose e gametogênese, identificando os gametas progenitores. Para isso, o professor poderá solicitar aos alunos que, após montarem o cariótipo, separem cada membro de um par cromossômico (carta) aleatoriamente em dois grupos. Assim, serão formados os gametas progenitores do cariótipo montado.

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ANEXO

ALTERAÇÕES CROMOSSÔMICAS NUMÉRICAS

As alterações ou mutações cromossômicas numéricas são variações observadas em indivíduos em relação ao número diploide padrão da espécie. Estas alterações são classificadas como Euploidia, quando a variação envolve o conjunto completo de cromossomos ou Aneuploidia quando afetam parcialmente o conjunto de cromossomos, ou seja, afetam pares cromossômicos.

ANEUPLOIDIAS

As aneuploidias podem envolver tanto a perda, quanto o acréscimo de cromossomos no complemento cariotípico. Elas são classificadas como:

- Nulissomia, quando ocorre a perda de um par de cromossomos homólogos (2n-2);
- Monossomia resulta da perda de um cromossomo em um par de cromossomos homólogos (2n-1);
- Trissomia, é o acréscimo de um cromossomo em um par de cromossomos homólogos (2n+1);
- Tetrassomia ocorre quando existem quatro cópias de um mesmo cromossomo (2n+2).

Além destas, as aneuploidias também podem afetar os cromossomos determinantes do sexo, ocorrendo tanto o excesso quanto à perda de cromossomos.

As aneuploidias podem surgir por não ocorrer disjunção de cromossomos durante as divisões mitótica e/ou meiótica, seja no momento da separação de cromossomos homólogos na meiose ou de cromátides irmãs, neste caso, podendo ocorrer tanto na mitose quanto na meiose II. A meiose tem especial importância, pois durante a formação dos gametas (Gametogênese) uma célula diploide (2n) é reduzida ao estado haploide (n), podendo eventualmente originar gametas Aneuploides. Assim, o gameta portador de uma Aneuploidia, quando fertilizado, resultará em um zigoto com o número de cromossomos diferente do valor diploide padrão para a espécie.

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9. CONSIDERAÇÕES FINAIS

Desde o início dos estudos citogenéticos em aves nos anos 50, uma ideia de conservadorismo do cariótipo das aves foi estabelecida. Os trabalhos apresentados nesta tese demonstraram que as variações ocorrem e que este pensamento deve ser observado com atenção. É verdade que a maioria das espécies de aves estudadas até o presente, tem mostrado um número de cromossomos pouco variável entre 78 e 82 cromossomos, o que inclui 50% das espécies com cariótipos conhecidos. No entanto este conservadorismo numérico não necessariamente reflete no conservadorismo estrutural dos cromossomos, e trabalhos recentes têm demostrado a ocorrência de rearranjos entre e intracromossosomicos e sua relação com a especiação.

É esperado que as variações cromossômicas sejam acumuladas a medida que a distância evolutiva entre as espécies aumenta em relação ao cariótipo ancestral. No entanto, as variações cromossômicas numéricas são observadas mesmo em espécies que possuem estreita relação filogenética. No capítulo I, por exemplo, foi apresentado o cariótipo das espécies *Megaceryle torquata* e *Chlorocer-yle americana*, ambas pertencentes a ordem Coraciformes, família Alcedinidae. Com o uso de somente métodos de citogenética clássica, foi observado que *M. torquata* e *C. americana*, divergem no número de cromossomos de 2n= 84 para 2n= 94, respectivamente. As comparações realizadas entre os cariótipos evidenciaram que a origem desta variação provavelmente deve se a fissões cêntricas, e com isso os cariótipos mostran-se bastante heterogêneos na morfologia e no tamaho dos macrocromossomos.

Esta tese, também destacou a importância de realizar os experimentos de hibridização *in situ* fluorescente (FISH) e pintura cromossômica, pois eles permitiram identificar diferenças estruturais importantes entre os macrocromossomos. No capítulo II, apresentamos o cariótipo da espécie *Trogon surrucura surrucura* (Trogoniformes) que tem um número diploide de 2n= 82, muito semelhante ao observado na maioria das espécies de aves. No entanto a pintura cromossômica com as sondas do *Gallus gallus* e *Leucopternis albicollis*, evidenciaram que este cariótipo foi derivado por fissões, fusões e inversões. Estes rearranjos certamente tem um papel importante na especiação das aves, visto que o acúmulo destas diferenças entre as espécies pode resultar no isolamento reprodutivo.

No capítulo III, é explorado o papel dos sítios cromossômicos do 45S rDNA. Apesar da maioria das espécies preservar o 45S rDNA em um par de microcromossomos, novamente foi observado que variações têm ocorrido mesmo em espécies intimamente relacionadas. As origens destas variações foram exploradas através de comparações ancoradas em uma filogenia existente e foi visto que processos recorrentes de duplicação resultaram em variações do número de cromossomos portadores do cluster 45S rDNA. Enquanto que fusões foram responsáveis pela redistribuição do cluster 45S rDNA de um ancestral localizado em microcromossomos para diferentes macrocromossomos.

No capítulo IV, considerando a relevância dos resultados encontrados nos métodos citogenéticos. Foi construído um banco de dados citogenéticos para as aves, no qual buscou-se resgatar trabalhos clássicos de descrições cariotipicas que se somaram ao que se pode considerar como a nova era da citogenética de aves, a pintura cromossômica. Assim, espera-se que as informações disponibilizadas forneçam uma visão global deste conhecimento, bem como os pesquisadores possam identificar as principais lacunas e estimular o desenvolvimento de novos estudos.

Finalmente no capítulo V, o jogo “Baralho mutante” que foi desenvolvido nesta tese, representa uma alternativa interessante para o ensino das Aneuploidias. Além de proporcionar um momento de descontração entre os colegas, o jogo estimula o desenvolvimento de estratégias e permite a participação ativa dos alunos na construção do conhecimento.

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