UNIVERSIDADE FEDERAL DO PAMPA

MARCELO SANTOS DE SOUZA

CARACTERIZAÇÃO CITOGENÔMICA DE PASSERIFORMES E CHARADRIIFORMES: UMA ANÁLISE DA EVOLUÇÃO CARIOTÍPICA NA ESTRUTURA ORGANIZACIONAL DOS MICROCROMOSSOMOS DAS AVES

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Ricardo José Gunski

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I. Título.

Dedico esta tese com todo meu carinho a meu filho Vinícius de Andrade de Souza.

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À medida que finalizo esta jornada, torna-se indispensável expressar minha profunda gratidão a cada um de vocês que deixaram uma marca permanente na busca deste sonho. **A Deus**, por ser a bússola inabalável em minha jornada, guiando-me através de desafios. Sua presença foi a luz que nunca se apagou, mesmo nos momentos mais obscuros. À minha mãe, **Clenir**, cujo amor incondicional e sacrifícios foram a fundação sobre a qual construí meus sonhos. Mãe, sua força e dedicação são minhas maiores inspirações. Não há palavras suficientes para expressar minha gratidão por tudo o que você é e tudo o que fez por mim. À memória do meu pai, **Valdemar Polini**, cujo legado de determinação e integridade continua a guiar-me. Pai, embora você não esteja aqui fisicamente, sua presença é sentida em cada passo que dou. Espero tê-lo feito orgulhoso. Ao meu filho amado, **Vinícius**, a luz da minha vida. Você é a razão pela qual busco ser a melhor versão de mim mesmo. Ver o mundo através dos seus olhos tem sido o maior presente, por mais longe que possamos estar um do outro. Obrigado por ser minha maior motivação. À minha irmã, **Sabrina**, obrigado por cada palavra de encorajamento. Sua fé em mim nunca vacilou, mesmo quando a minha própria fé tremia. À minha gata, **Jade**, minha companheira silenciosa (as vezes nem tanto), trazendo conforto e alegria nos dias mais árduos e nas noites mais longas. Ao meu orientador, **Prof. Dr. Ricardo Gunski**, por ser não apenas um mentor, mas uma verdadeira inspiração. Sua paixão pela ciência e dedicação à excelência são qualidades que busco aplicar em minha própria jornada acadêmica. Lhe agradeço do fundo de meu coração. À **Prof. Dra. Analía**, obrigada por expandir meus horizontes e me desafiar a ir além do que eu pensava ser capaz. Muito obrigado por tudo! Ao meu coorientador e amigo, **Prof. Dr. Rafael Kretschmer**, sua amizade, paciência e orientação foram além do que palavras podem expressar. Você foi essencial em cada etapa deste processo. Muito obrigado! Ao **Prof. Dr. Fabiano**, obrigado por cada palavra de apoio, e pela amizade que incentiva desenvolvimento pessoal de todos que estão ao seu redor. À minha melhor amiga, **Dra. Suziane Barcellos**, por estar sempre ao meu lado em cada passo deste caminho. Sua amizade é um tesouro, e suas palavras sempre foram meu farol nos momentos em que a dúvida trazia escuridão a minha alma. Sua amizade é, verdadeiramente, um dos grandes pilares que sustentaram minha jornada. À minha amiga **Victoria Tura**, por todos momentos compartilhados, que foram um alívio bem-vindo nos períodos mais estressantes desta jornada. Aos **colegas e amigos do laboratório DGA**, Nairo, Paulo, Diego, Tiago, Vitor, Ibrain, e Teilor por criarem um ambiente de trabalho que é ao mesmo tempo desafiador, acolhedor e divertido. À querida amiga **Roseli**, por sua amizade sincera e apoio constante. Suas palavras de encorajamento foram um bálsamo para a alma. Ao **Alef**, por todos os momentos de descontração ou estudo. Sua amizade foi essencial para manter meu equilíbrio. Agradeço de coração por cada ensinamento, cada gesto e por estar sempre ao meu lado. Sou profundamente grato por cada momento, cada conselho sábio, e pela sinceridade que sempre me ofereceu. Obrigado por ser esse ponto de luz e por me fazer lembrar de viver plenamente e não apenas existir. Ao **Junior**, mais do que um amigo, você se tornou um irmão nesta jornada. Sua lealdade, humor inigualável e apoio constante foram fundamentais para mim. Nos momentos de dúvida, sua presença amiga e seu incentivo sincero me ajudaram a manter o foco e a perseverança. As risadas, as vitórias e perdas, e inclusive as frustrações tornaram esta caminhada muito mais leve. Sua amizade é um tesouro inestimável que guardarei sempre comigo. A todos vocês, e tantos outros que contribuíram para a realização deste sonho, **minha eterna gratidão!**

"Se você sabe que está no caminho certo, se você tem esse conhecimento interno, então ninguém pode desviá-lo, não importa o que eles digam."

Barbara McClintock

RESUMO

As aves têm sido objetos de admiração e fascínio humano por sua notável diversidade em vários aspéctos, essas criaturas oferecem perspectivas cruciais sobre a evolução da vida na Terra. Desde suas formas mais primitivas até as modernas, a história evolutiva das aves reflete uma vasta gama de processos adaptativos ao longo de milhões de anos. As aves se distribuem por uma ampla variedade de ambientes, desde terras continentais até ecossistemas marinhos, demonstrando sua extraordinária capacidade de evolução e adaptação. Apesar dos avanços significativos nas últimas décadas, a compreensão da organização genômica e da evolução cariotípica das aves ainda é limitada. Para abordar essas questões, diferentes técnicas, incluindo citogenética clássica e molecular, têm sido empregadas. As aves apresentam um cariótipo bimodal, composto por dois grupos distintos de cromossomos: macrocromossomos e microcromossomos. A presença desses microcromossomos representa um desafio para os estudos citogenéticos, tornando este grupo um dos menos conhecidos em termos cromossômicos. Nesse trabalho, utilizou-se a técnica de BAC-FISH para investigar a organização e o status evolutivo dos microcromossomos em espécies de aves das ordens Passeriformes e Charadriiformes. Os resultados revelaram um alto grau de conservação na evolução do cariótipo dos passeriformes, apesar de serem considerados um grupo altamente derivado na filogenia das aves. Por outro lado, nas espécies de Charadriiformes analisadas, cada uma mostrou um caminho evolutivo distinto, com eventos de reorganização dos microcromossomos. Também foram descritos dois novos números cromossômicos para as espécies *Calidris canutus* (2n = 92) e *Troglodytes aedon* (2n = 76). Esses achados destacam as diferenças evolutivas entre as duas ordens de aves, sugerindo que os Charadriiformes possam estar sujeitos a pressões seletivas mais variadas ou intensas que levam a uma maior diversidade na organização cromossômica.

Palavras-Chave: Cariótipo; Microcromossomos; Evolução cromossômica; Citogenética Molecular; BAC-FISH.

ABSTRACT

Birds have long been subject of human admiration and fascination for their remarkable diversity in many ways, these creatures offer crucial insights into the evolution of life on Earth. From their most primitive forms to modern ones, the evolutionary history of birds reflects a wide range of adaptive processes over millions of years. Birds are distributed across a wide variety of environments, from continental lands to marine ecosystems, demonstrating their extraordinary capacity for evolution and adaptation. Despite significant advances in recent decades, the understanding of the genomic organization and karyotypic evolution of birds is still limited. To address these issues, different techniques, including classical and molecular cytogenetics, have been employed. Birds have a bimodal karyotype, made up of two distinct groups of chromosomes: macrochromosomes and microchromosomes. The presence of these microchromosomes represents a challenge for cytogenetic studies, making this group one of the least unknown in chromosomal terms. In this study, the BAC-FISH technique was used to investigate the organization and evolutionary status of microchromosomes in bird species from the orders Passeriformes and Charadriiformes. The results revealed a high degree of conservation in the evolution of the karyotype of the Passeriformes, despite the fact that they are considered a highly derived group in the phylogeny of birds. On the other hand, in the Charadriiformes analyzed species, each showed a distinct evolutionary path, with microchromosome reorganization events. Two new chromosome numbers were also described for the species Calidris canutus (2n = 92) and Troglodytes aedon (2n = 76). These findings highlight the evolutionary differences between the two orders of birds, suggesting that the Charadriiformes may be subject to more varied or intense selective pressures that lead to greater diversity in chromosome organization.

Keywords: Karyotype; Microchromosomes; Chromosome evolution; Molecular cytogenetics; BAC-FISH.

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LISTA DE ABREVIATURAS E SIGLAS

2n - Número diploide

BACs - Cromossomos artificiais bacterianos

CCA - *Calidris canutus*

CEUA - Comissão de Ética no Uso de Animais

DNA - Ácido desoxirribonucleico

FISH - Hibridização Fluorescente *in situ*

GGA - *Gallus gallus*

JJA - *Jacana jacana*

KCl - Cloreto de potássio

ml - Mililitro

PAK - Putative Avian Ancestral Karyotype

SISBIO - Sistema de Autorização e Informação em Biodiversidade

WCP – Whole Chromosome Probes

ZAU - *Zenaida auriculata*

µl - Microlitro

SUMÁRIO

1 INTRODUÇÃO

1.1 As Aves

Por milênios as aves têm sido elementos de curiosidade e admiração humana, isso se deve à sua notável diversidade morfológica, comportamental e especialmente por seus padrões de coloração sempre muito admirados (Futuyama, 2021). Contudo, para além desses atributos estéticos, elas representam importantes evidências da evolução da vida na Terra. Desde as formas mais primitivas até as aves modernas, sua história evolutiva reflete incontáveis processos de adaptação. As características singulares das aves não surgiram do nada, nessa perspectiva, são o resultado de milhões de anos de evolução, moldadas por processos como: seleção natural, deriva genética e adaptação radiativa (Darwin, 1859; Mayr, 1999; Futuyama, 2021).

Os animais pertencentes à Classe Aves são considerados membros de um grupo taxonômico foco de extensas pesquisas, com estimativas de que mais de 95% da diversidade global de espécies já tenham sido catalogadas e descritas taxonomicamente. Entre os tetrápodes, as aves se destacam como o grupo mais diverso, englobando mais de 10.500 espécies (Gill et al., 2022). Este é um conjunto taxonômico monofilético que está organizado em 40 ordens distintas dentro da subclasse Neornithes. Nessa classificação, atualmente identificam-se três grupos também monofiléticos: as Paleognatas, os Galloanserae e as Neoaves (Jarvis et al., 2014). Esses animais possuem uma imensa distribuição geográfica que inclui uma surpreendente variedade de ambientes ao redor do globo, desde terras continentais até ecossistemas marinhos, demonstrando assim a extraordinária capacidade de evolução e diversificação desses organismos.

Apesar de avanços consideráveis nas últimas décadas, a organização genômica e a evolução cariotípica das aves ainda são pouco compreendidas (Degrandi et al., 2020). Diversos fatores contribuem para essa lacuna de conhecimento, tais como, a complexidade do genoma em questão e a dificuldade de obtenção de dados cromossômicos de alta qualidade (Utsunomia et al., 2022). Diante disso, diferentes abordagens têm sido empregadas para compreender melhor a organização genômica e a evolução do cariótipo dessa classe. Abordagens de citogenética clássica e molecular, por exemplo, têm sido ferramentas valiosas na

investigação da estrutura e do comportamento evolutivo dos cromossomos desses animais (Ellegren, 2013). Através de técnicas da citogenética clássica (Ex. bandeamentos cromossômicos), e molecular (Ex. hibridização fluorescente in situ - FISH), é possível identificar características morfológicas dos cromossomos, padrões de bandas e a localização de sequências específicas, tornando possível entender melhor a organização do cariótipo das aves (O'Connor et al., 2024).

1.2 Origem e classificação filogenética das aves

Oriundos da subordem de dinossauros bípedes Theropoda, um dos grupos mais diversificados e longevos de vertebrados que surgiram em nosso planeta, sendo estes os únicos descendentes sobreviventes até os dias atuais, as aves distinguem-se por suas características únicas, resultado de milhões de anos de evolução. Estes animais estão todos inseridos dentro da Classe Aves e por consequência é considerado um grupo monofilético, ou seja, todas as aves descendem de um ancestral comum (Prum et al., 2008).

Na atualidade, os estudos filogenéticos levam em consideração dados oriundos de diversas áreas de estudo que tornam os resultados da classificação mais precisos. Porém, de modo geral há diversas características que sustentam o status monofilético para a Classe Aves. Diversas características morfológicas compartilhadas por todas as aves, como penas, ossos pneumatizados, bicos sem dentes e asas, fornecem fortes evidências de uma ancestralidade comum. O estudo do desenvolvimento embrionário destes animais também apresenta semelhanças marcantes, reforçando a ideia de que todas as aves compartilham sua origem em um ancestral comum (Varricchio e Jackson, 2016).

Do ponto de vista paleontológico, as evidências fósseis da existência de aves desde o Cretáceo Superior são comprovadas por um rico registro fóssil, oriundo principalmente da China, que incluem: Jeholornis - um fóssil de 120 milhões de anos que é considerado a primeira ave com asas completamente desenvolvidas e Confuciusornis - um fóssil de 125 milhões de anos que apresenta penas longas e elaboradas (Dongsheng et al., 2010). Entre outros, registros fósseis de aves primitivas como estes, demonstram a transição gradual entre dinossauros terópodes e aves modernas (Figura 1).

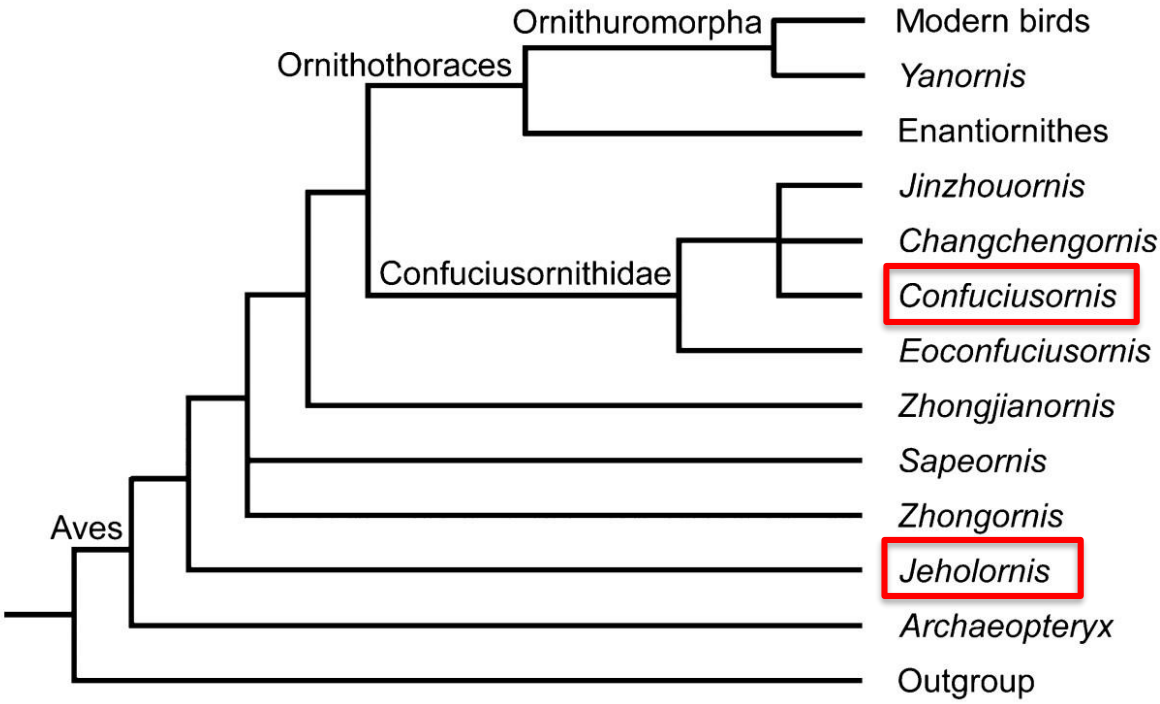

Figura 1. Relações filogenéticas das aves basais. As marcações em vermelho indicam alguns clados de aves ancestrais oriundas da China continental já descritas pela paleontologia (Adaptado de Dongsheng et al., 2010).

De forma sintetizada a classe Aves se organiza da seguinte forma: existem duas subclasses principais, Paleognathae - abrange aves que não voam, como avestruzes, emus e rheas e caracteriza-se por um osso esterno plano, palato sem forame infra-orbital e asas vestigiais, e as Neognathae - Inclui todas as aves voadoras e algumas aves não voadoras, como pinguins que apresentam um esterno com crista, palato com forame infra-orbital e asas adaptadas para o voo.

A subclasse Neognathae é subdividida em duas infraclasses: Galloanserae - abrangendo as galinhas, faisões, patos, gansos e aves relacionadas a estes que se caracterizam por um bico ceroso, pés com membranas e um sistema de siringe vocal complexo, e Neoaves - que inclui a grande maioria das aves modernas, como passeriformes, aves de rapina, papagaios, garças entre outras que apresentam um bico córneo diversificado, pés com diversas adaptações e uma variedade imensa de vocalizações.

Estudos filogenéticos contínuos utilizando dados morfológicos, moleculares e fósseis continuam a refinar a compreensão das relações evolutivas dentro da classe Aves (Figura 2), revelando novas descobertas sobre a história evolutiva e a diversificação desse grupo de animais.

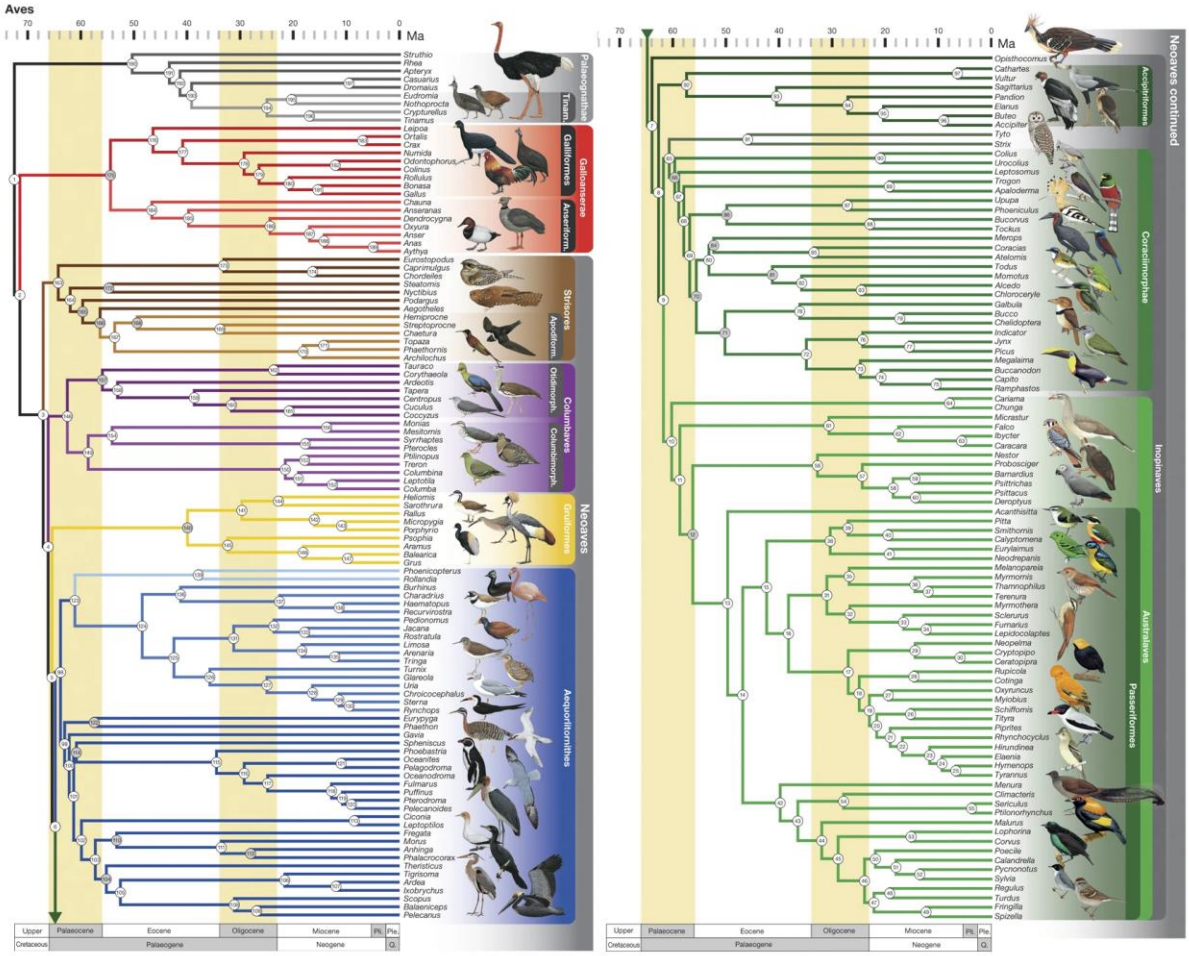

Figura 2. Relações filogenéticas das aves modernas (Adaptado de Prum et al., 2015).

1.3 O cariótipo típico das aves

As aves são caracterizadas por certa singularidade em sua configuração cariotípica, elas possuem um cariótipo bimodal, composto por dois grupos de cromossomos bem distintos, macrocromossomos (geralmente os 10 primeiros pares) e microcromossomos (geralmente a partir do 11° par). Esse segundo grupo de cromossomos, são pequenos em tamanho, com morfologia puntiforme de difícil distinção e geralmente presentes em grande número (Griffin et al., 2007; Degrandi et al., 2020; de Souza et al., 2023). Considerando o tamanho médio do genoma das aves, os microcromossomos comportam ~25% do genoma total, esse grupo de elementos geralmente detêm três quartos do número de cromossomos e cerca de

metade dos genes ativos do genoma desses animais (Waters et al., 2021). Considera-se ainda, a presença de microcromossomos no cariótipo das aves um desafio para os estudos citogenéticos e, consequentemente, torna este clado um dos menos conhecidos em termos cromossômicos onde, apenas ~ 10% de todas as espécies da Classe possuem alguma descrição relativa a seu cariótipo, geralmente apenas o número diploide, com descrições cromossômicas morfológicas parciais e alguns bandeamentos cromossômicos (Degrandi et al., 2020).

Dentro dos tetrápodes, as aves representam o táxon com a mais elevada quantidade de cromossomos, geralmente apresentando 2n ~ 80 (Damas et al., 2019). Por serem um grupo monofilético, com um cariótipo também considerado evolutivamente conservado, essas características típicas estão presentes em todas as aves Paleognatas (aves basais) e na maioria das Aves Neognatas (Aves modernas, Galloanseres e Neoaves) (Degrandi et al., 2020; O'Connor et al., 2024). Entretanto, apesar da alta percentagem de espécies com cariótipos típicos, com o aumento do número de espécies estudadas, é possível observar algumas com cariótipos atípicos, seja pelo alto número diploide e grande quantidade de microcromossomos, tais como os Piciformes (Kretschmer et al., 2021a) ou pelo contrário, um baixo número diploide e reduzido número de microcromossomos, como é possível observar nos Falconiformes (De Oliveira et al., 2013).

Com o desenvolvimento de técnicas e analises mais modernas, especialmente à luz dos avanços recentes na pesquisa genômica, as aves se tornaram um grupo de extrema importância sendo um grupo modelo ideal para estudos focados na compreensão da evolução do genoma (Kapusta e Suh, 2017). No entanto, uma parte essencial para o conhecimento da estrutura do genoma desse grupo, ainda é negligenciada devido a fatores limitantes como técnicas adequadas às peculiaridades do genoma e da organização cariotípica do grupo.

1.4 Origem e composição estrutural dos microcromossomos

Os microcromossomos das aves foram presumivelmente formados a partir de fissão cromossômica. Notavelmente, o alto número de cromossomos das aves deve-se, consequentemente, à presença de muitos microcromossomos. De acordo com Rodionov (1996), os microcromossomos têm, em média, a metade do tamanho dos macrocromossomos, no entanto, tais elementos podem apresentar tamanhos

extremamente reduzidos em comparação com a média proposta (Figura 3), sendo a maioria deles consideravelmente menor que 20 Mb (Burt, 2002; Hillier et al. 2004), uma característica genética que surgiu no ancestral das aves (Burt, 2002; Organ et al 2008; Kapusta e Suh, 2017). No entanto dados disponíveis apontam para a presença de determinados microcromossomos em táxons ainda mais antigos que o ancestral comum das aves, o que possivelmente influencia a presença desses elementos em outros grupos de vertebrados terrestres. (Takagi and Sasaki, 1974; Burt, 2002). Embora sejam muito menores em tamanho, a composição estrutural dos microcromossomos é semelhante aos macrocromossomos, estando presente neles os telômeros e centrômeros, induzindo esses elementos a se comportarem de forma semelhante aos macrocromossomos durante a mitose e a meiose (Burt, 2002).

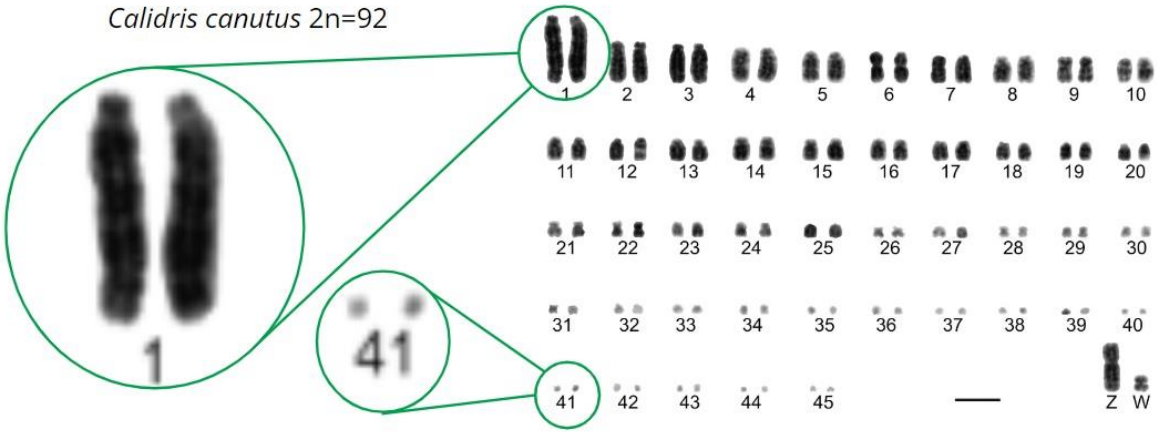

Figura 3: Comparação da divergência de tamanho entre macro e microcromossomos do cariótipo de *Calidris canutus*. Nos detalhes ampliados estão o par cromossômico 1 (macrocromossomos) e o par cromossômico 41 (microcromossomos). Barra de escala, 5 µm.

Os microcromossomos das aves têm taxas de recombinação meiótica mais altas que as apresentadas pelos macrocromossomos (em média, uma vez a cada 12 Mb contra uma vez a cada 30 Mb) (Rodionov et al. 1992; Organ et al 2008; Ellegren e Galtier, 2016). Isso é quase cinco vezes a taxa observada nos cromossomos de mamíferos e sugere que os microcromossomos são um fator fundamental na geração de variabilidade genética.

Em contraste com os macrocromossomos, os microcromossomos tendem a possuir alto teor de GC e CpG, íntrons pequenos, baixa densidade de TEs e reduzida quantidade e diversidade de elementos repetitivos (Burt, 2002; Ellegren, 2010; Organ

et al., 2011; Kapusta e Suh 2017). Essas propriedades genômicas sugerem que os microcromossomos das aves são menos complexos do que o restante do genoma, levando em consideração o seu conteúdo. No entanto, a seleção eficiente devido às altas taxas de recombinação sugestiona indagações sobre a evolução e a manutenção desses cromossomos pequenos e pobres em sequências repetitivas no genoma desse grupo (Ellegren e Galtier, 2016; Kapusta e Suh, 2017).

Os microcromossomos são 2 a 3 vezes mais densos em genes do que os macrocromossomos (Smith et al. 2000; Ellegren e Galtier, 2016), isso significa que possuem em comparação com cromossomos maiores, uma maior quantidade de genes em relação ao seu tamanho físico. A densidade gênica refere-se ao número de genes presentes em um comprimento específico de DNA, normalmente medido em genes por milhão de pares de bases (Mb). Basicamente, ela informa o quão compactado estão os genes em um genoma. A densidade de genes pode variar significativamente entre diferentes organismos e até mesmo dentro dos cromossomos do mesmo organismo. Os genomas menores geralmente têm densidades gênicas mais altas, pois precisam reunir mais informações funcionais em um espaço limitado, esse é o caso das aves, pois apresentam genomas reduzidos em tamanho (Hughes e Friedman, 2008).

A densidade gênica nas aves fornece informações importantes sobre os seguintes aspectos: i. Organização do genoma: ajudando na compreensão da eficiência organizacional gerada pela evolução no genoma de um organismo; ii. Pressões evolutivas: a alta densidade gênica pode sugerir uma forte pressão seletiva para agrupar mais genes em um genoma menor; iii. Diferenças funcionais: As variações na densidade gênica entre os cromossomos de um organismo podem indicar funções especializadas para regiões específicas, sendo um exemplo é a diferença entre a densidade gênica de macro e microcromossomos (Srinivas et al., 2020). Dessa forma essa importante característica nos microcromossomos desempenha papéis cruciais em organismos específicos, particularmente nas aves. Um exemplo interessante é a presença de genes ligados ao desenvolvimento e variação das penas em um par de microcromossomo (par 25), o que em uma visão mais ampla pode refletir diretamente na seleção sexual devido as características fenotípicas associadas a estes genes (Ng e Li, 2018).

1.5 Citogenética e citogenômica de aves

Com o avanço no desenvolvimento de técnicas modernas para o estudo genético, temos o surgimento da citogenômica que traz abordagens mais específicas para estudos detalhados, principalmente no que refere a organização genômica.

Uma das técnicas mais utilizada nas pesquisas de citogenética molecular é a pintura cromossômica com sondas de cromossomos inteiros (Whole Chromosome Probes – WCP). Essa abordagem utiliza a hibridização por FISH com sondas geradas a partir de macrocromossomos de algumas espécies de aves. Até o presente momento foram utilizadas sondas cromossômicas derivadas de seis diferentes espécies: *Gallus gallus* (Galliformes), *Leucopternis albicollis* (Accipitriformes), *Burhinus oedicnemus* (Charadriiformes), *Gyps fulvus* (Accipitriformes), *Zenaida auriculata* (Columbiformes), e *Myiopsitta monachus* (Psittaciformes) (Shetty et al., 1999; Nie et al., 2009; de Oliveira et al. 2010; Nie et al., 2015; Kretschmer et al., 2018a; Furo et al., 2020), porém a maioria dos experimentos de pintura cromossômica foram realizados utilizando sondas de *G. gallus* (GGA).

Os resultados das hibridizações com sondas de GGA em espécies Paleognatas, revelou a conservação da maioria dos macrocromossomos de *G. gallus*, com exceção do cromossomo 4, que hibridiza dois pares distintos em espécies Paleognatas (Griffin et al., 2007). Este mesmo padrão de hibridização com pontuais alterações, foi descrito para a maioria das espécies Neognatas (Griffin et al., 2007; Kretschmer et al., 2018b). Nesse sentido, pôde ser proposto um possível cariótipo ancestral das Aves (Putative Avian Ancestral Karyotype – PAK), que apresenta a conservação estrutural dos cromossomos de GGA1-3, 5-9, enquanto o cromossomo GGA4 representaria dois pares (4 e 10 ancestrais). A presença da fusão entre os cromossomos 4 e 10 ancestrais representa um caractere convergente na linhagem dos Galliformes e em poucas espécies de Neoaves (Griffin et al., 2007). Dessa forma, é possível identificar grupos sintênicos conservados em diferentes ordens de aves, bem como a reorganização genômica de alguns grupos como as Ordens Falconiformes, Psittaciformes e Charadriiformes (de Oliveira et al., 2013; Furo et al., 2015; Nie et al., 2015; Kretschmer et al., 2018b). Embora tenham sido alcançados avanços significativos com os dados de pintura cromossômica, esta apresenta algumas barreiras técnicas para estudos ainda mais específicos como a capacidade

limitada de detectar pequenos rearranjos cromossômicos ou de analisar a organização dos microcromossomos.

1.6 Utilização de sondas de BAC para o estudo de microcromossomos das aves

Os microcromossomos das aves, muito pequenos e difíceis de distinguir por sua morfologia, foram por muito tempo considerados apenas componentes da heterocromatina, no entanto, esses elementos são altamente preservados e estão presentes ao longo da evolução de várias linhagens, incluindo as aves, répteis e até mamíferos (Waters, 2021).

A alta densidade de genes e a taxa de recombinação desses elementos são características que sempre chamaram atenção no cariótipo das aves. Contudo, mesmo com o conhecimento da importância dos microcromossomos, poucos estudos foram realizados com eles e uma das causas dessa inconsistência de dados era justamente a falta de técnicas adequadas para as análises. Nesse sentido, sondas de BACs (em tradução livre: cromossomos artificiais bacterianos), foram desenvolvidas com o propósito de preencher essa lacuna de conhecimento.

As sondas BACs de microcromossomos de aves são sequências selecionadas e clonadas nos cromossomos artificiais bacterianos sendo isoladas, amplificadas e marcadas diretamente por "*nick translation*". Para facilitar a visualização dos resultados dos experimentos é possível observar marcações fluorescentes nas cores vermelha (Texas red-12-dUTP) ou verde (FITC-fluoresceína-12-UTP). A hibridização ocorre em metáfases de acordo com as técnicas de FISH descritas por Damas e colaboradores (2017). É importante ressaltar que existem duas bibliotecas de sondas de BAC para microcromossomos de aves, a primeira produzida a partir do genoma de *G. gallus* (CH261), e a segunda produzida a partir do genoma de *Taeniopygia guttata* (TGMCBA). Ambas bibliotecas possuem sequências referentes aos microcromossomos de GGA10-28, exceto GGA16 (Damas et al., 2017).

Independentemente do fato de utilizar sondas de BAC de bibliotecas de *G. gallus* ou *T. guttata*, os resultados sempre devem ser comparados com GGA, uma vez que essa espécie representa o estado do cariótipo ancestral. Geralmente a maioria das sondas de BAC tem origem na biblioteca de *G. gallus* porém, para alguns microcromossomos, os BACs da biblioteca de *G. gallus* não hibridizam com muito

sucesso em todas as espécies de aves, nesses casos, são usadas as sondas de BAC da biblioteca de *T. guttata*.

Para facilitar a identificação de possíveis rearranjos cromossômicos envolvendo microcromossomos, considera-se o seguinte: (i) nenhum rearranjo se ambas as sondas de BAC para cada microcromossomo produzissem sinais de FISH no mesmo microcromossomo e com um tamanho de microcromossomo; (ii) é considerado um evento de fissão quando ambas as sondas de BAC para cada microcromossomo produzirem sinais de FISH em microcromossomos diferentes; e (iii) considera-se um evento de fusão quando as sondas destinadas a um microcromossomo hibridizam em um macrocromossomo (de Souza et al., 2022).

Assim, o surgimento dos BACs pode ser considerado o principal avanço nos estudos dos microcromossomos nos últimos tempos, e possibilita a identificação de rearranjos intercromossômicos e intracromossômicos envolvendo microcromossomos (Lithgow et al., 2014). Esses estudos ainda são escassos, porém, sugerem a estabilidade evolutiva na organização dos microcromossomos das aves, exceto em algumas espécies das ordens Falconiformes, Psittaciformes, Cuculiformes, Suliformes e Caprimulgiformes, nas quais foram encontradas fusões envolvendo microcromossomos (O'Connor et al., 2024).

2 JUSTIFICATIVA

2.1 Escolha das Ordens

A organização cariotípica dos microcromossomos em aves continua sendo um campo de pesquisa intrigante, com importantes implicações para a compreensão da diversidade cromossômica e história evolutiva da classe Aves. A escolha dos modelos de estudo é crucial para obter avanços significativos nesse campo. As ordens Passeriformes e Charadriiformes representam opções excelentes por motivos filogenéticos e cariotípicos.

Passeriformes, um grupo filogenético com pouca variação cromossômica e imensa diversidade de espécies (mais de 6.000 espécies), representa a maior ordem de aves e são caracterizados por um cariótipo relativamente conservado. A maioria das espécies descritas pela citogenética possui 2n = ~80. Essa estabilidade torna os

Passeriformes um modelo ideal para estudos comparativos da organização cariotípica dos microcromossomos, sendo que em algumas espécies já se conhece a organização cariotípica dos macrocromossomos.

Charadriiformes com mais de 380 espécies, em contraste com os Passeriformes, apresenta maior variabilidade no número cromossômico, variando entre 2n = 42 a 98. Essa diversidade, juntamente com sua posição filogenética mais basal entre as aves modernas, torna os Charadriiformes um bom modelo para a comparação e possível elucidação das dinâmicas evolutivas dentro da organização dos microcromossomos das aves.

Ao comparar a organização cariotípica de Passeriformes e Charadriiformes, podemos ter perspectivas sobre os padrões de homologia entre microcromossomos e as relações entre diferentes clados. Esclarecendo quais possíveis mecanismos de reestruturação cromossômica, como inversões, fusões e fissões, contribuíram para a diversidade cromossômica desses grupos, bem como compreender as possíveis relação entre a organização cariotípica e a adaptação evolutiva de diferentes linhagens de aves. Dessa forma, a escolha de Passeriformes e Charadriiformes como modelos de estudo para a organização cariotípica e evolutiva dos microcromossomos nas aves é fundamentada em suas características filogenéticas e cariotípicas distintas, oferecendo uma oportunidade para desvendar os possíveis mecanismos evolutivos que moldaram a diversidade cromossômica nesses grupos e consequentemente contribuir para a compreensão da história evolutiva da classe Aves.

2.2 Definição das espécies a serem coletadas

O estudo citogenético em aves ainda hoje é um desafio do ponto de vista técnico. Para obter as metáfases, principal material de estudos cromossômicos, é necessário coletar tecidos vivo, dessa forma são exigidas várias condições específicas que acabam limitando muito a possível variedade de espécies. Por se tratar do grupo de animais vertebrados mais rico em espécies, para que a amostragem seja representativa, precisamos necessariamente coletar animais em seus ambientes de vida, pois muitos deles não permitem a criação em zoológicos ou criatórios devido principalmente suas especificidades alimentar e reprodutiva, ou seja, são obrigatoriamente animais de vida livre. Dessa forma, os animais foram coletados na natureza.

Foi estipulado que deveriam ser utilizados cerca de 3 a 4 espécies para cada ordem, observando um mínimo de 1 a 2 indivíduos de cada espécie para que o impacto ambiental fosse o menor possível. Todos os procedimentos de coleta e manejo dos animais foram realizados de acordo com as licenças e protocolos exigidos para pesquisas utilizando animais vivos.

Considerando o exposto, não tivemos a possibilidade de estipular de antemão quais espécies seriam utilizadas, apenas alguns pressupostos como: cada uma das espécies a serem coletadas deveriam ser de diferentes famílias ou clados e preferencialmente fêmeas (sexo heterogamético). No decurso das coletas a campo foi possível atingir os objetivos coletando as seguintes espécies: Passeriformes - *Myiodynastes maculatus*, *Molothrus bonariensis*, *Troglodytes aedon*, e *Sporophila caerulescens*. Charadriiformes: *Calidris canutus*, *Jacana jacana*, e *Vanellus chilensis*.

É importante destacar que todos os procedimentos experimentais, material e métodos utilizados serão descritos nos capítulos 1 e 2 deste documento.

3 OBJETIVOS

3.1 Objetivo geral

Contribuir para a compreensão da evolução cariotípica na Classe Aves com o foco na organização dos microcromossomos de espécies das Ordens Passeriformes e Charadriiformes.

3.2 Objetivos específicos

- Identificar a ocorrência de rearranjos envolvendo microcromossomos através de hibridização *in situ* fluorescente com sondas de BAC de *G. gallus* e *T. guttata* em quatro espécies de Passeriformes: *Myiodynastes maculatus*, *Molothrus bonariensis*, *Troglodytes aedon*, e *Sporophila caerulescens* (Figura 1: a, b, c, d, respectivamente).

- Identificar a ocorrência de rearranjos envolvendo microcromossomos através de hibridização *in situ* fluorescente com sondas de BAC de *G. gallus* e *T. guttata* em três espécies de Charadriiformes: *Calidris canutus*, *Jacana jacana*, e *Vanellus chilensis* (Figura 1: e, f, g, respectivamente).

- Identificar e confirmar informações descritas na literatura sobre número diploide (2n) e morfologia cromossômica de cada espécie a ser estudada.

- Avaliar o status evolutivo da organização dos microcromossomos nas espécies elencadas para o estudo.

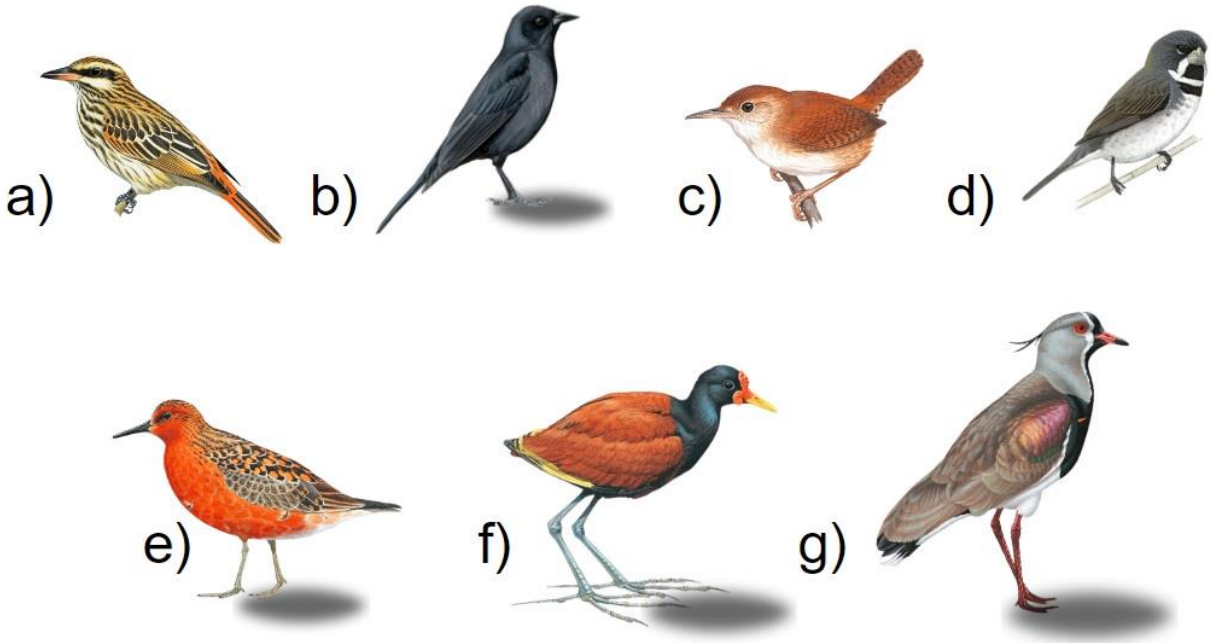

Figura 1: Ilustração das espécies utilizadas nesse estudo. Passeriformes a) *Myiodynastes maculatus* (Tyrannidae), b) *Molothrus bonariensis* (Icteridae), c) *Troglodytes aedon* (Troglodytidae), e d) *Sporophila caerulescens* (Thraupidae). Charadriiformes: e) *Calidris canutus* (Scolopacidae), f) *Jacana jacana* (Jacanidae), e g) *Vanellus chilensis* (Charadriidae). Imagem adaptada de https://www.birds.cornell.edu/

CAPÍTULO I

ORGANIZAÇÃO ALTAMENTE CONSERVADA DOS MICROCROMOSSOMOS DE AVES PASSERIFORMES REVELADA POR MEIO DA ANÁLISE BAC-FISH

Marcelo Santos de Souza, Suziane Alves Barcellos, Victoria Tura, Vera Lúcia Bobrowski, Analía Del Valle Garnero, Ricardo José Gunski, Darren K. Griffin e Rafael Kretschmer

RESUMO

As aves Passeriformes são amplamente reconhecidas por sua notável diversidade, com mais de 5.700 espécies descritas até o momento. Como a maioria das espécies de aves, elas possuem um cariótipo característico das aves modernas, que inclui um cariótipo bimodal composto por alguns pares de macrocromossomos e muitos pares de microcromossomos. Embora o cariótipo seja tipicamente 2n = 80, o número diploide pode variar muito atipicamente, indo de 56 a aproximadamente 100 cromossomos. Neste estudo, nosso objetivo foi compreender a extensão da conservação da estrutura organizacional do cariótipo em quatro espécies desse grupo usando cromossomos artificiais bacterianos via hibridização fluorescente *in situ* (BAC-FISH) com sondas de microcromossomos de *Gallus gallus* ou *Taeniopygia guttat*a (homólogos a GGA10-28, exceto GGA16). Examinando o complemento cromossômico de quatro espécies de passeriformes - *Myiodynastes maculatus*, *Molothrus bonariensis*, *Troglodytes aedon* e *Sporophila caerulescens*. Neste estudo, descrevemos um novo número cromossômico para *Troglodytes aedon*. Por meio de experimentos de FISH, pudemos observar o mesmo padrão de organização de microcromossomos do ancestral comum das aves. Como resultado, propomos um novo número diploide para o *Troglodytes aedon* e confirmamos o status de conservação da organização dos microcromossomos desse grupo, o que pode ter conferido vantagens evolutivas a esse clado.

Palavras-chave: Aves; número diploide; organização do cariotípica; citogenética molecular

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HIGHLY CONSERVED MICROCHROMOSOMAL ORGANIZATION IN PASSERIFORMES BIRDS REVEALED VIA BAC-FISH ANALYSIS

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Simple Summary: The Passeriformes order (songbirds) is incredibly diverse in terms of number of species and morphological and ecological diversification, comprising around 60% of all bird species. Despite considerable diversity, the genome organizational structure (i.e., the number and pattern of chromosomes) within Passeriformes is highly conserved, with a chromosome number that remains close to 80 in nearly all species studied. These characteristics raise interesting questions and stimulate curiosity about the genome evolution of this group. Therefore, this study aimed to analyze the organization of the smallest chromosomes (microchromosomes) in four Passeriformes species to understand whether they were rearranged during evolution. This has only recently become possible using fluorescent probes called bacterial artificial chromosomes (BACs) and a technique called fluorescence in situ hybridization (FISH). Our results confirm that the songbirds studied did not rearrange their microchromosomes to any great extent, and this may have contributed to their overall evolutionary success.

Abstract: Passeriformes birds are widely recognized for their remarkable diversity, with over 5700 species described so far. Like most bird species, they possess a karyotype characteristic of modern birds, which includes a bimodal karyotype consisting of a few pairs of macrochromosomes and many pairs of microchromosomes. Although the karyotype is typically 2n = 80, the diploid number can atypically vary greatly, ranging from 56 to approximately 100 chromosomes. In this study, we aimed to understand the extent of conservation of the karyotype's organizational structure within four species of this group using Bacterial Artificial Chromosomes via Fluorescence in Situ Hybridization (BAC-FISH) with microchromosome probes from Chicken (*Gallus gallus*) or Zebra Finch (*Taeniopygia guttata*) per microchromosomes (GGA10-28, except GGA16). By examining the chromosome complement of four passerine species—the Streaked Flycatcher (*Myiodynastes maculatus*), Shiny Cowbird (*Molothrus bonariensis*), Southern House Wren (*Troglodytes aedon*), and Double-collared Seedeater (*Sporophila caerulescens*)—we discovered a new chromosome number for Southern House Wren. Through FISH experiments, we were able to observe the same pattern of microchromosome organization as in the common ancestor of birds. As a result, we propose a new diploid number for Southern House Wren and confirm the conservation status of microchromosome organization, which may confer evolutionary advantages to this group.

Keywords: Aves; diploid number; karyotype organization; molecular cytogenetic

1. Introduction

Passeriformes, also known as songbirds, passerine, or perching birds, are the largest Neornithes (modern bird) order among birds and are renowned for their remarkable phenotypic diversity. This clade comprises two groups, Suboscines (Tyranni; Old World and New World Lineages) and Passeri (Oscine; Songbird), and accounts for approximately 60% of all existing bird species, with an estimated 5700 living species [1,2]. The Passeriformes karyotype shares the same classical pattern as most birds, with a diploid number (2n) ranging around 78–80 chromosomes. However, the bimodal karyotypic organization makes it difficult to characterize the several pairs of microchromosomes using classical cytogenetic approaches [3,4]. Despite this

challenge, the variation in diploid number within the group ranges from 2n = 56 for Red-winged Pytilia (*Pytilia phoenicoptera*) [5] to 2n = 96–100 for the Amethyst Sunbird (*Chalcomitra amethystina*) [6–9]. As in all birds, female Passeriformes are heterogametic, possessing a pair of distinct sex chromosomes (ZW), while males are of the homogametic sex (ZZ). The Z chromosome is typically conserved in size, usually located between the third and fourth pairs, but its morphology can be variable.

The bimodal karyotype, which comprises macrochromosomes and microchromosomes, is a typical characteristic of birds. This karyotype was established mostly before the divergence of birds and turtles and has been present in its current form in the lineage of Theropod dinosaurs for 240–250 million years; some of the microchromosomes however originated in the karyotype of ancestral vertebrates around 400 million years ago [3,10]. The reconstructed genome organization of the vertebrate ancestor demonstrated that bird microchromosomes correspond directly to the protochromosomes of the ancestors of gnathostomata [11], suggesting that they remained considerably stable throughout avian evolution. Comparative chromosome painting experiments using Chicken (*Gallus gallus*, GGA) probes in fluorescence in situ hybridization (FISH) experiments allowed for the identification of homologous synthetic blocks (HSBs) conserved in bird karyotypes, indicating high conservation and low rates of interchromosomal rearrangements compared to the Putative Ancestral Karyotype (PAK) of birds, even when very distant species are compared phylogenetically [12]. Regarding the PAK of birds, Passeriformes exhibit a fission of the first ancestral chromosomal pair (GGA1) [13–21]. Although the use of GGA probes has proven to be efficient in detecting interchromosomal rearrangements, it is limited in most cases to the macrochromosomes.

While interchromosomal rearrangements involving microchromosomes are relatively uncommon in birds, certain orders, such as Psittaciformes, Falconiformes, and Cuculiformes, have been found to exhibit this type of rearrangement more frequently than others [3,22]. However, despite detailed analysis of multiple bird orders, no interchromosomal rearrangements involving microchromosomes have been detected and shared among the analyzed orders, not even among closely related species [22,23]. These findings suggest that convergent evolution involving microchromosome rearrangements is an exceedingly rare occurrence in the class Aves.

The goal of this study was to review the karyotype and diploid number of four Passeriformes bird species. Additionally, the study aimed to analyze the organizational structure of microchromosomes from these species through BAC-FISH experiments. The study also examined how these characteristics impact the evolution of chromosomes in this group. By comparing the microchromosomes of different species, this study has shed light on the chromosome evolution of Passeriformes birds.

2. Materials and Methods

2.1. Species, Chromosome Preparation and Karyotype Description

According to SISBIO 61047-4 ICMBio, animals were collected in their natural environment (Table 1) and the samples were obtained with the approval of the Universidade Federal do Pampa's ethics committee (CEUA 019/2020). The sex was determined via cytogenetics. Skin biopsies or feather pulp samples were taken from each individual to establish fibroblast cell cultures and to obtain chromosome preparations. Cells were cultured in flasks (25 cm2) filled with Dulbecco's Modified Eagle's Medium (DMEM-GIBCO, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, GIBCO/Thermo Fisher Scientific, Burlington, MA, USA) and 1% penicillin (10,000 units/mL)/streptomycin (10,000 µg/mL) (GIBCO/Thermo Fisher Scientific, Burlington, MA, USA) and incubated at 37 ◦C [24]. When the cells formed a sub confluent monolayer, the medium was removed, followed by two washes with 1xPBS (Sigma-Aldrich, St. Louis, MO, USA), then 1 mL of trypsin 0.25% EDTA (Sigma-Aldrich, St. Louis, MO, USA) was added, and finally incubation at 37 ◦C for 1 min. Once the cells were released from the flask, a cell culture medium with FBS was added to stop the effect of trypsin. Metaphase chromosomes were obtained according to standard procedures involving colchicine exposure (1 h, 37 ◦C), hypotonic treatment (0.075 M KCl, 15 min, 37 ◦C), and methanol/acetic acid (3:1) fixation.

Table 1. Passeriformes species description list. The column N refers to the specimen quantities sampled and the specimen sex. RS = Rio Grande do Sul State.

Common Name	Scientific Name	Family	Suborder	N and Sex	Locality in Brazil
Streaked Flycatcher	*Myiodynastes maculatus*	Tyrannidae	Tyranni	2 ♀	Porto Vera Cruz-RS
Shiny Cowbird	*Molothrus bonariensis*	Icteridae	Passeri	1 ♂ and 1 ♀	São Gabriel-RS
Southern House Wren	*Troglodytes aedon*	Troglodytidae	Passeri	1 ♂ and 2 ♀	São Gabriel-RS
Double-collared Seedeater	*Sporophila caerulescens*	Thraupidae	Passeri	2 ♂	São Gabriel-RS

A direct chromosome preparation method was also used for Double-collared Seedeater and Southern House Wren, in which embryonic cells were dissociated with 2 mL of trypsin 0.25% EDTA (Sigma-Aldrich, St. Louis, MO, USA) for approximately 10 min; samples were soon after placed in 10 mL of RPMI 1640 (GIBCO/Thermo Fisher Scientific, Burlington, MA, USA), pre-warmed to 37 ◦C, and then 3 drops of 0.05% colchicine were added, followed by incubation for 1 h at 37 ◦C before hypotonic treatment for 20 min and fixation with methanol/acetic acid (3:1) [25].

After harvesting chromosomes, the cell suspension was dropped onto clean glass slides, air-dried, and stained with 5% Giemsa (Sigma-Aldrich, St. Louis, MO, USA) in a pH 6.8 phosphate-buffered saline. To determine diploid number and chromosome morphology, we analyzed at least 30 metaphases. Chromosomal morphology and karyotype organization were determined according to Guerra [26].

2.2. Bacterial Artificial Chromosomes (BACs) FISH Experiments

In this study we used Chicken and Zebra Finch probes (Supplementary Materials Table S1) because they are model species for several biological studies, including cytogenetics [12,27]. Isolation, amplification, labeling, and hybridization of clonal BACs were performed following the protocol described by O'Connor et al. [28]. Two BAC probes from the Chicken (CH261) or Zebra Finch (TGMCBA) genomic library per microchromosomes (GGA10-28 except for GGA16) were applied for FISH cross-mapping. The BACs were positioned as close as possible to each end (short and long arms) of each microchromosome tested. The majority of BAC probes utilized in this study were derived from the Chicken. However, the Chicken BACs were not consistently effective across all bird species for certain chromosomes [29]. In such instances, BAC probes sourced from the Zebra Finch were employed. We did not examine the GGA16 or the 29–38 chromosomes because there are no BAC probes for these chromosomes. The results of FISH experiments were confirmed by analyzing

at least 10 metaphases per slide. Adobe Photoshop 7.0 software was used for final image processing.

To detect potential chromosomal rearrangements, we utilized the following criteria from de Souza et al. [30]: (i) if both BAC probes per microchromosome produce FISH signals on the same microchromosome with a size consistent with that of a microchromosome, then no rearrangement has occurred and the state is considered to be conservative; (ii) if both BAC probes generate positive FISH signals on different microchromosomes, this indicates a fission event; and (iii) if a probe designed for a microchromosome hybridizes to a macrochromosome, this indicates a fusion event.

3. Results

3.1. The Karyotype Description

The karyotype of the Streaked Flycatcher has 80 chromosomes: 9 pairs of macrochromosomes and 31 pairs of microchromosomes (Figure 1a). All remaining autosomes are telocentric or punctiform with unidentifiable morphology, except for the second, fifth, and sixth pairs, which have acrocentric morphology. The Z chromosome has metacentric morphology, and the W is telocentric, like the majority of autosomes. The species Shiny Cowbird has 2n = 80 with 9 pairs of macrochromosomes and 31 pairs of microchromosomes (Figure 1b). The first pair is submetacentric; from the second to eighth pairs, the morphology is acrocentric; and from the ninth pair onward, the chromosomes present telocentric or punctiform morphology. The Z and W sex chromosomes are also telocentric. The Southern House Wren karyotype has 76 chromosomes: 9 pairs of macrochromosomes and 29 pairs of microchromosomes (Figure 1c). The first and fifth pairs are submetacentric; the second, third, fourth, and sixth are acrocentric, and the remaining autosomes are telocentric or punctiform. The Z chromosome has submetacentric morphology, and the W chromosome has metacentric morphology. The karyotype of Double-collared Seedeater has 78 chromosomes: 9 pairs of macrochromosomes and 30 pairs of microchromosomes (Figure 1d). The first pair is submetacentric, the second and third pairs are acrocentric,

and from the fourth pair on, macro- and microchromosomes are telocentric or have punctiform morphology. The Z chromosome from this species is metacentric.

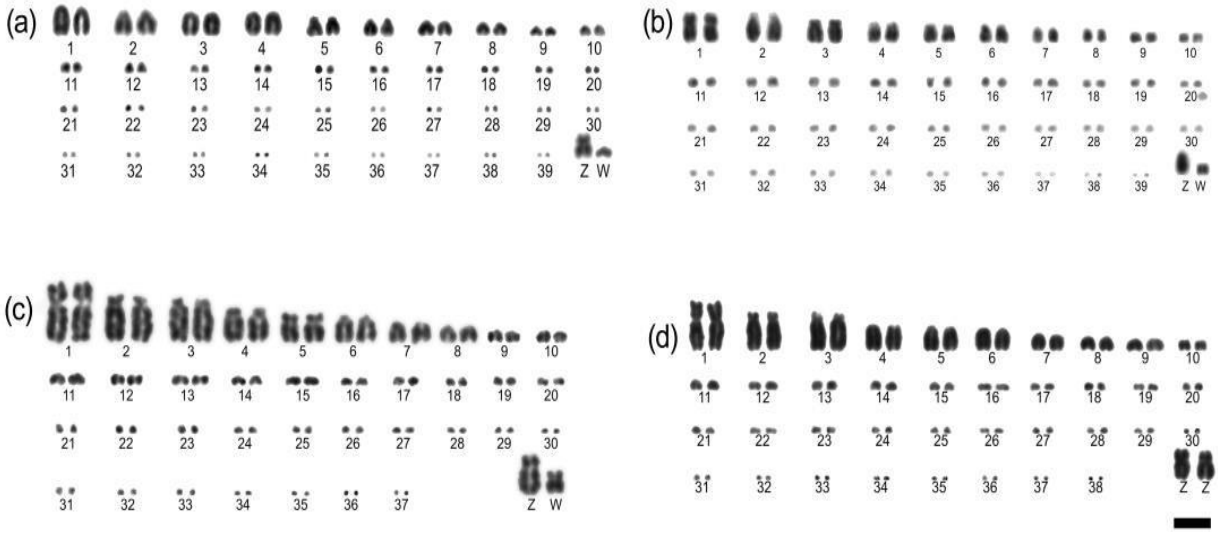

Figure 1. Chromosomic complement organized into complete karyotypes: (**a**) Streaked Flycatcher (2n = 80), (**b**) Shiny Cowbird (2n = 80), (**c**) Southern House Wren (2n = 76), and (**d**) Double-collared Seedeater (2n = 78). Scale bar, 5 µm.

3.2. Bacterial Artificial Chromosomes Fluorescence in Situ Hybridization (BAC-FISH) Experiments

For each microchromosome tested (GGA 10–28, except 16), no hybridization signals were found on different microchromosomes, which would indicate fission-type rearrangements (Figure 2a–d). Additionally, no positive hybridization signals were detected on macrochromosomes, which would indicate fusion-type rearrangements (Figure 2a–d). Therefore, our BAC-FISH analysis revealed that the microchromosome organization pat- tern in the species studied is highly conserved, with no evidence of interchromosomal rearrangements involving the microchromosomes tested. While the diploid numbers of the Double-collared Seedeater and Southern House Wren were found to be lower than that of the common ancestor, no fusions involving microchromosomes were observed.

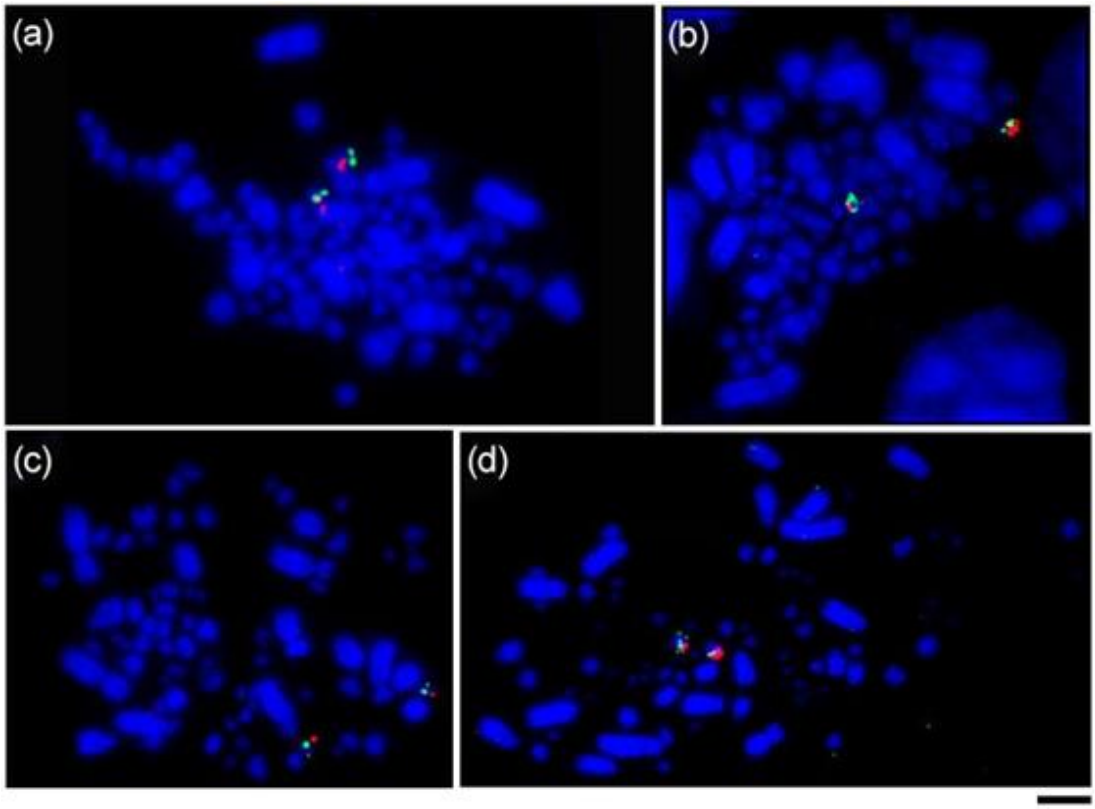

Figure 2. Examples of FISH experiments using Chicken (CH261) or Zebra Finch (TGMCBA) bacterial artificial chromosome (BAC) probes in Passeriformes. FISH results for the Streaked Flycatcher: (**a**) chromosome 13 TGMCBA-266G23 (red) and CH261-115I12 (green). FISH results for the Shiny Cowbird: (**b**) chromosome 20 TGMCBA-250E3 (red) and TGMCBA-375I5 (green). FISH results for the Southern House Wren: (**c**) chromosome 26 CH261-186M13 (red) and CH261-170L23 (green). FISH results for the Double-collared Seedeater: (**d**) chromosome 24 CH261-103F4 (red) and CH261-65O4 (green). Scale bar, 5 µm.

4. Discussion

The results presented in this study reveal a remarkable level of conserved microchromosomal organization across four species of Passeriformes birds. Our findings support previous studies [4,31,32] regarding the karyotype descriptions of the Streaked Flycatcher, Shiny Cowbird, and Double-collared Seedeater. However, we discovered a new diploid number for the Southern House Wren; while de Lucca and Waldrigues [33] first described its diploid number as 2n = 68, our results show it as 2n = 76. It is noteworthy that our findings indicate that the examined species possess typical bird diploid numbers, considering that most birds (around 61%) have diploid numbers between 76 and 82 [34].

The diploid number (2n) is a fundamental piece of information in the fields of genetics and cytogenetics, providing insight into the genome organization of all eukaryotic organisms. Nevertheless, describing this information in the case of birds poses distinct challenges due to their unique characteristics [35]. Birds typically have a high 2n count and a large number of microchromosomes, many of which have indistinguishable morphology (appearing as small dots (punctiform) under a microscope). As a result, accurate determination of bird karyotypes requires the analysis of many metaphase cells with high-quality preparation. Fortunately, advancements in microscopy and imaging technology have im- proved visualization, allowing for more accurate identification of the diploid number in bird species that have already been karyotyped. For instance, the karyotype of the Southern House Wren has been reviewed here, leading to a proposal of a new diploid number.

Our molecular cytogenetic characterization, which utilized BAC FISH microchromosomes probes from Chicken and Zebra Finch, demonstrated that all the microchromosomes tested in four Passeriformes species are conserved as complete units. This finding reinforces previous research indicating a high degree of conservation of microchromosomes in Passeriformes as well as in most avian species [23,28,36,37]. According to Burt [10], the distinct genomic characteristics exhibited by microchromosomes, including elevated GC content; reduced repeats; and increased gene density play a significant role in preserving these chromosomes as whole units in avian karyotypes. Among the Passeriformes, an exception to this pattern is observed in the Yellow-olive Flycatcher (*Tolmomyias sulphurescens*, 2n = 60), which underwent significant karyotypic reorganization involving both the macrochromosomes and microchromosomes [37]. However, we cannot entirely rule out the possibility of microchromosome fusions occurring in the species investigated, as we were not able to analyze microchromosomes 16 and 29–38 due to a lack of probes for these chromosomes. Considering that the putative ancestral karyotype (PAK) of birds is characterized by a 2n of 80, it seems plausible that fusion events have been responsible for the decrease in diploid numbers observed in the Southern House Wren (2n = 76) and Double-collared Seedeater (2n = 78). Specifically, it is possible that two fusions were involved in the reduction in diploid numbers in the Southern House Wren, while one fusion event was involved in the Double-collared Seedeater. Recently, Chicken probes for chromosomes 16 and 29–38 have been published [38,39], and

future studies will provide additional insight about the evolution of these chromosomes in birds.

Previous studies on Passeriformes demonstrated a high degree of conservation in their macrochromosomes [28,36,37]. This is illustrated in Figure 3, which highlights that fusion events have only been observed in a limited number of species. However, research conducted through in situ [18,19,40,41] and in silico [42] studies revealed that intrachromosomal rearrangements occur frequently in Passeriformes. Hence, we propose that the karyotypes of Passeriformes have evolved primarily through intrachromosomal rearrangements, while macro and microchromosomes remain highly conserved. Thus, the absence of interchromosomal rearrangements observed in the majority of the analyzed Passeriformes species may be linked to the evolutionary success of this group, which represents one of the most diverse and highly derived clades within Aves [36,43].

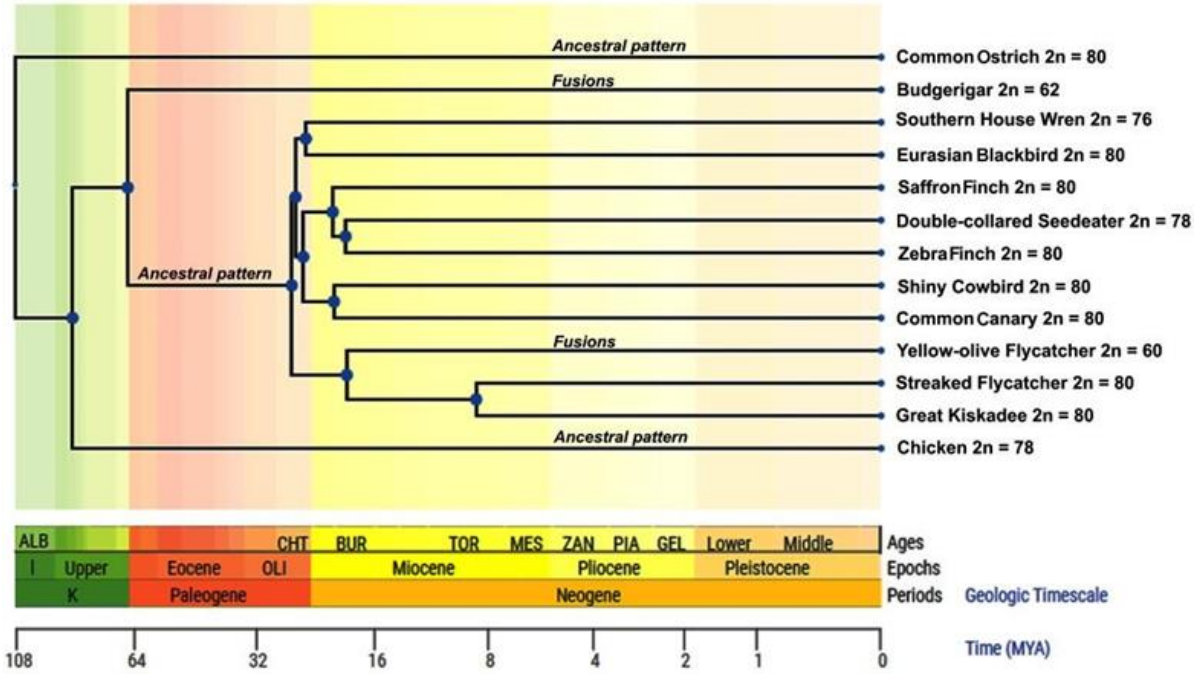

Figure 3. Chromosomal rearrangements in Passeriformes and the outgroups Struthioniformes (Common Ostrich, *Struthio camelus*), Galliformes (Chicken, *Gallus gallus*), and Psittaciformes (Budgerigar, *Melopsittacus undulatus*) were analyzed with BACs clones corresponding to the ancestral microchromosomes 11–28, except 16. The diploid numbers were sourced from avian chromosome databases [34]. The phylogenetic tree was sourced from TimeTree databases (http://www.timetree.org, accessed on 5 March 2023) [44].

4. Conclusions

In our study, we reviewed the diploid number and analyzed the microchromosome organization in four Passeriformes bird species. Our findings confirm previous studies, but we also discovered a new diploid number for the Southern House Wren (2n = 76), emphasizing the importance of analyze a large number of high-quality chromosome preparation to accurately determine diploid number in birds. Our BAC-FISH experiments revealed that all tested microchromosomes are conserved as whole units in the analyzed species, supporting the literature's findings on the high degree of conservation of these structures in Passeriformes. However, we cannot entirely exclude the possibility of microchromosome fusions, and comparative analysis with avian PAK suggests that fusions may have reduced the diploid numbers of the Southern House Wren and Double-collared Seedeater. Passeriformes karyotypes have mainly evolved through intrachromosomal rearrangements, which appear to be much more frequent than interchromosomal rearrangements, maintaining the organizational structure of microchromosomes and the 2n highly conserved in these species. The absence of observable interchromosomal rearrangements may have contributed to the evolutionary success of this feature in Passeriformes, contributing to making them one of the most diverse tetrapod groups on the planet in terms of number of species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/birds4020020/s1, Table S1: List of BACs applied to Streaked Flycatcher, Shiny Cowbird, Southern House Wren, and Double-collared Seedeater.

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Institutional Review Board Statement: The study was approved by the Institutional Ethics Committee of Universidade Federal do Pampa-CEUA 019/2020 and SISBIO 61047-4–ICMBio, for studies involving animals.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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CAPÍTULO II

BAC-FISH DE MICROCROMOSSOMOS REVELA DIFERENTES PADRÕES DE ORGANIZAÇÃO GENÔMICA EM TRÊS ESPÉCIES DE CHARADRIIFORMES

Marcelo Santos de Souza, Suziane Alves Barcellos, Michelly da Silva dos Santos, Ricardo José Gunski, Analía del Valle Garnero, Edivaldo Herculano Corrêa de Oliveira, Rebecca E. O'Connor, Darren K. Griffin e Rafael Kretschmer

RESUMO

Os microcromossomos, antes considerados elementos sem importância do genoma, representam blocos de construção fundamentais do cariótipo das aves. As aves limícolas (Charadriiformes) compreendem uma ampla variedade de aproximadamente 390 espécies e são consideradas um valioso grupo modelo para estudos biológicos. Apesar dessa variedade, a análise citogenética ainda é muito escassa nessa ordem, e assim, o objetivo deste estudo foi fornecer informações sobre o cariótipo de Charadriiformes, com ênfase na evolução dos microcromossomos em três espécies de aves limícolas - *Calidris canutus*, *Jacana jacana* e *Vanellus chilensis* - combinando abordagens clássicas e moleculares. O mapeamento através de FISH utilizou duas sondas BAC para cada microcromossomo, GGA10-28 (exceto GGA16). Os experimentos revelaram diferentes padrões de organização dos microcromossomos nas espécies investigadas. Assim, enquanto em *C. canutus* encontramos dois microcromossomos envolvidos em fusões cromossômicas, eles estavam presentes como pares únicos em *V. chilensis*. Também foi descrito um novo número cromossômico para o *C. canutus* (2n = 92). Portanto, este estudo contribuiu para a compreensão da organização e evolução do genoma de três espécies de aves limícolas.

Palavras-chave: microcromossomo; cariótipo; ave; genômica comparativa; citogenética molecular

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MICROCHROMOSOME BAC-FISH REVEALS DIFFERENT PATTERNS OF GENOME ORGANIZATION IN THREE CHARADRIIFORMES SPECIES

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Simple Summary: Numerous tiny (micro)chromosomes are a characteristic feature associated with birds, being found in smaller numbers in other organisms and absent in many, such as mammals. Al- though microchromosomes constitute a large portion of the genome in birds, data on them pertaining to comparative studies between birds are still scarce. This is the case in shorebirds (Charadriiformes), a group with a great variety of species. The aim of this study was to provide insight regarding the evolution of the microchromosomes of three species of shorebirds—the red knot (*Calidris canutus*), the wattled jacana (*Jacana jacana*), and the southern lapwing (*Vanellus chilensis*). The experiments are referred to as cross-species fluorescence in situ hybridization (FISH) mapping using probes called bacterial artificial chromosomes (or BACs), two (one labelled in red and one labelled in green) for every microchromosome. The results thus appear as the microchromosome with one green and one red end, revealing different patterns of organization over evolutionary time. In the red knot, they fuse together, but in the southern lapwing, they hardly change. We also described a new chromosome number for the red knot (92 in total). In conclusion, this study contributed to the understanding of microchromosomes organization and evolution of three shorebird species.

Abstract: Microchromosomes, once considered unimportant elements of the genome, represent fundamental building blocks of bird karyotypes. Shorebirds (Charadriiformes) comprise a wide variety of approximately 390 species and are considered a valuable model group for biological studies. Despite this variety, cytogenetic analysis is still very scarce in this bird order. Thus, the aim of this study was to provide insight into the Charadriiformes karyotype, with emphasis on microchromosome evolution in three species of shorebirds—*Calidris canutus*, *Jacana jacana*, and *Vanellus chilensis*—combining classical and molecular approaches. Cross-species FISH mapping applied two BAC probes for each microchromosome, GGA10–28 (except GGA16). The experiments revealed different patterns of microchromosome organization in the species investigated. Hence, while in *C*. *canutus,* we found two microchromosomes involved in chromosome fusions, they were present as single pairs in *V*. *chilensis*. We also described a new chromosome number for *C*. *canutus* (2n = 92). Hence, this study contributed to the understanding of genome organization and evolution of three shorebird species.

Keywords: microchromosome; avian karyotype; bird; BAC; FISH; comparative genomics; molecular cytogenetics

1. Introduction

The order Charadriiformes, commonly known as shorebirds, comprises approximately 390 species, divided into 13 families [1]. According to Baker et al. [2], the morphological analysis of the fossils with molecular studies suggests that this group originated during the Cretaceous period between 79 and 102 million years ago. Phylogenetic studies support three major clades: Lari (gulls, auks, and allies plus buttonquails), Scolopaci (sandpipers, jacanas, and allies), and Charadrii (plovers, oystercatchers, and allies) [2]. This order is a monophyletic clade where the genus *Vanellus* from clade Charadrii is more basal than the genera *Tringa* and *Jacana* from clade Scolopaci [3]. Considering the great diversity in the number of species, shorebirds are an excellent model group to investigate several biological questions, such as morphology, ecological diversification, and phylogenetic relationships [4].

Regarding genome organization, the majority of reports on shorebirds are based on classical cytogenetics, limited in most cases to conventional staining with Giemsa (reviewed in Degrandi et al. [5]). However, these studies revealed that shorebirds have an exceptional range of diploid numbers, ranging from 2n = 42 in *Burhinus oedicnemus* [6] to 2n = 98 in *Gallinago gallinago* [7], indicating that interchromosomal rearrangements played important role in the evolutionary history of this group. Hence, considering that the typical avian karyotype has approximately 2n = 80 chromosomes [8], shorebirds represent an excellent model for studying chromosome evolution.

The first detailed studies focused on chromosome organization among shorebirds came from chromosome painting data using different sets of paints: *Gallus gallus* [6,9], *B. oedicnemus* [6,10–12], *Leucopternis albicollis* [9], and *Zenaida auriculata* [13]. These studies revealed extensive chromosome reorganization in some species, while others retained a conserved karyotype, similar to the putative avian ancestral karyotype. For instance, in *B. oedicnemus* (Clade Charadrii), chromosome reorganization involved mainly chromosome fusions [6], while in *Jacana jacana* (Clade Scolopaci), the process was mediated by chromosome fusions and fissions [13]. In *Actitis macularius* (Clade Scolopaci), several fissions involving macrochromosomes were described [11]. In *Larus argentatus* (Clade Lari), only fusions of macrochromosomes with microchromosomes were detected [10]. On the other hand, *Charadrius collaris* and *Vanellus chilensis*, both included in the Clade Charadrii, have a typical avian karyotype [9,12].

The data obtained from chromosome painting contributed to our knowledge about chromosome evolution in shorebirds; however, they were limited to the macrochromosomes in most of the reports (this is still true of most avian karyotype studies). Although *B*. *oedicnemus* provides insights about rearrangements involving microchromosomes, the exact role of these small elements in the rearrangements were not identified [10–12]. An alternative approach to investigate the microchromosome organization is bacterial artificial chromosomes (BACs) derived from chicken and zebra finch. These probes have been used in several avian orders, but interchromosomal rearrangements involving the microchromosomes were found only in a few avian orders [14–18]. Among shorebirds, BAC FISH is limited to *Scolopax rusticola* (Clade Scolopaci), where no evidence of interchromosomal rearrangements involving microchromosomes was found [16].

The presence of so many microchromosomes is a peculiar characteristic for nearly all birds. This feature possibly evolved around 250 million years ago [19,20]. The avian karyotype is characterized by containing around 2n = 80, among those 40 pairs, 30 pairs are usually microchromosomes with size ranging between 0.5 and 2.5 µm [21]. Some studies suggest that the common ancestor of birds presented microchromosomes in its karyotype, which possibly arose from chromosome fissions [22,23]. The presence of these tiny elements in the bird genome for such a long period of time implies an evolutionary success of these vertebrates [20,22,23].

Considering that information on cross-species chromosome mapping in shorebirds is limited to macrochromosomes, further studies are necessary to improve our understanding of the role of microchromosomes in the karyotype organization of these birds. Hence, in this study, we explored the microchromosome organization in three shorebirds species using chicken and zebra finch BACs. The aim was to improve our knowledge of its karyotype, especially regarding microchromosomes. Our results revealed a different pattern of microchromosome organization in each investigated species. We also performed a comparative analysis with related Charadriiformes and other birds.

2. Materials and Methods

2.1. Animals' Collection and Chromosome Preparation

Samples (Table 1) were collected from individuals in their natural environment according to the permission of SISBIO 61047-4–ICMBio and the experiments were approved by the ethics committee from Universidade Federal do Pampa (CEUA 019/2020). For each individual, skin biopsies or feather pulp samples were collected to establish fibroblast cell culture in order to obtain chromosome preparations. Cells were cultured in flasks (25 cm2) with DMEM cell culture media (GIBCO), supplemented with 15% fetal bovine serum (GIBCO) and 1% penicillin (10,000 units/mL)/streptomycin (10,000 µg/mL) (GIBCO), and incubated at 37 ◦C [24]. Metaphase chromosomes were obtained according to standard procedures involving exposure to colcemid (1 h, 37 ◦C), hypotonic treatment (0.075 M KCl, 15 min, 37 ◦C), and fixation with methanol/acetic acid (3:1). *V. chilensis* species was also sampled by the direct chromosome preparation method, where embryo cells were dissociated by 2 mL of trypsin 0.25% EDTA for approximately 10 min, then the sample was placed in 10 mL

of RPMI 1040 medium pre warmed at 37 ◦C with three drops of colchicine 0.05% and incubated for 1 h at 37 ◦C, followed by hypotonic treatment and fixation [25].

Table 1. List of the avian species investigated and the approaches used. Brazilian States: RS, Rio Grande do Sul; PA, Pará.

Species	Sex	Locality	Macrochromosomes Study	Microchromosomes Study
Calidris canutus	Female	Belém, PA, Brazil	-	Present study
Jacana jacana	Female	São Gabriel, RS, Brazil	Kretschmer et al. [13]	Present study
Vanellus chilensis	Male	São Gabriel, RS, Brazil	Kretschmer et al. [9]; Pinheiro et al. [12]	Present study

2.2. Karyotype Description

After chromosome harvesting, the cell suspension was dropped onto clean glass slides and air-dried, following the staining with Giemsa 5% in phosphate buffer with pH 6.8. To determine the diploid chromosome number and chromosomal morphologies, we analyzed at least 30 metaphases. Chromosomal morphology and karyotype ordering were determined according to Guerra [26].

2.3. FISH Experiments Using Chicken and Zebra Finch Bacterial Artificial Chromosomes (BACs)

Two BAC probes from chicken (*Gallus gallus*, CH261) or zebra finch (*Taeniopygia guttata*, TGMCBA) per microchromosomes (GGA10–28, except GGA16) were applied for cross- species FISH mapping in *Calidris canutus*, *Jacana jacana*, and *Vanellus chilensis* (Supplementary Materials Table S1). The BAC clone isolation, amplification, labeling, and hybridization were performed following the protocol described by O'Connor et al. [16]. The FISH results were confirmed by analyzing at least 10 metaphases per experiment. Images were captured with a CCD camera and SmartCapture (Digital Scientific UK) system, coupled to an Olympus BX61 epifluorescence microscope. Final image processing was performed using Adobe Photoshop 7.0. Regardless of the fact that we used BAC probes from chicken and zebra finch libraries, the results were compared with chicken, once it represents the ancestral state. Most of the BAC probes used were obtained from chicken, but, for

some microchromosomes, the chicken BACs did not hybridize successfully in all bird species [27]; in these cases, we used BAC probes from the zebra finch. In order to identify the chromosomal rearrangements, we considered the following: (i) no rearrangements if both BAC probes for each microchromosome produced FISH signals in the same microchromosome and with a size of micro; (ii) fission event when both BAC probes for each microchromosome produced FISH signals in different microchromosome; and (iii) fusion event when probes intended for a microchromosome hybridized to a macrochromosome.

3. Results

3.1. Karyotype Description

The karyotype of *Jacana jacana* (2n = 82) and *Vanellus chilensis* (2n = 78) was found to be consistent with previous studies [9,13]. However, we found a diploid number for *Calidris canutus* (2n = 92), which was different to 2n = 90, as previously described [28]. Our results showed that most autosomes are acrocentric, except for pairs 6 and 9, which are metacentric and submetacentric, respectively. The smallest microchromosomes are telocentric, the Z sex chromosome is a submetacentric macrochromosome with the size between the first and the second pairs, and the W sex chromosome corresponds to a small metacentric element with a size between pairs 20 and 21 (Figure 1).

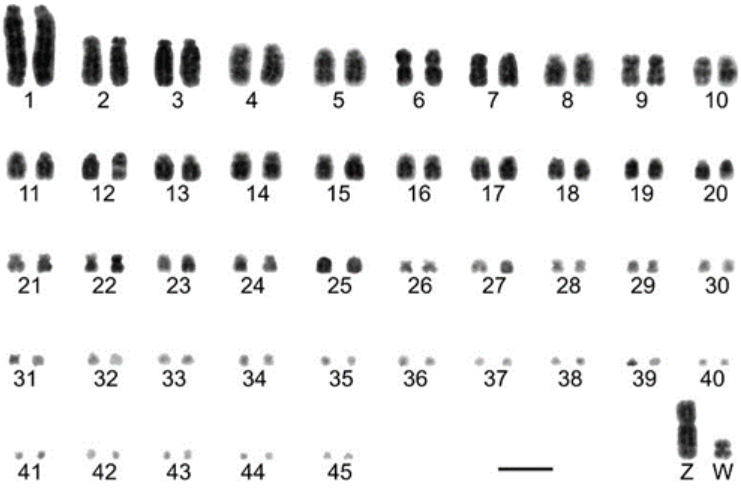

Figure 1. Conventionally stained complete karyotypes of *Calidris canutus* with 2n = 92. Bar 5 µm.

3.2. Fluorescence In Situ Hybridization (FISH) Experiments

The BAC FISH revealed different patterns of microchromosome organization in the species investigated. In *Calidris canutus*, two microchromosomes were involved in chromosome fusions (GGA 12 and GGA14). GGA12 probes produced signals in a medium macrochromosome, indicating the fusion with other microchromosome or a segment from a macrochromosome (as a result of fission events). GGA 14 hybridized on a macrochromosome, indicating a fusion of this chromosome with a macrochromosome. In *Jacana jacana*, the results revealed the conservation of the microchromosomes tested as one individual pair each; however, a gap in pair 8, previously described by Kretschmer et al. [13], remained unresolved, indicating that smaller chicken chromosomes were involved in that fusion. In contrast, there was no evidence of rearrangements involving microchromosomes in *Vanellus chilensis* (Figure 2). Table 2 summarizes the BAC FISH results in the three shorebirds investigated.

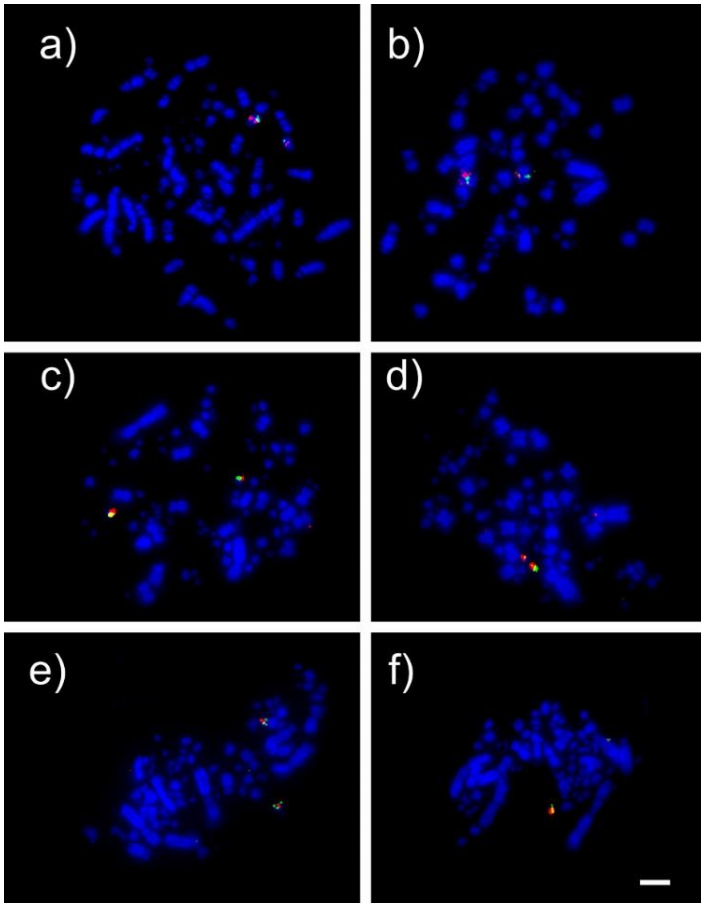

Figure 2. Examples of FISH experiments using chicken (GGA) bacterial artificial chromosome (BAC) probes in shorebirds. FISH results in *C. canutus*: chromosome 12 CH261-60P3 (red) and CH261-4M5 (green) (**a**) and chromosome 14 CH261-122H14 (red) and CH261-69D20 (green) (**b**). FISH results in *J. jacana*: chromosome 18 CH261-72B18 (red) and CH261-60N6

(green) (**c**) and chromosome 26 CH261- 170L23 (red) and CH261-186M13 (green) (**d**). FISH results in *V. chilensis*: chromosome 17 CH261-42P16 (red) and TGMCBA-375I5 (green) (**e**) and chromosome 28 CH261-72A10 (red) and CH261-64A15 (green) (**f**). Scale bar 5 μm.

Table 2. Microchromosome correspondence between chicken and three shorebirds: *Vanellus chilensis* (VCH), *Jacana jacana* (JJA), and *Calidris canutus* (CCA).

Chicken Chromosomes	Species		
	VCH	JJA	CCA
GGA10 *	9	12	Micro
GGA11	11	16	Micro
GGA12	12	17	Fusion
GGA13	13	18	Micro
GGA14	14	19	Fusion
GGA15	15	20	Micro
GGA16	No data	No data	No data
GGA17	17	22	Micro
GGA18	18	23	Micro
GGA19	19	24	Micro
GGA20	20	25	Micro
GGA21	21	26	Micro
GGA22	22	27	Micro
GGA23	23	28	Micro
GGA24	24	29	Micro
GGA25	25	30	Micro
GGA26	26	31	Micro
GGA27	27	32	Micro
GGA28	28	33	Micro

* The chromosomal correspondence to GGA10 of *V*. *chilensis* (VCH) and *J*. *jacana* from Kretschmer et al. [9] and Kretschmer et al. [13], respectively.

4. Discussion

BAC probes from chicken and zebra finch microchromosomes are a powerful tool to delineate chromosome homologies and to identify chromosomal rearrangements. This study thus contributed to the understanding of microchromosomes organization and evolution in shorebirds by investigating the karyotypes of *Calidris canutus*, *Jacana jacana*, and *Vanellus chilensis*. The karyotypes of *J*. *jacana* and *V*. *chilensis* have been previously described as 2n = 82 and 2n = 78, respectively [9,13], which was confirmed by our results. In addition, we found a new diploid number for *C*. *canutus*. It was previously described as 2n = 90 [28], but we found 2n = 92, owing to an additional pair of microchromosomes; however, the discrepancy between the new diploid number is

probably due to technics limitations, which is a common mistake in avian classic cytogenetics because of its high number of very small microchromosomes.

Our molecular cytogenetic characterization using BAC FISH from the microchromosomes of chicken and zebra finch on metaphases of three shorebirds species confirmed that most of the chicken microchromosomes are conserved as entire units, as already reported in previous studies that demonstrated the high degree of conservation of microchromosomes in birds [16,29]. Interestingly, each species investigated here illustrated different microchromosome involvements in rearrangements. For instance, we found evidence of their involvement in interchromosomal rearrangements in *C*. *canutus* and *J*. *jacana*, while in *V*. *chilensis*, they were conserved as single pairs. In our study, we did not investigate

The intrachromosomal rearrangements, as we used only two BAC probes per microchromosome. Nevertheless, these rearrangements in microchromosomes are very important features in bird genome owing to its capability of generating phenotypic differentiation, as reported for *Calidris pugnax*, where different mating phenotypes are described due to an intrachromosomal rearrangement on microchromosome 11 [30,31].

Analyzing the karyotype of *V*. *chilensis* and *C*. *collaris*, Pinheiro et al. [12] found differences in the microchromosomes with FISH signals using *Burhinus oedicnemus* probes corresponding to microchromosomes. These authors suggested that the variation in the number of signals was indicative of the considerable number of rearrangements involving microchromosomes in *V*. *chilensis*. However, our results disregard interchromosomal rearrangements involving the microchromosomes in this species. It is likely that the misinterpretation of FISH results by these authors was because of the background signal produced as a result of hybridization to repetitive sequences.

Although we did not find fusions involving microchromosomes in *J*. *jacana*, a previous study revealed a gap in pair 8 of this species, which was proposed as the result of a fusion between a microchromosome and a segment from the ancestral chromosome 5 (GGA5) [13]. This fusion remained unresolved, once none of the probes used in our study produced signals in the chromosome 8 of *J*. *jacana*. Nevertheless, it is important to highlight the fact that BAC probes corresponding to the

chicken chromosome 28–39 have not been developed so far. Hence, a plausible explanation still relies on a possible fusion of a microchromosome pair within this range (pairs 28–39).

In contrast, in *C*. *canutus*, microchromosome pairs 12 and 14 were involved in fusions. According to Kretschmer et al. [17], these microchromosomes are more likely to undergo interchromosomal rearrangements in birds. Fusion patterns differ between lineages, as observed in Waters et al. [29]. A possible explanation for the fusion events in *C*. *canutus* could be the presence and location of some specific motifs of repetitive sequence insertions, such as transposable elements, as observed in Psittaciformes species [32]. Besides that, no evidence of the occurrence of fissions of microchromosomes was observed in our results, indicating that the increase in the diploid number in *C*. *canutus* was due to macrochromosome fissions or even a smaller microchromosome fission (microchromosomes between 28–39). Similar results were observed in *Scolopax rusticola*, which have 2n = 96, and only macrochromosome fissions were found [16,33]. However, in *S*. *rusticola*, no evidence of microchromosome fusions was found.

BAC probes from microchromosomes have been used in several bird orders and have significantly contributed to our knowledge about microchromosome organization and evolution [14,16–18,27,34,35]. Interchromosomal rearrangements involving these tiny chromosomes were found only in some orders, such as Falconiformes, Psittaciformes, Caprimulgiformes, Cuculiformes, Suliformes, and Passeriformes, always in species with a relatively low diploid number for birds (usually lower than 2n = 74), indicating that the decrease in the diploid number was due to microchromosome fusions. However, to the best of our knowledge, this is the first time that fusions involving microchromosomes were found in a species with a high diploid number (e.g., *C*. *canutus*, 2n = 92), indicating that this type of rearrangements is not limited to species with a low diploid number.

Until now, including our study, the microchromosomal dynamics in karyotype evolution have been investigated in detail in four shorebirds species, three from the clade Scolopaci, *S*. *rusticola* [16], *C*. *canutus*, and *J*. *jacana*, and one from the clade Charadrii, *V. chilensis*. Considering that no interchromosomal rearrangements involving the microchromosomes were found in *S*. *rusticola*, we propose that the

common ancestor for the clade Scolopaci had the ancestral pattern of microchromosome organization similar to *G. gallus*. After the divergence, each Scolopaci species has undergone different strategies in the microchromosome organization; that is, remained conserved as in *S*. *rusticola* or rearranged as in *C*. *canutus* and *J*. *jacana*. Similarly, the common ancestor for the clade Charadrii had the ancestral pattern once the microchromosome organization remained highly conserved in *V*. *chilensis*.

Previously, analyzing the macrochromosomes using chromosome painting, we pro- posed that, after divergence, each shorebird suborder underwent different chromosome rearrangements [13], which was later confirmed by others [12]. Here, we extended this hypothesis to microchromosomes as well.

5. Conclusions

Our results illustrate that homology mapping using BAC probes for microchromosomes is necessary to understand the dynamics of genome reorganization in birds. The results of chromosome painting for both macro and microchromosomes of shorebirds suggest that the karyotypical evolution of these birds involved different chromosomal strategies in each clade. It is also important to highlight that, although the species are closely related, we have found different microchromosome behavior for each shorebird. Furthermore, our results in *Calidris canutus* indicated that species with a high diploid number could also undergo microchromosomal fusions.

Supplementary Materials: The following supporting information can be downloaded at: https:// www.mdpi.com/article/10.3390/ani12213052/s1, Table S1: List of BACs applied to shorebird species.

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5 CONSIDERAÇÕES FINAIS

5.1 Estudo comparativo das Ordens Passeriformes e Charadriiformes

O estudo da evolução cromossômica em aves é um campo fascinante e cheio de desafios. No entanto, essa Classe de animais nos proporcionam a oportunidade de entender melhor a biodiversidade, diversidade genética e evolução. A cooperação entre pesquisadores foi essencial diante da complexidade desses estudos, pois ajudou a superar desafios teóricos e técnicos da pesquisa, ajudando a desenvolver habilidades essenciais como pesquisador. O ambiente colaborativo e sob orientação ofereceu uma plataforma rica para o treinamento e desenvolvimento deste doutorando durante os quatro anos de formação, proporcionando uma experiência imersiva na resolução de problemas, pois pode experimentar uma variedade de técnicas, metodologias e pontos de vista analíticos que são fundamentais para a pesquisa contemporânea em citogenética, genética e biologia evolutiva.

As sondas BAC dos microcromossomos das bibliotecas de GGA e TGU são uma ferramenta poderosa para delinear homologias cromossômicas e identificar a ocorrência de rearranjos cromossômicos. Este estudo vem para contribuir na compreensão da organização e evolução dos microcromossomos em aves Passeriformes e Charadriiformes. Os resultados apresentados revelam diferentes níveis de organização nos microcromossomos das espécies estudadas ampliando o conhecimento da organização dessas estruturas em ambas as ordens.

Os cariótipos e números diploide de *J. jacana* (2n = 82), *V. chilensis* (2n = 78), *M. maculatus* (2n = 80), *M. bonariensis* (2n = 80) e *S. caerulescens* (2n = 78) foram confirmados com os resultados deste trabalho da mesma forma como descritos anteriormente em outros estudos, (Carvalho, M. 1989; Christidis, L. 1990; Gunski et al., 2000; Kretschmer et al., 2015; Kretschmer et al., 2020;). Todavia, foram identificados novos números diploides para as espécies *C. canutus* (2n = 92) anteriormente descrito como 2n = 90 (Bian et al., 1993), e também para *T. aedon* (2n = 76) descrito por de Lucca e Waldrigues (1985) com 2n = 68.

Sobre a discordância encontrada no número diploide de ambas espécies é importante pontuar que uma característica notável na citogenética de aves é a constância nesse caractere, ou seja, a variação do número diploide dentro de uma

mesma espécie é um fato de rara ocorrência. Isso fortalece a conclusão de que possivelmente limitações técnicas poderiam ser responsáveis pela descrição incorreta realizada a mais de 30 anos, fazendo com que este não seja um erro incomum na citogenética clássica de aves, devido a presença de muitos microcromossomos.

A caracterização citogenética molecular usando FISH de sondas BAC dos microcromossomos de GGA e TGU em metáfases das espécies utilizadas neste trabalho confirmou que a maioria dos microcromossomos do PAK estão conservados como unidades inteiras, conforme já relatado em estudos anteriores que demonstraram o alto grau de conservação destes elementos na maioria das aves (O'connor et al., 2019), fato que se mostrou fortemente evidente nas espécies da ordem passeriformes, que possuem o número diploide relativamente conservado (2n = ~80). No entanto em Charadriiformes, apesar de a maioria dos microcromossomos testados apresentarem-se conservados, em comparação ao PAK, foi possível observar alguns rearranjos como fusões de microcromossomos em macrocromossomos ou cromossomos "médios" formando macrocromossomos. Nesse sentido é possível inferir que as espécies testadas da ordem Charadriiformes apresentaram distintos caminhos evolutivos, mantendo o estado conservado como em *V. chilensis*, ou reorganizado como em *J. jacana* e *C. canutus*.

A comparação dos resultados de ambas ordens pode expor um padrão interessante, relacionando a variação do número diploide a eventos de reorganização dos microcromossomos, apesar da inquestionável conservação da característica ancestral desses elementos do genoma das aves. Todavia, torna-se importante esclarecer que essa relação não é diretamente proporcional, ou seja, mesmo em cariótipos com alto número cromossômico, eventos de fusões, que levam a redução do número cromossômico, podem ocorrer, como observado em *C. canutus*, espécie que em teoria, esperava-se encontrar fissões cromossômicas. Contudo, mesmo não observando fissões nos cromossomos das espécies testadas, não podemos excluir a possibilidade desse evento, visto que as bibliotecas de sondas BAC utilizadas neste estudo não possuem sondas para todos os microcromossomos homólogos aos do PAK.

Como os eventos de reorganização nos microcromossomos das espécies testadas neste trabalho demostraram que rearranjos intercromossômicos tais como

fusões ou fissões são raros, é possível inferir que a evolução desses genomas, ocorre mais frequentemente por rearranjos intracromossômicos, tanto em macro quanto em microcromossomos, fato que já foi evidenciado por estudos *in situ* e *in silico* (Romanov et al., 2014; Rodrigues et al., 2017; Kretschmer et al., 2018b). Dessa forma, se propõem que os cariótipos de Passeriformes possivelmente evoluíram por meio de rearranjos intracromossômicos, enquanto os macrocromossomos e microcromossomos permaneceram altamente conservados em suas unidades, possivelmente presente já no ancestral comum deste grupo, e assim, a ausência de rearranjos intercromossômicos observada na maioria das espécies analisadas pode estar ligada ao sucesso evolutivo deste caractere nesta ordem, que representa um dos clados mais diversificados e altamente derivado da classe Aves.

Em Charadriiformes podemos inferir que o ancestral comum do clado Scolopaci possuía o padrão ancestral de organização dos microcromossomos em seu cariótipo ou seja homólogo ao PAK. Após a divergência, cada espécie de Scolopaci sofreu diferentes eventos de organização dos microcromossomos, isto é, permaneceu conservada, como em *S. rusticola* (O'connor et al., 2019), ou foi reorganizada, como em *C. canutus* e *J. jacana*. Da mesma forma, o ancestral comum do clado Charadrii possuía o padrão ancestral, uma vez que a organização dos microcromossomos permaneceu altamente conservada em *V. chilensis*. Assim, após a divergência, cada subordem de Charadriiformes passou por diferentes trajetórias de reorganização em seus microcromossomos.

5.2 Publicações relacionadas ao tema da pesquisa (Artigos em coautoria)

Durante os anos de 2020 a 2024, período em que o pesquisador Marcelo Santos de Souza esteve matriculado e realizando seu doutorado no Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal do Pampa, participou como autor principal ou como coautor de 13 publicações (anexados a este documento). Em sua maioria foram trabalhos aceitos em revistas internacionais com alta relevância e impacto principalmente na área de Genética e Citogenética. É importante destacar que a participação no desenvolvimento dessas pesquisas pode incluir diferentes etapas como: obtenção e processamento de amostras, realização de experimentos, análise de dados ou ainda, escrita e revisão de manuscritos.

5.3 Conclusão

O estudo da evolução cromossômica em aves é repleto de desafios, que abrem caminho para compreendermos a diversidade genética e evolutiva destes seres. Diante da complexidade desses estudos, a colaboração entre pesquisadores teve papel crucial, não apenas na superação de obstáculos técnicos e teóricos da pesquisa, mas também como ferramenta geradora de habilidades essenciais na formação dos envolvidos nela.

A pesquisa conduzida com o emprego da técnica BAC-FISH para a análise da organização e status evolutivo dos microcromossomos em espécies de aves das ordens Passeriformes e Charadriiformes proporcionou uma visão mais ampla sobre a evolução do cariótipo de grupos taxonômicos distintos de forma pioneira.

Os resultados obtidos revelaram um padrão surpreendentemente conservado na evolução do cariótipo das espécies de Passeriformes utilizadas neste trabalho, mesmo considerando a posição bastante derivada desse grupo na filogenia das aves. Este achado sugere a presença de mecanismos de estabilização cromossômica que têm sido eficazes na manutenção da estrutura cariotípica ao longo da evolução deste clado.

Por outro lado, as espécies de aves Charadriiformes examinadas exibiram um panorama de organização cariotípica mais heterogêneo, caracterizado por caminhos distintos de reorganização em alguns microcromossomos. Este resultado indica uma maior plasticidade na evolução cariotípica dentro deste grupo, com eventos de rearranjos cromossômicos que podem ter desempenhado um papel importante na adaptação e diversificação dessas espécies em diferentes ambientes ecológicos.

Esses achados contribuem significativamente para o entendimento da dinâmica evolutiva dos cariótipos das aves Passeriformes e Charadriiformes, fornecendo perspectivas importantes sobre os processos genômicos subjacentes à diversificação e especialização destes grupos. Esses resultados contribuem de forma significativa para o entendimento da dinâmica evolutiva do cariótipo das aves aqui estudadas, destacando a importância da técnica BAC-FISH como uma ferramenta valiosa para a investigação da organização e evolução dos microcromossomos deste grupo.

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8 LISTA DE PRODUÇÕES CIENTÍFICAS

Aqui está um índice com todas as publicações realizadas durante o período de doutorado do autor (2020 a 2024).

Genome
An International Journal
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Article

Understanding the chromosomal evolution in cuckoos (Aves, Cuculiformes): a journey through unusual rearrangements

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Abstract

The Cuculiformes are a family of over 150 species that live in a range of habitats, such as forests, savannas, and deserts. Here, bacterial artificial chromosome (BAC) probes (75 from chicken and 14 from zebra finch macrochromosomes 1–10 +ZW and for microchromosomes 11–28 (except 16)) were used to investigate chromosome homologies between chicken and the squirrel cuckoo (*Piaya cayana*). In addition, repetitive DNA probes were applied to characterize the chromosome organization and to explore the role of these sequences in the karyotype evolution of *P. cayana*. We also applied BAC probes for chicken chromosome 17 and Z to the guira cuckoo (*Guira guira*) to test whether this species has an unusual Robertsonian translocation between a microchromosome and the Z chromosome, recently described in the smooth-billed ani (*Crotophaga ani*). Our results revealed extensive chromosome reorganization with inter- and intrachromosomal rearrangements in *P. cayana*, including a conspicuous chromosome size and heterochromatin polymorphism on chromosome pair 20. Furthermore, we confirmed that the Z-autosome Robertsonian translocation found in *C. ani* is also found in *G. guira*, not *P. cayana*. These findings suggest that this translocation occurred prior to the divergence between *C. ani* and *G. guira*, but after the divergence with *P. cayana*.

Key words: birds, genome evolution, sex chromosomes, chromosomal rearrangements, heterochromatic polymorphism

Introduction

Cuculiformes is a group of birds commonly known as cuckoos exhibiting great diversity in morphology, ecology, and behavior (Shufeldt 1901; Payne 1997). There are ~150 species of cuckoos found worldwide (Gill et al. 2023), with a wide range of habitats, including forests, savannas, and deserts (Shufeldt 1901; Payne 1997). Five subfamilies are recognized among cuckoos, Crotophaginae, Neomorphinae, Centropodinae, Couinae, and Cuculinae (Sorenson and Payne 2005) (Fig. 1). Cuckoos are essential components in many ecosystems, being both predators of insects and other tiny animals as well as food for other birds and mammals and have cultural significance since many traditions and civilizations value their distinctive calls. Little research has hitherto focused on chromosomal studies in these species and most of these used conventional karyotyping methods (Waldrigues and Ferrari 1982; Waldrigues et al. 1983). Despite this, these investigations showed a significant range of karyotypes, with diploid numbers ranging from $2n = 64$ in *Crotophaga major* (Crotophaginae) (Waldrigues et al. 1983) to $2n = 90$ in *Piaya cayana* (Cuculinae) (dos Santos et al. 2020). Moreover, there have been many differences reported in chromosomal size and morphology, indicating various evolutionary chromosome rearrangements, including inversions, fusions, fissions, and translocations.

Molecular cytogenetic data in Cuculiformes are only available for *Guira guira* (Crotophaginae), *P. cayana*, and *Crotophaga ani* (dos Santos et al. 2020; Kretschmer et al. 2021). In *G. guira* and *P. cayana*, whole chromosomal painting probes derived from *Gallus gallus* (GGA) and *Leucopternis albicollis* were used to investigate the conservation of the syntenic groups corresponding to the avian ancestral macrochromosomes (GGA1-10) (dos Santos et al. 2020). This report highlighted fusion events in *G. guira*, bringing the ancestral diploid number

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Original Article

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Chromosomal Evolution of Suboscines: Karyotype Diversity and Evolutionary Trends in Ovenbirds (Passeriformes, Furnariidae)

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Keywords
Chromosome · Cytogenetics · Birds · Simple short repeats · Comparative genomic hybridization

Abstract
Furnariidae (ovenbirds) is one of the most diversified families in the Passeriformes order and Suboscines suborder. Despite the great diversity of species, cytogenetic research is still in its early stages, restricting our knowledge of their karyotype evolution. We combined traditional and molecular cytogenetic analyses in three representative species, *Synallaxis frontalis*, *Syndactyla rufosuperciliata*, and *Cranioleuca obsoleta*, to examine the chromosomal structure and evolution of ovenbirds. Our findings revealed that all the species studied had the same diploid number (2n = 82). Differences in chromosomal morphology of some macrochromosomes indicate the presence of intrachromosomal rearrangements. Although the three species only had the 18S rDNA on one microchromosome pair, chromosomal mapping of six simple short repeats revealed a varied pattern of chromosome distribution among them, suggesting that each species underwent different repetitive DNA accumulation upon their divergence. The interspecific comparative genomic hybridization experiment revealed that the Furnariidae species investigated carry centromeric regions enriched in similar repetitive sequences, bolstering the Furnariidae family's karyotype conservation. Nonetheless, the outgroup species *Turdus rufiventris* (Turdidae) demonstrated an advanced stage of sequence divergence with hybridization signals that were almost entirely limited to a few microchromosomes. Overall, the findings imply that Furnariidae species have a high degree of chromosomal conservation, and we could also observe a differentiation of repetitive sequences in both Passeriformes suborders (Suboscines and Oscines).

Introduction

Passeriformes is the largest avian order, with more than 6,000 widely distributed species that show an extraordinary morphological and ecological diversity [Ericson et al., 2014]. The order is divided into two suborders: Oscines (vocal learners), comprising 776 genera and roughly 80% of all Passeriformes species, and Suboscines (vocal non-learners), which has 284 genera,

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birds

MDPI

Communication

Highly Conserved Microchromosomal Organization in Passeriformes Birds Revealed via BAC-FISH Analysis

Marcelo Santos de Souza [1], Suziane Alves Barcellos [1], Victoria Tura [1], Vera Lúcia Bobrowski [2], Analía Del Valle Garnero [1], Ricardo José Gunski [1], Darren K. Griffin [3] and Rafael Kretschmer [2,*]

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Simple Summary: The Passeriformes order (songbirds) is incredibly diverse in terms of number of species and morphological and ecological diversification, comprising around 60% of all bird species. Despite considerable diversity, the genome organizational structure (i.e., the number and pattern of chromosomes) within Passeriformes is highly conserved, with a chromosome number that remains close to 80 in nearly all species studied. These characteristics raise interesting questions and stimulate curiosity about the genome evolution of this group. Therefore, this study aimed to analyze the organization of the smallest chromosomes (microchromosomes) in four Passeriformes species to understand whether they were rearranged during evolution. This has only recently become possible using fluorescent probes called bacterial artificial chromosomes (BACs) and a technique called fluorescence in situ hybridization (FISH). Our results confirm that the songbirds studied did not rearrange their microchromosomes to any great extent, and this may have contributed to their overall evolutionary success.

Abstract: Passeriformes birds are widely recognized for their remarkable diversity, with over 5700 species described so far. Like most bird species, they possess a karyotype characteristic of modern birds, which includes a bimodal karyotype consisting of a few pairs of macrochromosomes and many pairs of microchromosomes. Although the karyotype is typically 2n = 80, the diploid number can atypically vary greatly, ranging from 56 to approximately 100 chromosomes. In this study, we aimed to understand the extent of conservation of the karyotype's organizational structure within four species of this group using Bacterial Artificial Chromosomes via Fluorescence In Situ Hybridization (BAC-FISH) with microchromosome probes from Chicken (*Gallus gallus*) or Zebra Finch (*Taeniopygia guttata*) per microchromosomes (GGA10-28, except GGA16). By examining the chromosome complement of four passerine species—the Streaked Flycatcher (*Myiodynastes maculatus*), Shiny Cowbird (*Molothrus bonariensis*), Southern House Wren (*Troglodytes aedon*), and Double-collared Seedeater (*Sporophila caerulescens*)—we discovered a new chromosome number for Southern House Wren. Through FISH experiments, we were able to observe the same pattern of microchromosome organization as in the common ancestor of birds. As a result, we propose a new diploid number for Southern House Wren and confirm the conservation status of microchromosome organization, which may confer evolutionary advantages to this group.

Keywords: Aves; diploid number; karyotype organization; molecular cytogenetic

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animals

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Article

Microchromosome BAC-FISH Reveals Different Patterns of Genome Organization in Three Charadriiformes Species

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Simple Summary: Numerous tiny (micro)chromosomes are a characteristic feature associated with birds, being found in smaller numbers in other organisms and absent in many, such as mammals. Although microchromosomes constitute a large portion of the genome in birds, data on them pertaining to comparative studies between birds are still scarce. This is the case in shorebirds (Charadriiformes), a group with a great variety of species. The aim of this study was to provide insight regarding the evolution of the microchromosomes of three species of shorebirds—the red knot (*Calidris canutus*), the wattled jacana (*Jacana jacana*), and the southern lapwing (*Vanellus chilensis*). The experiments are referred to as cross-species fluorescence in situ hybridization (FISH) mapping using probes called bacterial artificial chromosomes (or BACs), two (one labelled in red and one labelled in green) for every microchromosome. The results thus appear as the microchrochromosome with one green and one red end, revealing different patterns of organization over evolutionary time. In the red knot, they fuse together, but in the southern lapwing, they hardly change. We also described a new chromosome number for the red knot (92 in total). In conclusion, this study contributed to the understanding of microchromosomes organization and evolution of three shorebird species.

Abstract: Microchromosomes, once considered unimportant elements of the genome, represent fundamental building blocks of bird karyotypes. Shorebirds (Charadriiformes) comprise a wide variety of approximately 390 species and are considered a valuable model group for biological studies. Despite this variety, cytogenetic analysis is still very scarce in this bird order. Thus, the aim of this study was to provide insight into the Charadriiformes karyotype, with emphasis on microchromosome evolution in three species of shorebirds—*Calidris canutus*, *Jacana jacana*, and *Vanellus chilensis*—combining classical and molecular approaches. Cross-species FISH mapping applied two BAC probes for each microchromosome, GGA10–28 (except GGA16). The experiments revealed different patterns of microchromosome organization in the species investigated. Hence, while in *C. canutus*, we found two microchromosomes involved in chromosome fusions, they were present as single pairs in *V. chilensis*. We also described a new chromosome number for *C. canutus* (2n = 92). Hence, this study contributed to the understanding of genome organization and evolution of three shorebird species.

Keywords: microchromosome; avian karyotype; bird; BAC; FISH; comparative genomics; molecular cytogenetics

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DIFERENTES AMBIENTES ESCOLARES E O PROCESSO DE APRENDIZAGEM DE CIÊNCIAS: UM ESTUDO DE CASO

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Resumo: No Brasil, costuma-se atribuir o conceito de bom às escolas privadas e de ruim às escolas públicas, mas poucos estudos compararam de fato diferenças entre ambas. O presente trabalho estudou o caso de duas escolas, uma pública e uma privada, buscando compreender como o ambiente escolar influencia o aprendizado de alunos do ensino fundamental. Foram realizadas intervenções em sala de aula com temas ligados à ciência e testes de conhecimento, que foram avaliados e analisados pela equipe executora, bem como comportamento e interesse dos alunos. Apesar do desempenho médio inferior, alguns alunos da rede pública mostraram qualidade acima da média, evidenciando a heterogeneidade da escola pública e seu potencial para formar bons cidadãos e futuros profissionais, desde que haja recursos para isso.

Palavras-chave: Educação. Escola Privada. Escola Pública. Ritmo de aprendizagem.

DNA

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Brief Report

Direct Chromosome Preparation Method in Avian Embryos for Cytogenetic Studies: Quick, Easy and Cheap

Suziane Alves Barcellos [1], Marcelo Santos de Souza [1], Victoria Tura [1], Larissa Rodrigues Pereira [1], Rafael Kretschmer [2,*], Ricardo José Gunski [1] and Analía Del Valle Garnero [1]

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Abstract: Avian cell culture is widely applied for cytogenetic studies, the improvement of which increasingly allows for the production of high-quality chromosomes, essential to perform both classical and molecular cytogenetic studies. Among these approaches, there are two main types: fibroblast and bone marrow culture. Despite its high cost and complexity, fibroblast culture is considered the superior approach due to the quality of the metaphases produced. Short-term bone marrow cultivation provides more condensed chromosomes but nonetheless is quicker and easier. In the search for a quicker, cheaper way to prepare metaphases without losing quality, the present work developed a novel, widely applicable protocol for avian chromosome preparation. Twenty-one bird embryos from distinct families were sampled: Icteridae, Columbidae, Furnariidae, Estrildidae, Thraupidae, Troglodytidae and Ardeidae. The protocol was based on a combination of modified fibroblast culture and bone marrow cultivation, taking the advantages of both. The results show that all species consistently presented good mitotic indexes and high-quality chromosomes. Overall, the application of this protocol for bird cytogenetics can optimize the time, considering that most fibroblast cultures take at least 3 days and often much longer. However, our protocol can be performed in 3 h with a much-reduced cost of reagents and equipment.

Keywords: Aves; cell cultivation; chromosomes; metaphases; method

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1. Introduction

Since the early 1900s, bird chromosomes have been investigated using classical and molecular approaches. Despite the fact that avian cytogenetics play an important role in evolutionary studies, less than 10% of all bird species have a karyotype description so far, and nearly all of these are only partial karyotypes [1]. Birds have a bimodal karyotype and a ZZ/ZW sex chromosome system [2]. One of the most remarkable characteristics in their karyotypes is the large number of microchromosomes, which encode a high rate of important genes [3]. Regarding diploid numbers, they display a wide range, from 40 to 142. Despite this variation, more than 50% of birds have between 78 and 82 chromosomes [1].

Working with wild birds is always challenging due to the limitations associated with animal sampling [4]. Even though some samples can be collected from birds in captivity, it is still hard to do this in some species without negatively affecting their health [5].

Avian cytogenetic studies require cell cultures that provide high-quality metaphases for chromosome analysis, as well as the application of classical and molecular techniques, such as karyotyping, chromosome banding and fluorescent in situ hybridization (FISH). In

cells

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Article

Interspecies Chromosome Mapping in Caprimulgiformes, Piciformes, Suliformes, and Trogoniformes (Aves): Cytogenomic Insight into Microchromosome Organization and Karyotype Evolution in Birds

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Citation: Kretschmer, R.; de Souza, M.S.; Furo, I.d.O.; Romanov, M.N.; Gunski, R.J.; Garnero, A.d.V.; de Freitas, T.R.O.; de Oliveira, E.H.C.; O'Connor, R.E.; Griffin, D.K. Interspecies Chromosome Mapping in Caprimulgiformes, Piciformes, Suliformes, and Trogoniformes (Aves): Cytogenomic Insight into Microchromosome Organization and Karyotype Evolution in Birds. *Cells* **2021**, *10*, 826. https://doi.org/10.3390/cells10040826

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Abstract: Interchromosomal rearrangements involving microchromosomes are rare events in birds. To date, they have been found mostly in Psittaciformes, Falconiformes, and Cuculiformes, although only a few orders have been analyzed. Hence, cytogenomic studies focusing on microchromosomes in species belonging to different bird orders are essential to shed more light on the avian chromosome and karyotype evolution. Based on this, we performed a comparative chromosome mapping for chicken microchromosomes 10 to 28 using interspecies BAC-based FISH hybridization in five species, representing four Neoaves orders (Caprimulgiformes, Piciformes, Suliformes, and Trogoniformes). Our results suggest that the ancestral microchromosomal syntenies are conserved in *Pteroglossus inscriptus* (Piciformes), *Ramphastos tucanus tucanus* (Piciformes), and *Trogon surrucura surrucura* (Trogoniformes). On the other hand, chromosome reorganization in *Phalacrocorax brasilianus* (Suliformes) and *Hydropsalis torquata* (Caprimulgiformes) included fusions involving both macro- and microchromosomes. Fissions in macrochromosomes were observed in *P. brasilianus* and *H. torquata*. Relevant hypothetical Neognathae and Neoaves ancestral karyotypes were reconstructed to trace these rearrangements. We found no interchromosomal rearrangement involving microchromosomes to be shared between avian orders where rearrangements were detected. Our findings suggest that convergent evolution involving microchromosomal change is a rare event in birds and may be appropriate in cytotaxonomic inferences in orders where these rearrangements occurred.

Keywords: avian cytogenomics; evolution; genome organization; FISH; chromosomal rearrangements

1. Introduction

Birds (class Aves) are the most diverse lineage of extant tetrapod vertebrates, comprising 10,806 extant species, divided into 40 extant avian orders [1]. Despite the extraordinary diversity in morphology, ecology and behavior [2], a high proportion of species analyzed so far showed karyotypes composed of about 80 chromosomes, consisting of a few large macrochromosomes (~10) and numerous microchromosomes (~30) [3–5]. This cytogenomic

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Cytogenetic Evidence Clarifies the Phylogeny of the Family Rhynchocyclidae (Aves: Passeriformes)

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Abstract: The phylogenetic position and taxonomic status of Rhynchocyclidae (Aves: Passeriformes) have been the subject of debate since their first description. In most models, Rhynchocyclidae represents a subfamily-level taxon placed within the Tyrant Flycatchers (Tyrannidae). Considering that this classification does not include cytotaxonomic characters, we tested the hypothesis that the chromosome organization of Rhynchocyclidae members differs from that of Tyrannidae. Hence, we selected two species, *Tolmomyias sulphurescens*, and *Pitangus sulphuratus*, representing Rhynchocyclidae and Tyrannidae, respectively. Results revealed a diploid number (2n) of 60 in *T. sulphurescens* and 2n = 80 in *P. sulphuratus*, indicating significant chromosomal differences. Chromosome mapping of *Gallus gallus* (GGA) and *Taeniopygia guttata* bacterial artificial chromosome (BAC) corresponding to chromosomes GGA1-28 (except 16) revealed that the genome evolution of *T. sulphurescens* involved extensive chromosome fusions of macrochromosomes and microchromosomes. On the other hand, *P. sulphuratus* retained the ancestral pattern of organization of macrochromosomes (except the centric fission involving GGA1) and microchromosomes. In conclusion, comparing our results with previous studies in Tyrant Flycatchers and allies indicates that *P. sulphuratus* has similar karyotypes to other Tyrannidae members. However, *T. sulphurescens* does not resemble the Tyrannidae family, reinforcing family status to the clade named Rhynchocyclidae.

Keywords: phylogenetic relationships; chromosomal rearrangements; cytotaxonomy; passerines; tyrant flycatchers

1. Introduction

The phylogenetic position and taxonomic status of the flycatcher lineage named Rhynchocyclidae (Aves: Passeriformes) have been debated since their proposition. In most classifications, it represents a subfamily placed within the Tyrant Flycatchers (Tyrannidae), composing the most diverse Neotropical family of suboscine passerines [1]. Tyrannidae "lato sensu" exhibits high degrees of morphological, ecological, and behavioral diversity, drawing the attention of several phylogenetic studies [1–5]. However, some aspects of their relationships and classification remain controversial. In a recent study of a massive

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Comparative cytogenetics in three species of Wood-Warblers (Aves: Passeriformes: Parulidae) reveal divergent banding patterns and chromatic heterogeneity for the W chromosome

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Absract. Chromosomal rearrangements are an important process in the evolution of species. It is assumed that these rearrangements occur near repetitive sequences and heterochromatic regions. Avian karyotypes have diverse chromosomal band patterns and have been used as the parameters for phylogenetic studies. Although the group has a high diversity of species, no more than 12% has been analyzed cytogenetically, and the Parulidae family are extremely underrepresented in these studies. The aim of this study was to detect independent or simultaneous chromosomal rearrangements, and also to analyze chromosomal banding convergences and divergences of three Wood-Warblers species (*Myiothlypis leucoblephara, Basileuterus culicivorus,* and *Setophaga pitiayumi*). Our CBG-band results reveal an unusual W sex chromosome in the three studied species, containing a telomeric euchromatic region. The GTG and RBG bands identify specific regions in the macrochromosomes involved in the rearrangements. Cytogenetic data confirm the identification of speciation processes at the karyotypic of this group.

Keywords: chromosomal evolution, karyotype, diploid number, chromosomal banding, constitutive heterochromatin.

INTRODUCTION

The Avian Class is characterized by a bimodal karyotype, composed of many pairs of microchromosomes and just a few macrochromosomes (Christidis 1990). The Class presents several patterns of chromosomal bands. In CBG-banding, species of Passeriformes usually reveal the W chromosome

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Research Article
Evolutionary Genetics

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

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Abstract

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

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

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Introduction

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

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

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

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Research Article
Animal Genetics

Comparative analyses of three swallow species (Aves, Passeriformes, Hirundinidae): Insights on karyotype evolution and genomic organization

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Abstract

Despite the richness of species in the Hirudinidae family, little is known about the genome organization of swallows. The *Progne tapera* species presents genetic and morphological difference when compared to other members of the same genus. Hence, the aims of this study were to analyze the chromosomal evolution of three species *Progne tapera*, *Progne chalybea* and *Pygochelidon cyanoleuca* - by comparative chromosome painting using two sets of probes, *Gallus gallus* and *Zenaida auriculata*, in order to determine chromosome homologies and the relationship between these species. All karyotypes exhibited 76 chromosomes with similar morphology, except for the 5th, 6th and 7th chromosome pairs in *P. cyanoleuca*. Additionally, comparative chromosome painting demonstrated the same hybridization pattern in the two *Progne*, which was similar to the putative avian ancestral karyotype, except for the centric fission in the first pair, as found in other Passeriformes. Thus, these data display a close relationship between the *Progne* species. Although *P. cyanoleuca* demonstrated the same fission in the first pair of the ancestral syntenic (GGA1), it also showed an additional chromosomal rearrangement for this species, namely a fusion with a microchromosome in the seventh pair.

Keywords: Homology, molecular cytogenetics, fluorescent *in situ* hybridization, Hirundinidae.

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Introduction

The order Passeriformes is one the most diverse within the class Aves, including around 6000 species (Del Hoyo *et al.*, 2011). As the other members from this class, it presents small genome, high chromosomal number, a few pairs of macrochromosomes and several microchromosome pairs. Furthermore, birds have a sexual system ZZ/ZW, where the female is heterogametic (Griffin *et al.*, 2007; Barcellos *et al.*, 2019).

The Hirundinidae family (Aves: Passeriformes), commonly known as swallows, comprises approximately 84 species (Dickinson, 2003; Sheldon *et al.*, 2005). These birds are well known worldwide due to their cosmopolitan habits, behavior and ecology (Sheldon *et al.*, 2005). Moreover, they are migratory and insectivorous. Due to the scarcity of food resources in winter, swallows tend to fly several miles to find food and a safe place to stay during this season (Sigrist, 2013).

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Progne tapera (Linnaeus, 1766), *Progne chalybea* (Gmelin, 1789) and *Pygochelidon cyanoleuca* (Vieillot, 1817) have similar karyotypical organization with the same diploid number (2n=76) and distribution of repetitive DNA. Furthermore, recent studies with these species found an interesting characteristic, an enlarged W chromosome (Barcellos *et al.*, 2019). Despite recent research, the cytogenetics of swallows is still poorly defined.

Cross-species chromosome painting has been applied widely for evolutionary biology studies and karyotype evolution (Ferguson-Smith and Trifonov, 2007; Ellegren, 2010) and, in particular, to identify chromosomal homo-

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Research Article
Evolutionary Genetics

Novel insights into chromosome evolution of Charadriiformes: extensive genomic reshuffling in the wattled jacana (*Jacana jacana*, Charadriiformes, Jacanidae)

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Abstract

The order Charadriiformes comprises three major clades: Lari and Scolopaci as sister group to Charadrii. Until now, only three Charadriiformes species have been studied by chromosome painting: *Larus argentatus* (Lari), *Burhinus oedicnemus* and *Vanellus chilensis* (Charadrii). Hence, there is a lack of information concerning the third clade, Scolapaci. Based on this, and to gain a better understanding of karyotype evolution in the order Charadriiformes, we applied conventional and molecular cytogenetic approaches in a species belonging to clade Scolopaci - the wattled jacana (*Jacana jacana*) - using *Gallus gallus* and *Zenaida auriculata* chromosome-specific probes. Cross-species evaluation of *J. jacana* chromosomes shows extensive genomic reshuffling within macrochromosomes during evolution, with multiple fission and fusion events, although the diploid number remains at high level (2n=82). Interestingly, this species does not have the GGA7-8 fusion, which was found in two representatives of Charadrii clade, reinforcing the idea that this fusion may be exclusive to the Charadrii clade. In addition, it is shown that the chromosome evolution in Charadriiformes is complex and resulted in species with typical and atypical karyotypes. The karyotypic features of Scolopaci are very different from those of Charadrii and Lari, indicating that after divergence, each suborder has undergone different chromosome rearrangements.

Keywords: Charadrii, karyotype, Avian genome, comparative mapping.

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Introduction

Charadriiformes comprises 19 families with approximately 370 species (Gill and Donsker, 2017) and is divided into 3 clades: Lari (gulls, auks and their allies, along with buttonquails), Scolopaci (sandpipers, jacanas and allies), and Charadrii (plovers, oystercatchers and allies) (Baker *et al.*, 2007). Species of this order have been the subject of numerous studies, addressing topics such as systematics, behavior, diseases and cytogenetics (Baker *et al.*, 2007; Nie *et al.*, 2009; Bahl *et al.*, 2013; Kretschmer *et al.*, 2015b; Jackson *et al.*, 2017). Cytogenetics has shown the occurrence of a wide range of diploid numbers, from 2n=42 to 98 in *B. oedicnemus* (Nie *et al.*, 2009) and *Gallinago gallinago* (Hammar, 1970), respectively. However, the exact nature of the chromosomal rearrangements that took place in the

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Da teoria à prática: a utilização de oficinas didáticas no processo de ensino e aprendizagem para alunos do ensino médio

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RESUMO

A utilização de oficinas didáticas em sala de aula é um método eficiente, que pode auxiliar o aluno no processo de aprendizagem, interligando teoria e prática motora. Esta pesquisa teve como principal objetivo avaliar de forma quantitativa e qualitativa os múltiplos conhecimentos disseminados através do uso de oficinas didáticas em sala de aula, tendo como receptora uma escola pública. Os acadêmicos que ministraram a atividade buscaram analisar as principais dificuldades de aplicação deste método. Resultados demonstraram que, de acordo com a lista presencial e avaliação quantitativa de contextualização, a oficina didática teve bons níveis de aceitação entre os alunos. Através desta prática e análise dos questionários aplicados, podemos concluir que temas pouco abordados em sala de aula, tais como: cultivo *in vitro* e nutrição vegetal, tornaram-se mais compreensíveis pelos alunos por intermédio desta contextualização. Demonstrando a importância de atividades práticas para o processo crítico e reflexivo dos alunos.

PALAVRAS-CHAVE: Escolarização. Iniciação Científica. Interdisciplinaridade.