Universidade Federal de São Carlos
Centro de Ciências Biológicas e da Saúde

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Cytogenomic studies in birds focusing on the differentiation of the ZZ/ZW sex chromosome system

São Carlos
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Bachelor's Degree Final Project submitted as a requirement for obtaining the degree of Bachelor of Biotechnology from the Universidade Federal de São Carlos

Supervisor: Prof. Dr. Marcelo de Bello Cioffi

São Carlos
2025

I dedicate this work to my family, especially my parents Rosa and Edmilton, and my sister, Geovana, for all their support and understanding during my degree.

Resumo

Uma das particularidades das Aves é a presença de um sistema estável de cromossomos sexuais ZZ/ZW, que teve origem em um ancestral aproximadamente 70 milhões de anos atrás. O grupo é igualmente conhecido por exibir cariótipos que apresentam um nível de conservação relativamente elevado em relação ao número diploide (2n), geralmente próximo de 80 cromossomos, sendo caracterizado por 10 pares de macrocromossomos e 30 pares de microcromossomos. O cromossomo W das aves é caracterizado por frequentemente apresentar um acúmulo de sequências repetitivas, tais como DNA satélite, microssatélites e elementos transponíveis, resultantes dos processos de supressão da recombinação, que provocam sua diferenciação morfológica e o acúmulo desses elementos ao longo do tempo. Os DNAs satélites (satDNAs), por sua vez, têm sido isolados e analisados em várias linhagens, evidenciando, cada vez mais, sua contribuição para a evolução do cariótipo em geral e para os cromossomos sexuais, sendo o conjunto de todos os satDNAs de uma espécie denominado Satelitoma. Neste âmbito, este trabalho teve por objetivo combinar análises citogenéticas, genômicas e bioinformáticas no estudo da evolução e diferenciação dos cromossomos sexuais em Aves. Para tal, selecionamos dois modelos de estudo (denominados A e B). No modelo A, selecionamos duas espécies da família Turdidae (*Turdus leucomelas* e *Turdus rufiventris*), ambas com 2n preservados, que apresentam números de cromossomos de 80 e 78, respectivamente. Realizou-se, então, o isolamento do satelitoma de *T. leucomelas*, seguido por experimentos de hibridização fluorescente in situ (FISH) nas duas espécies com o objetivo de identificar a conservação e a divergência entre as espécies, bem como seu papel na diferenciação dos cromossomos sexuais. Adicionalmente, realizamos Bandamento C e estudos de hibridização genômica comparativa (CGH), nos quais identificamos uma configuração atípica do sistema sexual ZZ/ZW em T. leucomelas, evidenciando uma via alternativa para a evolução do cromossomo W. No modelo B, selecionamos dois representantes da família Cariamidade (*Cariama cristata* e *Chunga burmeisteri*), os quais apresentam cariótipos incomuns, com 108 e 106 cromossomos, respectivamente, sendo o cromossomo Z o maior componente dos cariótipos. Realizamos comparações entre experimentos de FISH dos satélites em *C. cristata* e análises in silico correspondentes. Além disso, realizamos a comparação entre os dois Satelitomas isolados e analisamos a proporção de elementos repetitivos presentes no cromossomo Z, com o objetivo de compreender de que maneira esses elementos, combinados com os rearranjos cromossômicos, podem estar associados ao seu crescimento em tamanho. Os achados proporcionaram uma compreensão mais aprofundada sobre a evolução dos cromossomos sexuais em aves, demonstrando que a evolução dos cromossomos Z e W é consideravelmente mais complexa do que se pensava anteriormente, englobando rearranjos cromossômicos e alterações genômicas que se divergem do padrão ancestral.

Palavras-chave: Citogenômica, cromossomos sexuais, Satelitoma, FISH.

Abstract

One of the peculiarities of birds is the presence of a stable system of ZZ/ZW sex chromosomes, which originated in an ancestor approximately 70 million years ago. The group is also known for exhibiting karyotypes that present a relatively high level of conservation in relation to the diploid number (2n), generally close to 80 chromosomes, characterized by 10 pairs of macrochromosomes and 30 pairs of microchromosomes. The W chromosome of birds is characterized by frequently presenting an accumulation of repetitive sequences, such as satellite DNA, microsatellites and transposable elements, resulting from the processes of recombination suppression, which cause their morphological differentiation and the accumulation of these elements over time. Satellite DNAs (satDNAs), in turn, have been isolated and analyzed in several lineages, increasingly evidencing their contribution to the evolution of the karyotype and sex chromosomes, with the set of all satDNAs of a species being called Satelitoma. In this context, this work aimed to combine cytogenetic, genomic and bioinformatics analyses in the study of the evolution and differentiation of sex chromosomes in birds. For this purpose, we selected two study models (named A and B). In model A, we selected two species of the Turdidae family (named *Turdus leucomelas* and *Turdus rufiventris*), both with conserved 2n, 80 and 78, respectively. The isolation of the *T. leucomelas* satellitome was then performed, followed by fluorescence in situ hybridization (FISH) experiments in both species with the aim of identifying the conservation and divergence between the species, as well as their role in the differentiation of sex chromosomes. Additionally, we performed C-banding and comparative genomic hybridization (CGH) studies, in which we identified an atypical configuration of the ZZ/ZW sexual system in *T. leucomelas*, evidencing an alternative pathway for the evolution of the W chromosome. In model B, we selected two representatives of the Cariamidae family (*Cariama cristata* and *Chunga burmeisteri*), which present unusual karyotypes, with 108 and 106 chromosomes, respectively, with the Z chromosome being the largest component of both karyotypes. We compared FISH mapping of the satellites in *C. cristata* and corresponding in silico analyses. In addition, we compared the two isolated Satellitomes and analyzed the proportion of repetitive elements present in the Z chromosome, aiming to understand how these elements, combined with chromosomal rearrangements, may be associated with their growth in size. The findings provided a deeper understanding of the evolution of sex chromosomes in birds, demonstrating that the evolution of the Z and W chromosomes is considerably more complex than previously thought, encompassing chromosomal rearrangements and genomic alterations that diverge from the ancestral pattern.

Key-words: Cytogenomics, sex chromosomes, Satellitome, FISH.

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Introduction

1. INTRODUCTION

1.1 Birds: General considerations

At present, birds represent the most diverse class within Tetrapoda. (Gill et al. 2024). Approximately 11,000 extant species, referred to as Neornithes, are typically classified into three groups: Paleognaths (Tinamiformes and Struthioniformes), Galloanseres (Galliformes and Anseriformes), and Neoaves (all remaining orders) (Hackett et al. 2008; Jarvis et al. 2014; Prum et al. 2015). The typical karyotypes of birds exhibit a high diploid number (approximately 2n=80), characterized by multiple pairs of microchromosomes (around 30) and a limited number of macrochromosome pairs (approximately 10) (Griffin et al. 2007). Despite their small size, microchromosomes exhibit a high gene density, containing roughly fifty percent of the genes found in avian genomes. Their presence often hinders cytogenetic research, thus representing a group with undersampled chromosomal data (Kretschmer et al. 2018).

The Passeriformes order is the most biodiverse, encompassing over 6,500 species, which account for 59% of existing bird biodiversity. The Turdidae family is particularly emblematic due to its extensive representation across global urban and forest habitats (del Hoyo, 2019; Gill et al. 2024). The Pale-breasted Thrush (*Turdus leucomelas*) and the Rufous-bellied Thrush (*Turdus rufiventris*) (focus of the present project), are both endemic to the Americas and extensively distributed across various countries on the continent. These species are frequently observed in urban settings, and their melodious songs are notably prominent, with the Rufous-bellied being regarded as a symbolic bird of Brazil (Batista et al. 2020; Gill et al. 2024). They exhibit relatively conserved karyotypes compared to the ancestral 2n, with 2n=80 for *T. leucomelas* and 2n=78for *T. rufiventris* (Giannoni et al. 1990; Kretschmer et al. 2014).

Raptors are distinguished by their significant karyotypic diversity and atypical diploid numbers. Species such as *Falco biarmicus* (2n=52), *Harpia harpyja* (2n=58), and *Leucopternis albicollis* (2n=66) are noteworthy for their reduced diploid number, which is indicative of numerous fusion events that have influenced these extensively rearranged karyotypes (De Boer, 1975; Hoffmann et al. 1976; de Oliveira et al. 2005; de Oliveira et al. 2010). Despite the typically low 2n in these groups, cariamiforms are notable among raptors for possessing a high 2n, ranking among the highest chromosome numbers recorded in birds. The two extant species of cariamiforms are remnants of a formerly diverse group that underwent significant adaptations for enhanced size and carnivorous behavior, commonly referred to as terror birds (LaBarge et al. 2024; Degrange et al. 2024). The species *Cariama cristata* (Red-legged Seriema) and

Chunga burmeisteri (Black-legged Seriema) are the sole extant representatives of the family Cariamidae, exhibiting 2n = 108 in *C. cristata* and 2n = 106 in *C. burmeisteri* (Takagi and Sasaki 1980; Sasaki and Takagi 1981; Belterman and De Boer 1984). Both species are endemic to South America, with *C. burmeisteri* confined to a limited area in the continent's center, whereas *C. cristata* predominantly inhabits savanna regions with a broad distribution across the central and western parts of the continent (Brooks 2014).

1.2 Sex chromosomes and the integration of Cytogenetics and Genomics (Cytogenomics)

Similar to mammals, birds possess a stable sex chromosome system that originated from an autosomal pair around 70 million years ago (Nanda et al. 1999; Prum et al. 2015). Multiple hypotheses have been formulated to elucidate the mechanism of its ZW sex chromosome differentiation. The classical model posits that the development of a region or gene associated with sexual differentiation, along with the cessation of recombination between homologous chromosomes, results in the accumulation of repetitive elements and deletions, ultimately causing the degeneration of the sex-specific chromosome and subsequent differentiation from the ancestral homologous pair (Wright et al. 2016; Charlesworth, 2021). Nonetheless, there are instances where recombination is not entirely halted, permitting a substantial region of homology (homomorphism) to persist between the sex-determining chromosomes (Kamiya et al. 2012). In all Neornithes, the ZW sex chromosome system is characterized by males being the homogametic ZZ) and females the heterogametic (ZW) sex. In Paleognaths, excluding the order Tinamiformes, homomorphic sex chromosomes are present, exhibiting minimal differentiation between the Z and W chromosomes. Conversely, all other groups display a heteromorphic system, characterized by a medium sized Z chromosome and a heterochromatic, reduced W chromosome in the majority of species examined to date (Nanda et al. 1999; Stiglec et al. 2007; Prum et al. 2015). It is traditionally posited that the Z chromosome exhibits significant conservation across various avian lineages, a phenomenon corroborated by hybridizations in multiple species utilizing chromosome painting probes from the Z chromosome of *Gallus gallus* (Nanda et al. 2008; Nishida-Umehara et al. 2007). Like other groups, the avian sex-specific W chromosome is typically abundant in repetitive sequences, including satellite DNAs, microsatellites, and transposable elements. The rapid evolution of these sequences results in greater differentiation of the W chromosome among avian species and groups compared to the Z chromosome (Solari and Dresser, 1995; Zhou et al. 2014; Smeds et al. 2015).

Satellite DNAs (SatDNAs) are repetitive sequences prevalent in eukaryotes, distinguished by identical sequences repeated in tandem with variable copy numbers and repeat unit sizes. Cytogenomics holds the potential to elucidate genomes through the integration of sequencing techniques and cytogenetic analyses (Deakin and Ezaz, 2019). This study examined the satellite DNAs of a species (Satellitome) identified through bioinformatics and fluorescent in situ hybridization techniques to elucidate the evolution and chromosomal location of these elements (Ruiz-Ruano et al. 2016). Initially, it was determined that these sequences lacked functions and were categorized as "junk DNA" because they were not transcribed and did not encode proteins. Notwithstanding this, it has recently been established that these sequences serve multiple functions, ranging from structural roles for chromosomes to transcription (Garrido-Ramos, 2017). Satellite DNAs are consistently located in centromeric and telomeric regions, as well as clustered within heterochromatin, serving a crucial function in chromosomal speciation and the evolution of sex chromosomes (O´Neill et al. 2004; Plohl et al. 2012; Utsunomia et al. 2019; Shatskikh et al. 2020; Ferretti et al. 2020). Recent SAtellitome investigations have been undertaken across various taxa, including plants (Ruiz-Ruano et al. 2019; Gálvez-Galván et al. 2024), invertebrates (Ferretti et al. 2020; Cabral-de-Mello et al. 2021; Pereira et al. 2021; Camacho et al. 2022), and vertebrates such as fish (Silva et al. 2017; Utsunomia et al. 2019; Crepaldi et al. 2021; Deon et al. 2024; Kretschmer et al. 2022), crocodilians (Romanenko et al. 2022; Sales-Oliveira et al. 2024), and mammals (Valeri et al. 2021; Sena et al. 2020).

Objectives

2. OBJECTIVES

This work aimed to combine cytogenetic, genomic and bioinformatics analyses in the study of the evolution and differentiation of sex chromosomes in birds. For this purpose, we selected two study models: A) species with conserved karyotypes with a typical ZW system (*T. leucomelas* and *T. rufiventris*, Turdidae) and B) species with highly rearranged karyotypes *(C. cristata and C. burmeisteri*, Cariamidae*),* characterized by an atypical sexual system where the Z chromosome is the largest component of the karyotype.

Results and Discussions

3. RESULTS AND DISCUSSIONS

The results, discussion, and materials and methods sections were compiled in two chapters that correspond to two scientific papers, as presented below.

Chapter I

Satellitome analysis on the pale-breasted thrush *Turdus leucomelas* (Passeriformes; Turdidae) uncovers the putative co-evolution of sex chromosomes and satellite DNAs.

Souza GM, Kretschmer R, Toma GA, Oliveira AM, Deon GA, Setti PG, Santos RZ, Goes CAG, Garnero AV, Gunski RJ, Oliveira EHC, Porto-Foresti F, Liehr T, Utsunomia R, Cioffi MB. Satellitome analysis on the pale-breasted thrush *Turdus leucomelas* (Passeriformes; Turdidae) uncovers the putative co-evolution of sex chromosomes and satellite DNAs. **Scientific Reports** 14, 20656 (2024).

Abstract

Do all birds' sex chromosomes follow the same canonical one-way direction of evolution? We combined cytogenetic and genomic approaches to analyze the process of the W chromosomal differentiation in two selected Passeriform species, named the Pale-breasted Thrush *Turdus leucomelas* and the Rufous-bellied thrush *T. rufiventris*. We characterized the full catalog of satellite DNAs (satellitome) of *T. leucomelas*, and the 10 TleSatDNA classes obtained together with 16 microsatellite motifs were in situ mapped in both species. Additionally, using Comparative Genomic Hybridization (CGH) assays, we investigated their intragenomic variations. The W chromosomes of both species did not accumulate higher amounts of both heterochromatin and repetitive sequences. However, while *T. leucomelas* showed a heterochromatin-poor W chromosome with a very complex evolutionary history, *T. rufiventris showed* a small and partially heterochromatic W chromosome that represents a differentiated version of its original autosomal complement (Z chromosome). The combined approach of CGH and sequential satDNA mapping suggests the occurrence of a former W-autosomal translocation event, which had an impact on the W chromosome in terms of sequence gains and losses. At the same time, an autosome, which is present in both males and females in a polymorphic state, lost sequences and integrated previously W-specific ones. This putative W-autosomal translocation, however, did not result in the emergence of a multiple-sex chromosome system. Instead, the generation of a neo-W chromosome suggests an unexpected evolutionary trajectory that deviates from the standard canonical model of sex chromosome evolution.

Key-words: Molecular cytogenetics, evolution, neo sex chromosomes, translocation, satDNA.

Introduction

Nearly all bird species share the same ZZ/ZW sex chromosome system (an exception to this rule is described by (Gunski et al. 2017) which is widely regarded as a stable sex system with males (ZZ) and females (ZW) representing the homogametic and heterogametic sex, respectively (Griffin et al. 2007; Kretschmer et al. 2018). Both the Z and W sex chromosomes originated from an ancestral autosomal pair more than 110 Mya ago (Nanda et al. 1999; Graves et al. 2001;) In most species (except the Ratites where the sex chromosomes are homomorphic), the W chromosome is small and mostly heterochromatic, whereas the Z is typically preserved in both morphology and gene content (Ohno et al. 1967; Nanda and Schmid 2002; Nishida-Umehara et al. 2007; Nanda et al. 2008; Kretschmer et al. 2018). Despite the conservation of the Z chromosomes, they are subject to frequent intrachromosomal rearrangements, such as inversions, resulting in changes in their morphology within species (Griffin et al. 2007).

With over 6500 species, the order Passeriformes is the most varied group of birds (Gil et al. 2003). Despite this remarkable variety, only ~460 of these species, or 7.2% of the total in this order, have had their diploid number determined (Degrandi et al. 2020). Most Passeriform species have small W chromosomes that have experienced dynamic processes of constitutive heterochromatin accumulation and sequence elimination throughout their evolutionary history (Correia et al. 2009; Barbosa et al. 2013; Dos Santos et al. 2017). Among them, the genus *Turdus* (Thrushes) stands out as the most diverse one with 88 species (Gill et al. 2023). While all Thrushes share common morphological characteristics, they exhibit a wide range of plumage colorations and ecological adaptations. This plasticity allows them to thrive in diverse biomes, including savannahs, alpine areas, and both tropical and temperate forests (Del Hoyo et al. 2019).

Among the 88 species within the *Turdus* genus, 18 of them have had their karyotypes described, revealing a substantial degree of chromosome similarity among them. The diploid number (2n) ranges from 78 to 84, indicating slight variations (Degrandi et al. 2020). Molecular cytogenetics studies using chicken macrochromosome probes (GGA1-10) have been conducted on a limited scale, encompassing only four species: *T. merula*, *T. iliacus*, *T. rufiventris*, and *T. albicollis* (Guttenbach et al. 2003; Derjusheva et al. 2004; Kretschmer et al. 2014). These investigations unveiled a sole interchromosomal rearrangement, specifically the fission of the ancestral chromosome one (GGA1), which is a common characteristic observed among Passeriforms (Griffin et al. 2007; Degrandi et al. 2020). In *T. merula*, except for chromosome 16, which has remained unstudied, no evidence of interchromosomal rearrangements in

connection to the homologous chromosomes to GGA11-28 has been found in any of the microchromosomes (O´Connor et al. 2019).

In recent years, the integration of molecular cytogenetics techniques with *in silico* data derived from the Next Generation Sequence (NGS) and novel software pipelines have provided significant advances in the comprehension of intricate chromosome rearrangements (Furo et al. 2020; Huang et al. 2022; Höök et al. 2023) and in the evolution of sex chromosomes (Stöck et al. 2021; Stundlová et al. 2022). In particular, the characterization and *in situ* mapping of the satelitome, which is a catalog of the most representative satellite DNAs (satDNAs) in a genome (Ruiz-Ruano et al. 2016), is capable of highlighting transpositions and translocation events, giving insights into the framework of karyotype evolution and chromosome speciation (Potter et al. 2017; Garrido-Ramos, 2017; Ahmad et al. 2020; Talbert and Henikoff, 2022). Moreover, although still incipient in birds (however see Peona et al. 2023 and Kulak et al. 2022), investigations in other vertebrates, such as mammals (Sena et al. 2020; Valeri et al. 2021; Gutierrez et al. 2022) and amphibians (Gatto et al. 2018; Da Silva et al. 2023) demonstrated the fast-evolving nature of these *in tandem* repetitive DNAs and their putative role in the formation and composition of centromeres and in the evolution of sex chromosomes (Ferretti et al. 2020; Crepaldi et al. 2020).

Comparative genomic hybridization (CGH) (sometimes also referred as GISH) is a fine-scale molecular cytogenetic approach used to detect chromosomal rearrangements that has also been applied to discover the evolutionary origin and composition of sex chromosome systems (Traut et al. 1999; Symonová et al. 2015). This method allows us to recognize the high level of molecular differentiation of sex chromosomes, localize sex-specific chromosome regions, and track early stages of sex chromosome differentiation in several groups (De Freitas et al. 2018; Giardini et al. 2023). In this way, repetitive DNAs and comparative genomic hybridization mapping, are an attempt to advance toward the knowledge of the processes that have shaped the evolution of sex chromosomes.

Here, we selected two Passeriform species belonging to the Turdidae family, named the Pale-breasted thrush *T. leucomelas* and the Rufous-bellied thrush *T. rufiventris* to analyze the process of their W chromosomal evolution. In that regard, we compared the intragenomic differences (focusing on their repetitive DNA content) between males and females of each species and used cytogenetic and genomic methods to analyze their satDNA composition and their putative involvement in their W chromosomal evolution.

Results

The aim of our work was to characterize and map the satellite DNA sequences present in the species *T. leucomelas* (TLE) and then compare these sequences isolated in a similar species, *T. rufiventris* (TRU) First, we investigated and confirmed that the 2n for both species investigated were 2n=80 for *T. leucomelas* and 2n=78 for *T. rufiventris.* These results corroborated earlier information for these species (Kretschmer et al. 2014; Giannoni et al. 1990). The next step in delving deeper into the previously mentioned issues was to describe *T. leucomelas´* satellitome.

satDNA content of the T. leucomelas genome

After three iterations in TAREAN, 10 satDNA families (TleSatDNAs) were recovered. **Table 1** presents the general characteristics of the *T. leucomelas´* satellitome, such as the A+T content of the satellites, which ranged from 27.2% to 69.6%, with an average of 51.73%, and the length of the repeated units (RUL), which ranged between 21bp and 1644bp with 80% of the satDNAs families having monomers greater than 100bp. By aligning each *T. leucomelas* satDNA in the RM_Homology version 1 (https://github.com/fjruizruano/satminer) and Geneious software version 8.0 (https://www.geneious.com), a superfamily relationship (50% to 80% similarity) was observed between the satDNAs TleSat02-145 and TleSat05-21, which are considered a *high-order repeat* (HOR). The *repeat landscapes* generated are shown in **Supplementary Figure 1**. Comparing the satDNA catalogs of males and females, the presence of two satellites more abundant in females than in males was observed, these being TleSat06-645 (ratio of 3.94 between genders) and TleSat08-419 (ratio of 51.89 between genders) **(Table 1).**

Table 1 General features of *T. leucomelas* satellitome.

satDNA	RUL	Abundance (F)	Abundance (M)	Abundance (F/M)	A+T (%)
TleSat01-1220	1220	0.043067479	0.052959842	0.813210121	48
TleSat02-145	145	0.000925024	0.000867871	1.065853631	46.9
TleSat03-1644	1644	0.000871392	0.00093426	0.932706941	49.8
TeleSat 04-23	23	0.000508905	0.00088081	0.577765438	69.6
TleSat05-21	21	0.00048423	0.00047336	1.022953453	47.6
TleSat06-645	645	0.00046497	0.00011781	3.94678455	62.8
TleSat07-103	103	0.00033896	0.00048223	0.702890814	27.2
TleSat08-419	419	0.000211289	4.07E-06	51.89737354	60.4
TleSat09-638	638	0.000164396	0.00015275	1.076197143	41.2
TleSat10-426	426	0.000140907	0.00025902	0.543988135	63.8

RUL: Repeat unit length; F: female and M: male; A+T: Adenine and thymine content.

Minimmum spanning trees (MSTs)

We selected TleSat05-21 and TleSat07-103 to generate minimum spanning trees (MSTs) **(Figure 1).** These satDNAs were selected due to their monomer sizes (<150 bp), differential abundance between sexes (see **Table 1**), and clusterization after FISH results **(Figure 2).** TleSat05-21 doesn't demonstrate accumulation in sex chromosomes of *T. leucomelas* **(Figure 2),** and the MST is composed of six mainly haplotypes shared between males and females, following the observed ratio of abundance in males and females (**Table 1**). In contrast, TleSat07-103 shows a ratio of 0.70, with more abundance of this sequence in males than in females. The MST demonstrates a predominance of one haplotype, shared between sexes, and several less abundant haplotypes, and some of them are male-specific **(Figure 1),** despite the absence of FISH signals in the Z chromosome.

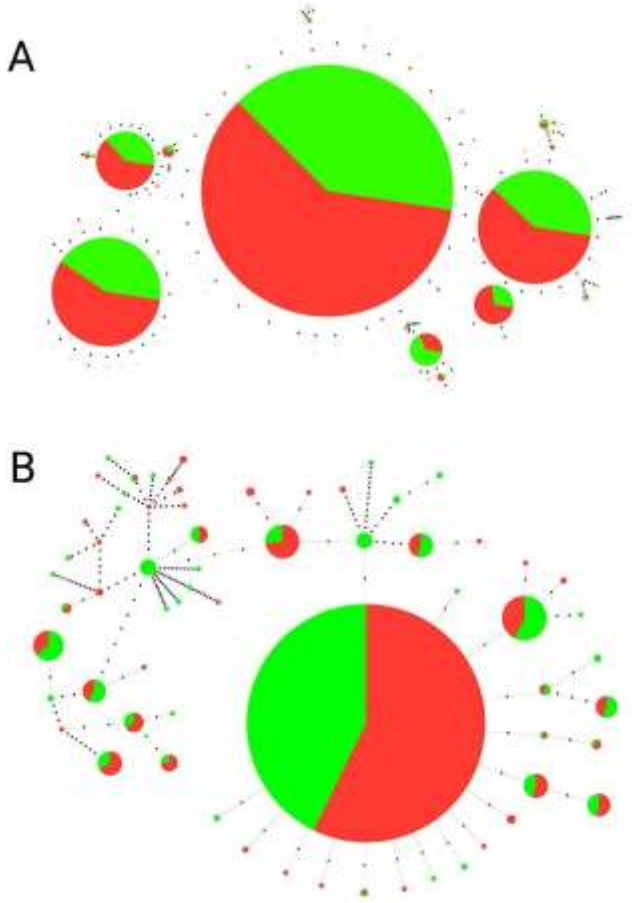


Figure 1. Linear MSTs of A) TleSat05-21 and B) TleSat07-103 obtained from female (red) and male (green) reads. Each circle represents one haplotype and the diameter is proportional to the abundance of the haplotype. Black dots represent a mutation event. Image from Souza et al. (2024).

Chromosomal distribution of TleSatDNAs and microsatellites

Following the in situ investigations, we found that, Except for TleSat02 and TleSat04, all the remaining TleSatDNAs showed positive signals on female chromosome metaphases of *T. leucomelas* **(Figure 2).** The TleSat01 displayed signals in the centromeric region of all chromosomes. TleSat03 was mapped in the centromeric region of two pairs of macrochromosomes, as well as in some microchromosomes. TleSat06 was located in the pericentromeric region of three macrochromosomes, one microchromosome, and on the W. TleSat05, TleSat07, and TleSat09 were exclusively mapped on microchromosomes, while TleSat10 was exclusively mapped on the pericentromeric region of the Z chromosomes (**Figure 2).** The TleSat06 and TleSat08 displayed a variable number of sites among the individuals, indicating a polymorphism related to the satellites, which may involve W-autosomal translocation events or also to transpositions of mobile elements.

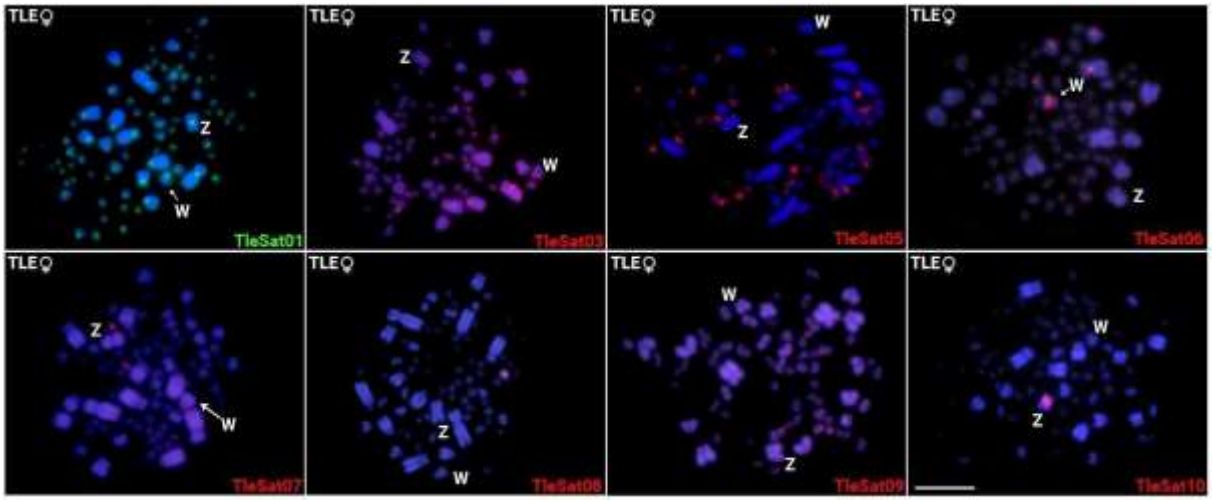


Figure 2. Chromosomal mapping of the eight TleSatDNAs hybridized on female metaphases of *T. leucomelas* (TLE ♀F01). The Z and W sex chromosomes are indicated. While the Z chromosome was identified by its distinct morphology (i.e: the only metacentric macrochromosome), the W chromosome was appropriately identified after a sequential hybridization with TleSat06, which provides a unique and distinctive pattern for this chromosome. Bar = 10 μm. Image from Souza et al. (2024).

In *T. rufiventris,* only six of the 10 TleSatDNA (TleSat01, TleSat05, TleSat06, TleSat07, TleSat08, and TleSat10) showed positive signals after *in situ* experiments (**Figure 3).** The TleSat01 and TleSat10 present the same pattern found in *T. leucomelas,* being mapped in all centromeres and solely on the Z chromosome, respectively. However, TleSat05, TleSat06, TleSat07, and TleSat08 showed different accumulations in *T. rufiventris.* Although TleSat05 and TleSat07 exhibited hybridization signals only in the microchromosomes, like in *T. leucomelas,* no signals for TleSat06 were observed in the W chromosome of this species. TleSat08 only displayed hybridization clusters in a few pairs of microchromosomes (**Figure 3).**

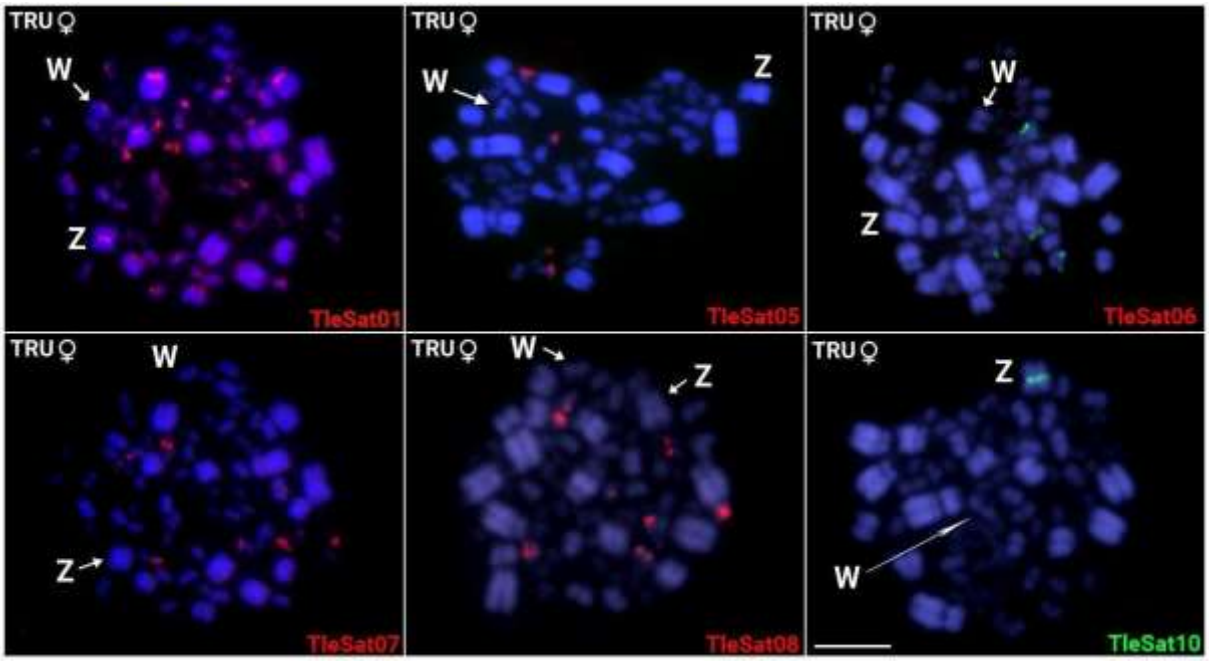


Figure 3. Chromosomal mapping of TleSatDNAs on metaphase plates of T. *rufiventris* (TRU ♀F01). While the Z chromosome was identified by its distinct morphology (i.e: the only metacentric macrochromosome), the W chromosome was appropriately identified after a sequential C-banding, which provides a unique and distinctive pattern for this chromosome. The Z and W sex chromosomes are indicated. Bar = 10 μm. Image from Souza et al. (2024).

Of the total of 16 microsatellites tested in both Pale-breasted Thrush and Rufous-bellied thrush, only two had positive hybridization signals in *T. leucomelas*, named $(GA)_{15}$ and $(CGG)_{10}$, which demonstrated clusters in one and three pairs of microchromosomes, respectively (**Figure 4).** On the other hand, *T. rufiventris* showed positive hybridization signals for three microsatellites, with $(CGG)_{10}$ displaying signals in three pairs of microchromosomes, while $(CAG)_{10}$ and $(CAT)_{10}$ both accumulated in the telomeric region of Z chromosomes **(Figure 4).**

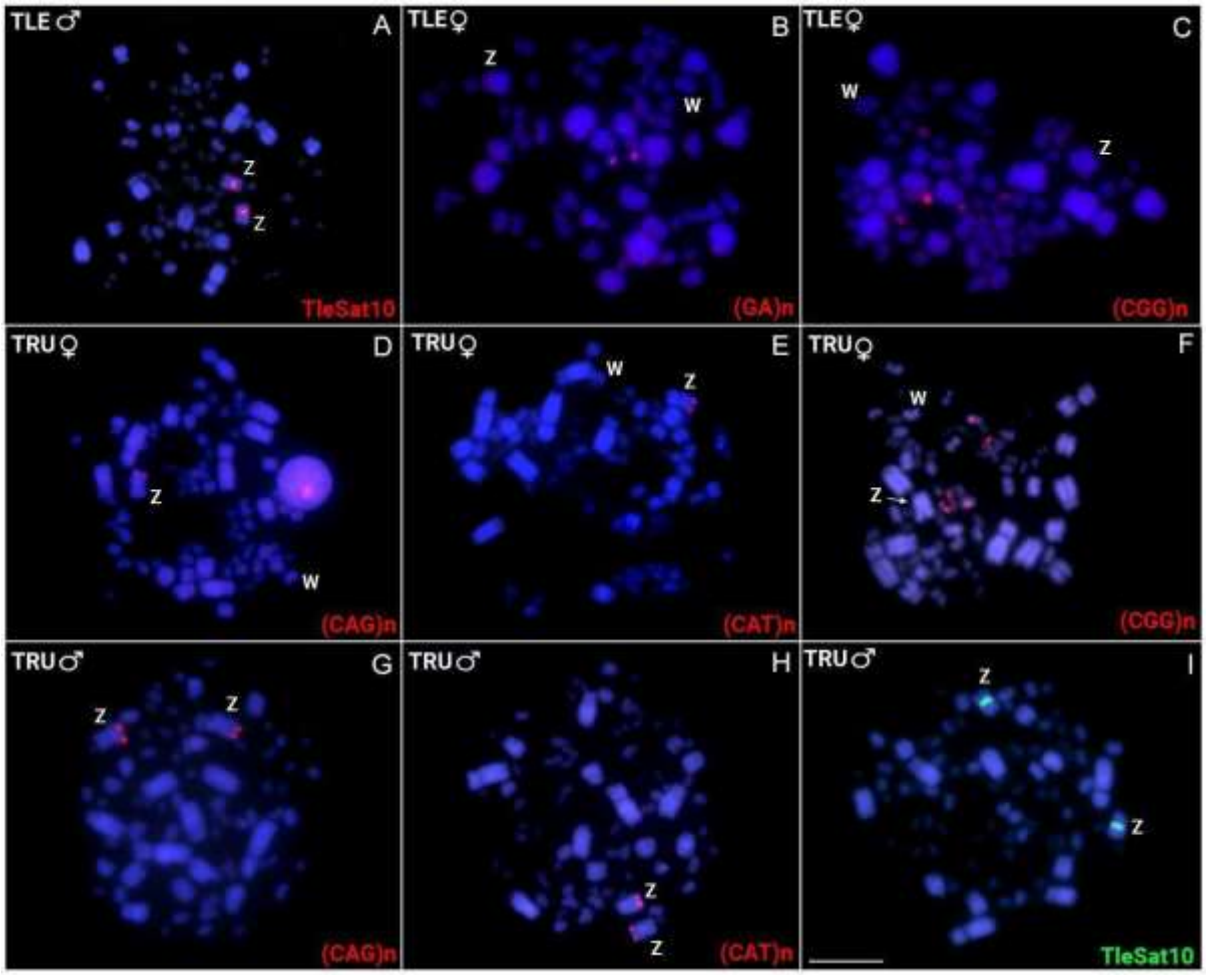


Figure 4 Metaphase plates of *T. leucomelas* TLE♂M01; TLE♀F01; *T. rufiventris* TRU♀F01 and TRU♂M01 highlighting the chromosomal mapping of microsatellites (B-H) and TleSat10 (A and I). Bar = 10 μm. Image from Souza et al. (2024).

Comparative Genomic Hybridization

Lastly, after examining specific sequences for each sex, we found overlapping signals in the pericentromeric regions of almost all chromosomes, except for an exclusive strong female-specific region on the W chromosome, coincident with a C-positive heterochromatic block **(Figure 5, Supplementary Figure 2E).** Contrarily, four distinct hybridization patterns were identified in the *T. leucomelas* individuals **(Figure 6, Supplementary Figures 2 and 3).** In addition to overlapping signals in the centromeric region of all male and female chromosomes, the accumulation of female-biased hybridization signals in the entire W chromosome and half of a small autosome was evidenced in the two females (TLE♀F01 and TLE♀F02) analyzed **(Figure 6D**, **Supplementary Figure 3).** In turn, three different hybridization patterns were found in the four males analyzed. In the TLE♂M01 only overlapping signals of the male and female gDNA probes were detected in the centromeric region of all chromosomes **(Figure 6A, Supplementary Figure 2A).** This same pattern was

also observed in the other three males, in addition to one copy (in TLE♂M02) or two copies (in both TLE♂M03 and TLE♂M04) of the same small autosome displaying female-biased hybridization signals **(Figure 6B-C, Supplementary Figure 2B-D).** We sampled individuals from different populations, thus ensuring that the patterns discovered were not exclusive to a specific population. In all cases, this small autosome also accumulated the TleSat06 and TleSat08 **(Figure 6).** The whole short arms of the W chromosome contain a weak C-positive heterochromatic block. On the other hand, prominent C-positive blocks are observed in the Z chromosome and in the short arms of the small autosome that exhibit the female-biased hybridization signals **(Figure 6).**

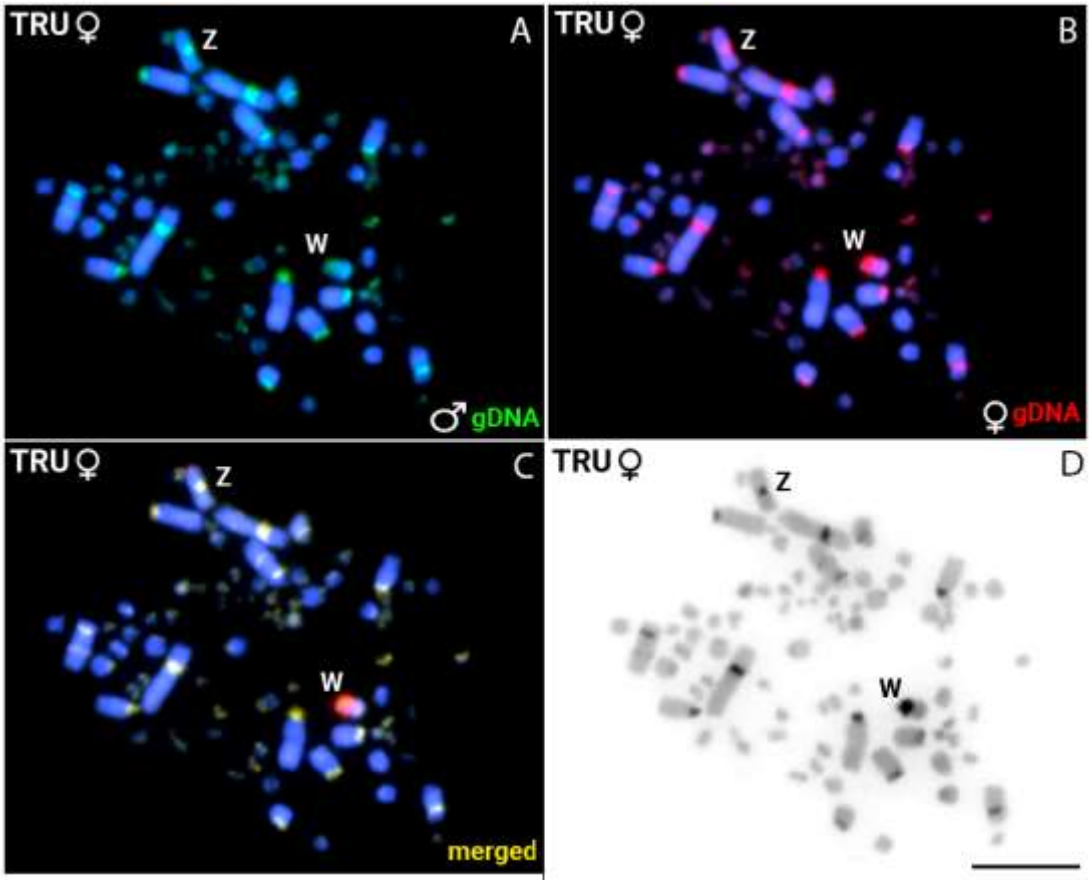


Figure 5. *Turdus rufiventris* male and female genomic DNA probes hybridized on female metaphase chromosomes of *T. rufiventris* (TRU♀F01) following the experimental design described in **Table 3**. The hybridization patterns of the probes derived from male (green), female (red), and the combined pictures are shown in (A), (B), and (C), respectively. The sequential C-banding highlighted a conspicuous C-positive heterochromatic block in the short arms of the W chromosome (D). Bar= 10 μm. Image from Souza et al. (2024).

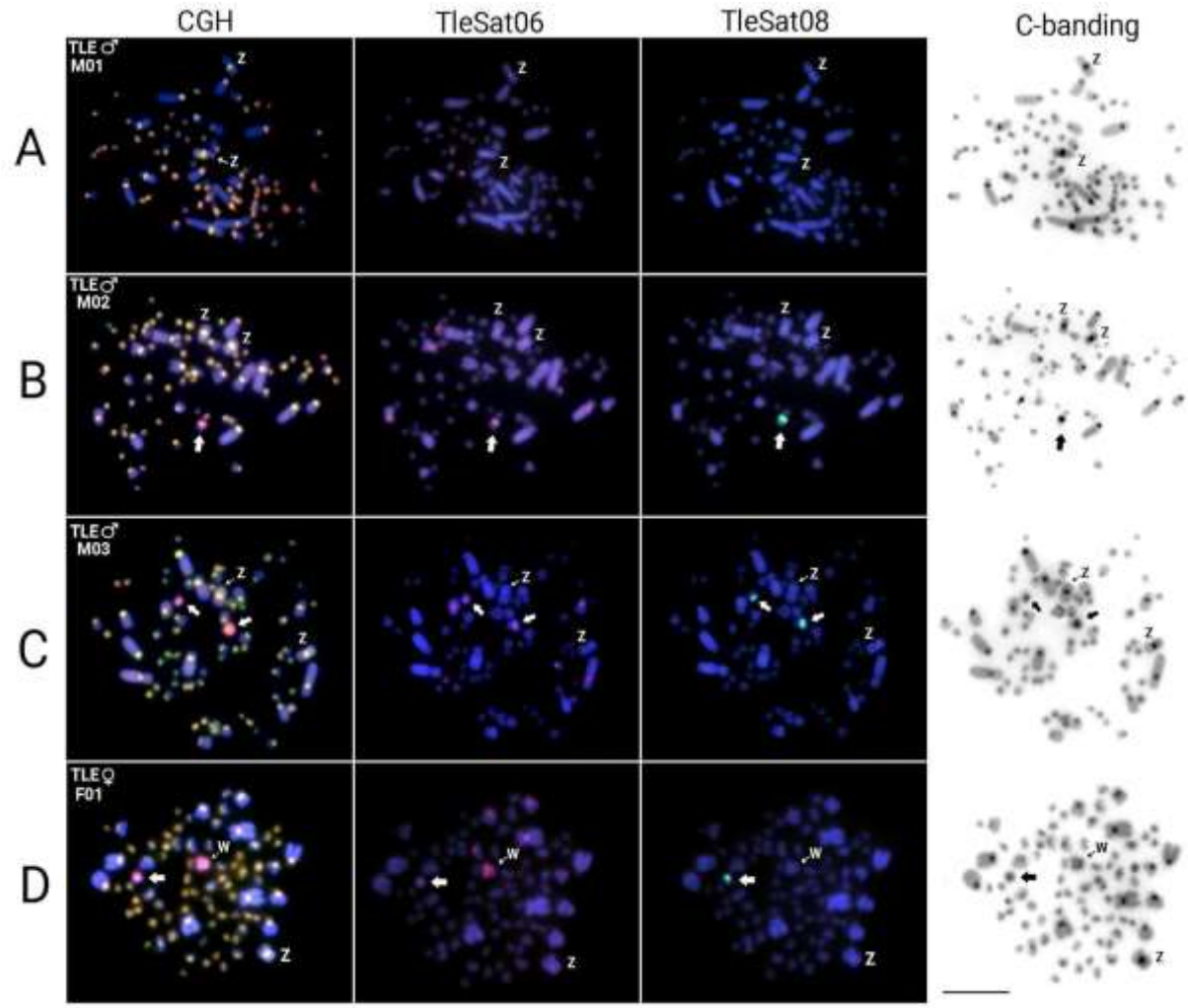


Figure 6 Intraspecific genomic hybridization (CGH) in *T. leucomelas* males: A) TLE♂M01; B) TLE♂M02; C) TLE♂M03 and female D) TLE♀F01 specimens following the experimental design described in **Table 3**. The merged images displayed in the CGH column were obtained from those present in Supplementary Figs. 2 and 3. After the CGH, chromosomes were sequentially mapped with TleSat06 (second column) and TleSat08 (third column) probes, and then C-banded (fourth column). The small autosomes displaying female-biased hybridization signals, the accumulation of Tlesat06 and TleSat08, and a conspicuous C-positive block are indicated by the arrowheads. Bar: 10 μm. Image from Souza et al. (2024).

Discussion

The diploid numbers of both species, *T. rufiventris* and *T. leucomelas*, have already been characterized in previous works, like the morphology of ZW sex chromosomes (Kretschmer et al. 2014; Giannoni et al. 2023). However, despite their similar sizes, no data on the molecular and heterochromatic content of their W chromosomes is currently available. Here, we provide further confirmation of the 2n number [i.e.: *T. leucomelas* (2n = 80) and *T. rufiventris* (2n = 78)] and molecular cytogenetic analyses. We showed that the W chromosome of *T. rufiventris* has a very strong C-positive band on its short arms **(Figure 5),** while the W chromosome of *T. leucomelas* exhibited a faint block of heterochromatin encompassing just its entire short arms **(Figure 6).** Although the occurrence of W chromosomes with unusual morphologies and scarce in heterochromatin has also been evidenced (Gerbault-Seureau et al. 2019; Costa et al. 2021), both these patterns seem atypical for W chromosomes of Passeriformes members, once most species up to now karyotyped have almost entirely heterochromatic W chromosomes, such as in the zebra finch (*Taeniopygia guttata*), the canary (*Serinus canaria*) (Dos Santos et al. 2017), and the Sooty-fronted Spinetail (*Synallaxis frontalis*) (Kretschmer et al. 2018).

To characterize the repetitive DNA fraction of these W chromosomes, we first isolated and characterized the satellitome of *T. leucomelas* and further *in situ* mapped the 10 TleSatDNAs obtained, together with 16 microsatellite motifs in both *T. leucomelas* and *T. rufiventris* species. The data confirm the previous findings obtained in some few bird species highlighting that avian satellites are usually composed of a small number of particularly large satDNAs rich in GC content (Peona et al. 2023). However, this is the first case where the satellitomes were mapped in their respective chromosomes. The MSTs produced in this work demonstrate a prevalence of shared haplotypes between males and females to TleSat05 and TleSat07, due to their presence in autosomal microchromosomes, as demonstrated by FISH. The presence of TleSat07 haplotypes exclusive to males, together with its higher frequency in males, implies the presence of Z chromosome clusters that are not visible by FISH, possibly because of the small array sizes **(Figures 1 and 2).**

Except for TleSat01, which is present in the centromeric region of all *T. leucomelas* and *T. rufiventris* chromosomes (probably representing their primary centromere component), and TleSat06, which accumulated exclusively in *T. leucomelas*' W chromosome, we did not detect any evidence of accumulation for the extant TleSatDNAs on the W chromosomes of both species **(Figures 2 and 3).** Similarly, none of the microsatellites examined in this study were accumulated on any W chromosomes **(Figure 4).** The heterogametic chromosomes (W and Y) tend to differentiate once recombination ceases and heterochromatization followed by the

accumulation of repetitive elements begins (Charlesworth, 2002; Poltronieri et al. 2014). In this pathway, Singh et al. (1980) proposed that the accumulation of satDNA sequences throughout the length of the sex-specific (Y and W) chromosome plays a significant role in generating its morphological differentiation from the X or Z, respectively. Likewise, microsatellite repeats are crucial for the differentiation of sex-specific chromosomes, as they may be the first type of repeat that accumulates during its early stages of differentiation (Cermak et al. 2008; Kubat et al. 2008). Accordingly, reports from various taxa show the accumulation of repetitive sequences specifically on the Y or W chromosomes, which are enriched in high-, middle-, and low-copy repetitive sequences and contain only a few functional genes (Pokorná et al. 2011; Poltronieri et al. 2014; Palacios-Gimenez et al. 2017). However, it is not a rule that most repetitive sequences are found exclusively in heterogametic chromosomes, as revealed by several groups (Cioffi et al. 2010a; 2010b; De Oliveira et al. 2017). Here, an exceptionally high number of repetitions accumulated on the Z chromosomes, including centromeric clusters of TleSat10 in both species as well as (CAG)n and (CAT)n in the terminal region of the q arms of the Z chromosomes of *T. rufiventris* **(Figures 2-4).** This scenario is unusual among birds since very few cases of repeat accumulation on the Z chromosomes were documented (De Oliveira et al. 2017; Furo et al. 2017; Barcellos et al. 2019; Kretschmer et al. 2021).

Instead, the great majority of TleSatDNAs was mapped in microchromosomes in both species. Experiments in other bird families, including Caprimulgidae and Picidae, have also demonstrated a high density of repetitive microsatellite and telomeric sequences in microchromosomes (International Chicken Genome Sequencing Consortium 2004; De Oliveira et al. 2017; De Souza et al. 2020). Similarly, in some species of turtles and lizards, the accumulation of these repeats in microchromosomes has also been shown (Yamada et al. 2005; Srikulnath et al. 2021).

In both thrush species, the conventional chromosomal analysis, C-banding, and repetitive DNA mapping pointed to a specific W chromosome arrangement that differs from the majority of avian species up to now analyzed (Dos Santos et al. 2017; Kretschmer et al. 2018b), since it does not reveal many repeated sequences or significant blocks of heterochromatin accumulating on these chromosomes. In addition, this particular scenario was shown to be even more complex when intraspecific CGH analyses were performed. While the *T. rufiventris* specimens presented the expected overall results after intraspecific-CGH experiments (i.e., the W chromosome showing the only particularly rich region in the female-biased hybridization signals), an unusual pattern was observed in the *T. leucomelas* individuals **(Figure 6).** In the two females of *T. leucomelas* (TLE♀F01 and TLE♀F02), besides the entire

W chromosome, half of a small autosome is also enriched by the female-biased hybridization signals. Except for the TLE♂M01 specimen, the other males show a polymorphic state for this same small autosome, i.e., with only one copy (TLE♂M02) or two copies (both TLE♂M03 and TLE♂M04) of those female-biased hybridization signals **(Figure 6, Supplementary Figures 2 and 3).** So, it is likely that this portion of the autosome enriched by the female-biased hybridization signals was originally part of the W chromosome. Therefore, the occurrence of a W-autosomal reciprocal translocation (not involved in the creation of a multiple-sex chromosome system) is one of the hypotheses that best explains this complex scenario, where the W chromosome both gained and lost sequences (Charlesworth et al. 1994), as well as the small autosome (also present in males) which incorporated both TleSat06 and TleSat08 (the latter, being previously W-specific and now present in its short arms) **(Figure 7).** Likely, this chromosomal rearrangement does not lead to a dosage composition problem for individuals exhibiting either the heteromorphic or homomorphic condition, as the translocated segment encompasses repetitive DNA sequences (as shown by our CGH-SatDNA-FISH analysis), which are usually transcriptionally silenced (Charlesworth et al. 1994; Jurka et al. 2007). However, we cannot exclude an alternative hypothesis, as the presence of repetitive sequences may significantly change due to various parameters, such as copy number variation (expansions and contractions), their genome location, and sometimes even as a result of transposition events and/or major chromosomal rearrangements (Charlesworth et al. 1994; Jurka et al. 2007; Biscotti et al. 2015).

How do sex chromosomes evolve? Up until recently, it was widely believed that the sex chromosomes followed a canonical one-way direction of evolution, which was proposed by gathering information from multiple independent works (Muller 1918; Ohno et al. 1967; Rice 1984; Charlesworth et al. 2014). This standard sex chromosome evolution model predicts that the Y and W chromosomes gradually differentiate and most of their genetic material is lost owing to a lack of recombination with the X or Z, respectively. This leads to the usual gradual loss of genes or gene function and structural modifications like deletions and heterochromatinization. As a result, the sex-specific chromosome might progressively shrink and ultimately be eliminated from the genome (White 1973; Graves 2006; Charlesworth 2015). At first glance, since all Neognathae species, with a few exceptions, have small and heterochromatic W chromosomes, this seemed to be also the standard path taken by all bird sex chromosomes (reviewed in (Pigozzi and Solari 1999)) (Wang et al. 2014). Besides, Ratite birds, which represent the basal avian lineage (paleognaths), present sex chromosomes at an early stage of differentiation, where Z and W chromosomes are still morphologically similar (Ogawa

et al. 1998; Nishida-Umehara et al. 1999; Nishida-Umehara et al. 2007; Tsuda et al. 2007). Our data in *T. leucomelas*, however, points to an unusual evolutionary pathway for the W chromosome that deviates from the standard canonical model of sex chromosome evolution.

Novel investigations (see for example (Perrin 2009; Furman et al. 2020; Meisel 2020; Perrin 2021; Kratochvíl et al. 2021)) are steadily describing new deviant models that differ from the canonical one-way direction of evolution. In particular, cases of new genetic material being added to the sex chromosomes are outstanding examples as they contradict the so-thought inevitable degeneration of the heteromorphic sex chromosome (Charlesworth 2015; Kratochvíl et al. 2021). In these models, new linkage groups can be created by extensive amplification of sequence copy number, brought by molecular drive, and/or sex chromosome-autosome translocations (Kratochvíl et al. 2021) In the latter, a reciprocal translocation (i.e., DNA segments are swapped mutually between chromosomes), results in two possible scenarios. The first, leads to a multiple-sex chromosome system, as the two linkage groups, being consequently whole chromosomes, remain in the form of the larger translocation product (e.g., Neo-sex chromosome) (Schubert and Lysak 2011). The second scenario, however, does not alter the 2n number, and consists of a non-homologous exchange between different sections of two or more chromosomes, thus generating independent Neo-chromosomes that share common DNA motifs (Schubert and Lysak 2011). Regarding W-autosome translocations, despite the unique multiple ♂$Z_1Z_1Z_2Z_2$/♀Z_1Z_2W sex chromosome system described (Gunski et al. 2017), recent studies have revealed the fusion of sex chromosomes and autosomes in different bird lineages, indicating that this type of rearrangements is more common than initially believed. For example, the fusion of ZW sex chromosomes with chromosome 11 has been observed in the ancestor of parrots (Huang et al. 2022). Additionally, in the parrot *Myiopsitta monachus*, chromosome 25 has been further fused to the sex chromosomes (Huang et al. 2022). In the cuckoo species *Crotophaga ani* a Robertsonian translocation between the microchromosome 17 and the Z chromosome was found (Kretschmer et al. 2021). Among songbirds (Sylvoidea), a series of papers have indicated that autosomal material had been integrated into both Z and W (Pala et al. 2012; Sigeman et al. 2019). Therefore, the evolution of bird W chromosomes is revealing more dynamic than previously thought as new data derived from cutting-edge sequencing and cytogenetic investigations (such as the ones described here) become available.

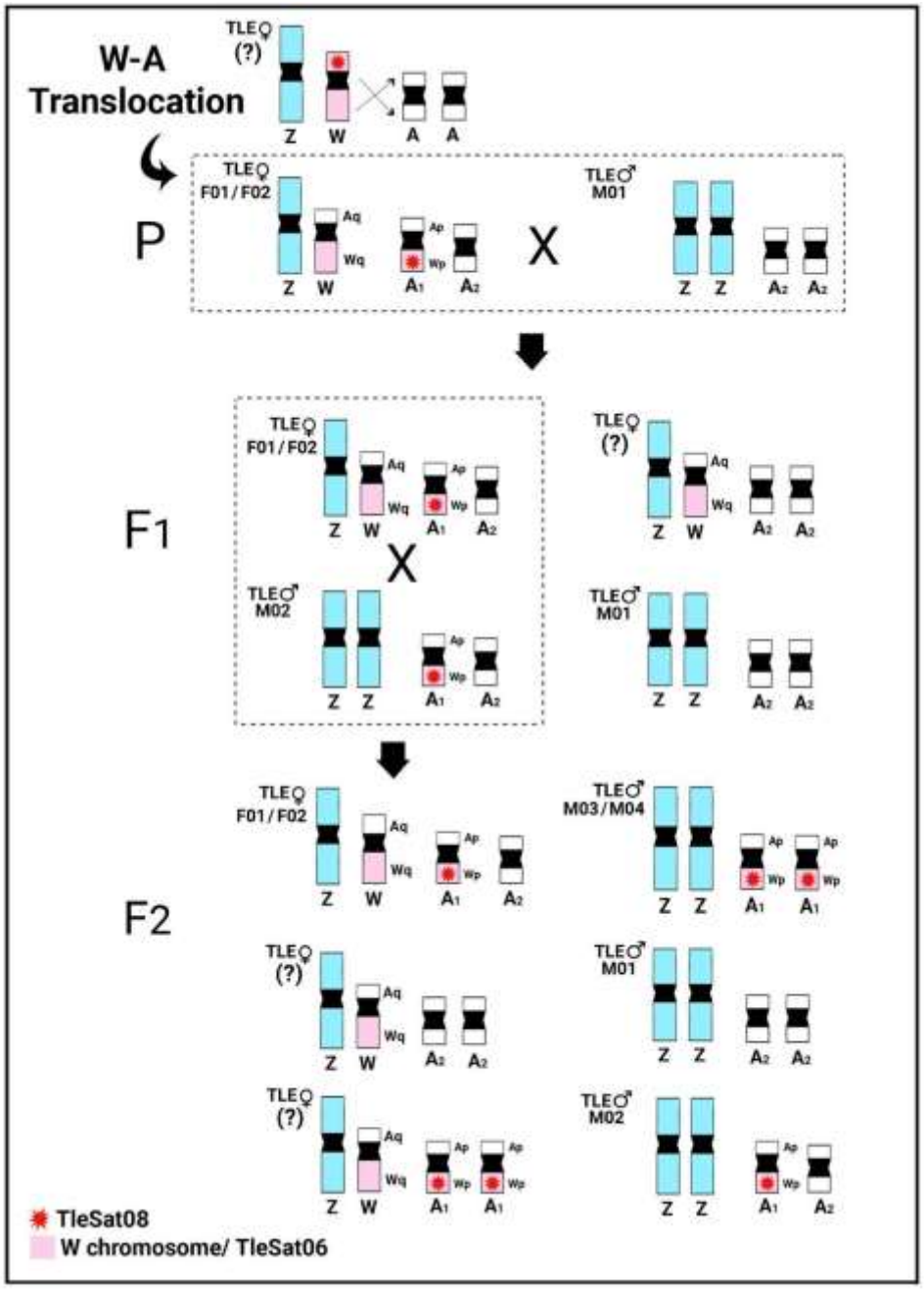


Figure 7. Idiogram representing one of the main hypotheses that involve a translocation event in *T. leucomelas* specimens and the resulting genotypes from possible crossings. An ancestral female undergoes a translocation between the short arms of the W chromosome and an autosome (A). As a result, the W chromosome acquired and lost sequences, while an autosome (A1) also lost sequences and integrated previously W-specific sequences, which included both TleSat06 and TleSat08, while its homologous remained untouched (A2). This pattern (ZW+A1A2) is observed in both *T. leucomelas* (TLE♀F01/F02). When crossed with a wild-type ZZ+A2A2 male (TLE♂M01), it produces an F1 offspring with four possible results: (i) ZW +A1A2 females (TLE♀F01/F02); (ii) ZW+A2A2 females (?); (iii) ZZ+A1A2 males (TLE♂M02), and (iv) ZZ+A2A2 males (TLE♂M01). A new crossing between TLE♀F01/F02 (ZW+A1A2) x TLE♂M02 (ZZ+A1A2) generates an F2 offspring with six possible results: (i) ZW +A1A2 females (TLE♀F01/F02); (ii) ZW+A2A2 females (?); (iii) ZW+A1A1 females (?); ZZ+A1A1 males (TLE♂M03/M04), and (iv) wild-type ZZ+A2A2 males (TLE♂M01). Individuals marked with (?) stand for those that we were unable to find in this work. Image from Souza et al. (2024).

Conclusions

Why have birds´ W chromosomes endured for more than 100 Myr? We demonstrate that its evolution could be far more complex than previously thought. We showed that the W chromosomes of both thrushes did not accumulate higher amounts of heterochromatin and repetitive sequences, as observed in most bird species. Besides, the W chromosome of the pale-breasted thrush, instead of representing a straightforward "degenerated" version of its earlier homologous Z chromosomes, may represent a dynamic “patchwork” that includes deletions and the integration of new genomic material as a result of chromosomal rearrangements with autosomes. Specific satDNA families were directly associated with these rearranged regions. These findings challenge the unidirectional evolutionary process of W chromosomes widely proposed for birds.

Methods

Sampling, Chromosomal preparation, and C-banding

The samplings of *T. leucomelas* and *T. rufiventris* were authorized by the Brazilian environmental agency ICMBio/SISBIO (Licenses 61047-4, 44173-1, and 68443-2) and SISGEN (A96FF09). Each individual of *T. leucomelas* and *T. rufiventris* was assigned a code (i.e., male - M; female -F) **(Table 2).** Mitotic chromosomes were obtained according to the protocols described by (Furo et al. 2017) and (Garnero and Gunski 2000), which utilized skin biopsies and bone marrow for fibroblast culture, respectively. The constitutive heterochromatin regions were evidenced following the protocol proposed by (Sumner 1972). All experiments followed the guidelines and were approved by the Ethics Committee on Animal Experimentation of the Federal University of Pampa (018/2014 and 019/2020). The authors complied with ARRIVE guidelines.

Table 2 List of analyzed species, with the indication of the respective collection location, sample number (N), sex of individuals collected, and the code applied for all analyzed individuals.

Species	Location	N	Individuals
Turdus leucomelas (TLE)	Porto Vera Cruz (RS) – Brazil	(02♀; 03♂)	F01, F02; M01, M03, M04
Turdus leucomelas (TLE)	Belém (PA) - Brazil	(--♀; 01♂)	M02
Turdus rufiventris (TRU)	São Gabriel (RS) – Brazil	(02♀; 02♂)	F01, F02, M01, M02

RS = Rio Grande do Sul; PA= Pará Brazilian States.

The genomic DNAs (gDNAs) from *T. leucomelas* and *T. rufiventris* individuals were extracted following the protocol described by (Sambrook and Russel 2001). DNA samples from *T. leucomelas*♀F01 and *T. leucomelas*♂M01 were sequenced using the BGISEQ-500 platform (paired-end 2x150bp) with a 3x coverage normally required for satellite assembly (Ruiz-Ruano et al. 2016; Utsunomia et al. 2019). The genomic reads obtained were deposited in the Sequence Read Archive (SRA) under accession numbers SRR26625300 (male) and SRR26625299 (female).

Bioinformatic analyses: Construction of T. leucomelas satellite DNA catalogs and additional analyses

The genomic libraries were subjected to a process of quality trimming using the software Trimmomatic version 0.36 (https://github.com/usadellab/Trimmomatic) (Bolger et al. 2014). After, the satellitome of a female of *T. leucomelas* was characterized using the TAREAN

tool (Novák et al. 2020), following the SatMiner pipeline (Ruiz-Ruano et al. 2016). Then, the outputs containing the putative consensus sequences of satDNAs were used to filter the genomic libraries using the software Deconseq version 0.4.3 (https://deconseq.sourceforge.net) (Schmieder and Edwards 2011), and other iterations of TAREAN were performed until no satDNAs were found. After the characterization of all consensus sequences, we filtered and removed other tandemly repeated elements, such as multigene families, and a homology search using RepeatMasker (Smit et al. 2020) was performed to group the sequences as the same variant (similarity greater than 95%), variants of the same satDNA (similarity between 80% and 95%), and superfamilies (similarity between 50% and 85%), following the patterns established by (Ruiz-Ruano et al. 2016). The abundance and divergence of each satDNA were estimated in females and males using RepeatMasker software version 3.0 (https://www.repeatmasker.org) (Smit et al. 2020), with a random selection of 2 x 5,000,000 reads. After that, satDNA families were named according to their abundance in *T. leucomelas*. Considering the particularities of the sex chromosome system of *T. leucomelas*, the quotient between the abundance of each satDNA in females and males (F/M) was calculated to verify putatively accumulated satDNAs in the sex chromosomes. TleSatDNAs were deposited in GenBank with accession numbers OR675141.1- OR675150.

Besides, we selected TleSat05-21 and TleSat07-103 to construct minimum spanning trees (MSTs). Only these satDNAs were selected due to technological limitations, in which it is only possible to use satDNAs whose monomer size is smaller than the read size (< 150 bp in this case). We extracted monomers of the cited satDNAs from genomic libraries of both sexes, followed by alignment of the reads against each satDNA, to select only full reads. After that, we discarded singletons using CD-Hit software version V4.8.1 (https://sites.google.com/view/cd-hit/home?authuser=0) (Fu et al. 2012). Finally, the MSTs were constructed using PHILOVIZ 2.0 software version 2.0 (https://www.phyloviz.net) (Nascimento et al. 2017), and Inkscape was utilized to produce the final image.

Primer design and amplification using Polymerase Chain Reaction (PCR)

A total of ten satDNA sequences (hereafter named TleSatDNAs) were isolated (**Table 1**), for which eight were designed primers (TleSat01, TleSat02, TleSat03, TleSat06, TleSat07, TleSat08, TleSat09, and TleSat10). The remaining two (TleSat04 and TleSat05) were synthesized and labeled with Cy3 at the 5' end by ThermoFisher (ThermoFisher Scientific), since they are smaller than 30bp. The PCR reactions followed the conditions optimized according to (Kretschmer et al. 2022). To confirm the amplification of each satDNA, the PCR

products were subjected to electrophoresis in a 1% or 2% agarose gel, and subsequently quantified by the ThermoFisher NanoDrop spectrophotometer (ThermoFisher Scientific).

Fluorescence in situ Hybridization (FISH)

All TleSatDNAs were labeled using a nick translation Kit from Jena Bioscience (Jena, Germany) incorporating the fluorophore Atto488-dUTP or Atto550-dUTP according to the instructions in the manufacturer's manual. Microsatellite sequences $(GAA)_{10}$, $(GAC)_{10}$, $(CGG)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(GAG)_{10}$, $(TAA)_{10}$, $(TAC)_{10}$, $(CAA)_{10,}$ $(GA)_{15}$, $(CA)_{15}$, $(GC)_{15}$, $(TA)_{15}$, $(C)_{30}$, and $(A)_{30}$ were labeled directly with Cy3 at the 5' end during synthesis (VBC Biotech, Vienna, Austria) and also used in the hybridization experiments. We performed the fluorescence *in situ* hybridization experiments following the protocol described by (Kretschmer et al. 2023). The slides were dehydrated in a 70%, 85%, and 100% ethanol solution and the metaphases were stained with 4',6-diamidino-2-phenylindole (DAPI).

Comparative Genomic Hybridization (CGH)

We performed intraspecific CGH in both *T. leucomelas* and *T. rufiventris* individuals following the experimental designs described in **Table 3**. For this purpose, gDNAs from males and females of each species were respectively labeled using a nick-translation labeling kit with Atto488-dUTP (green) and Atto550-dUTP (red), from Jena Bioscience (Jena, Germany). To block common genomic repetitive regions, we used Cot-1 DNA derived from the male gDNA of each species, produced according to (Zwick et al. 1997). Each hybridization was composed of 3µg of male-derived Cot-1 DNA and 500ng of each labeled male and female gDNAs. After using ethanol-precipitation, the pellet was air-dried and well mixed with 20µL of hybridization buffer (Denhardt's buffer, pH 7.0), composed of 50% formamide, 2% 2xSSC, 10% SDS, 10% dextran sulfate. The CGH experiments followed the methodology detailed in (Yano et al. 2017). After the CGH experiments, the *T. leucomelas* chromosomal slides were washed 3 times in a 4SSC-Tween solution at 42°C, and sequentially *in situ* mapped with TleSat06 (red) and TleSat08 (green) probes. Finally, the material was sequentially C-banded using the abovementioned probes and protocols.

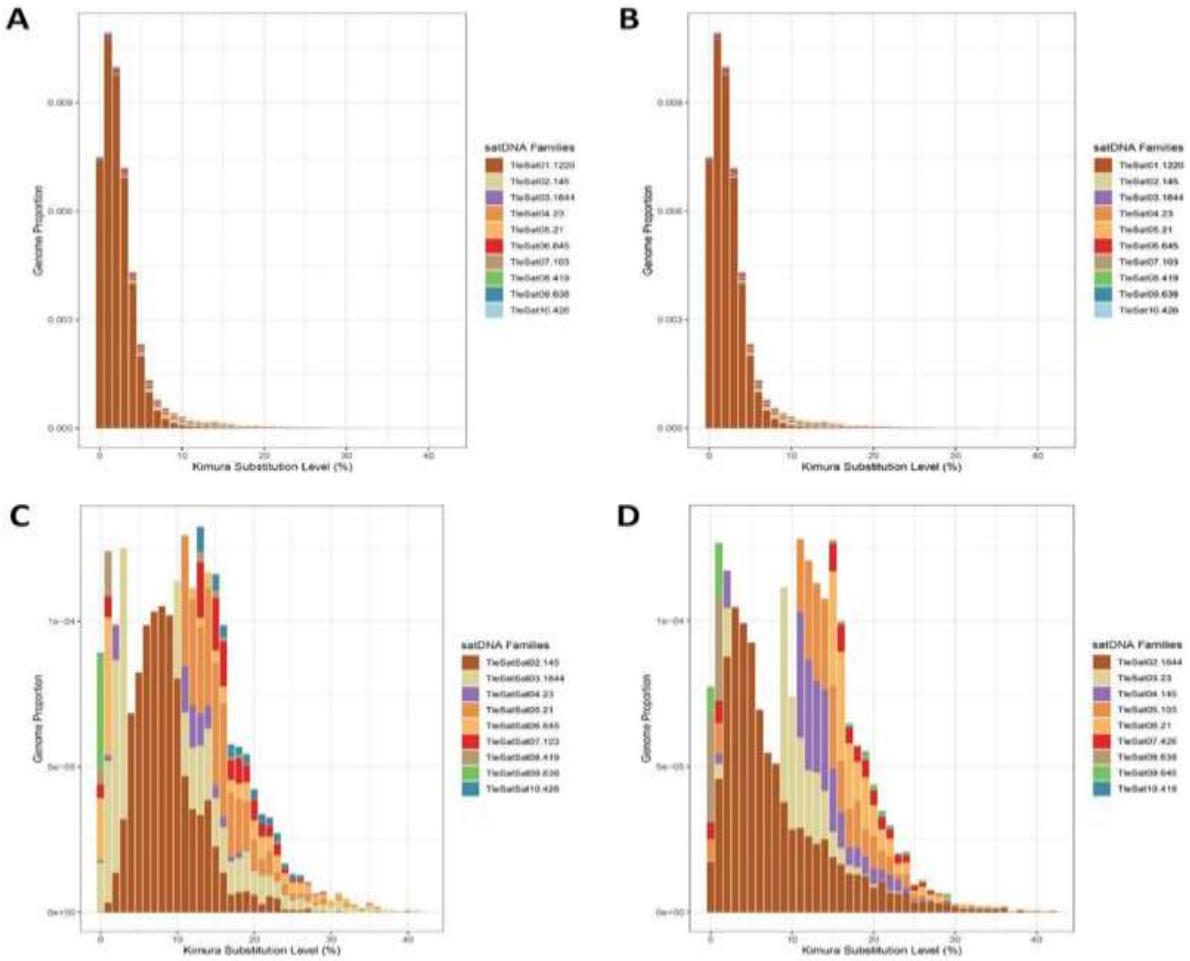
Table 3 The experimental design used for intraspecific comparative genomic hybridization indicating the chromosomal background, the gDNAs probes applied, and the corresponding results.

Chromosomal background	**gDNA probes**	**Results**
TRU ♀F01	♀F01 x ♂M01	Fig. 5
TRU ♀F02	♀F02 x ♂M01	Data not shown
TRU ♂M01	♀F01 x ♂M01	Sup Fig. 2E
TRU ♂M02	♀F01 x ♂M02	Data not shown
TLE ♂M01	♀F01 x ♂M01	Fig. 6A and Sup Fig. 2A
TLE ♂M02	♀F01 x ♂M02	Fig. 6B and Sup Fig. 2B
TLE ♂M03	♀F01 x ♂M03	Fig. 6C and Sup Fig. 2C
TLE ♂M04	♀F01 x ♂M04	Sup Fig. 2D
TLE ♀F01	♀F01 x ♂M01	Fig. 6D and Sup Fig. 3A
TLE ♀F02	♀F02 x ♂M01	Sup Fig. 3B

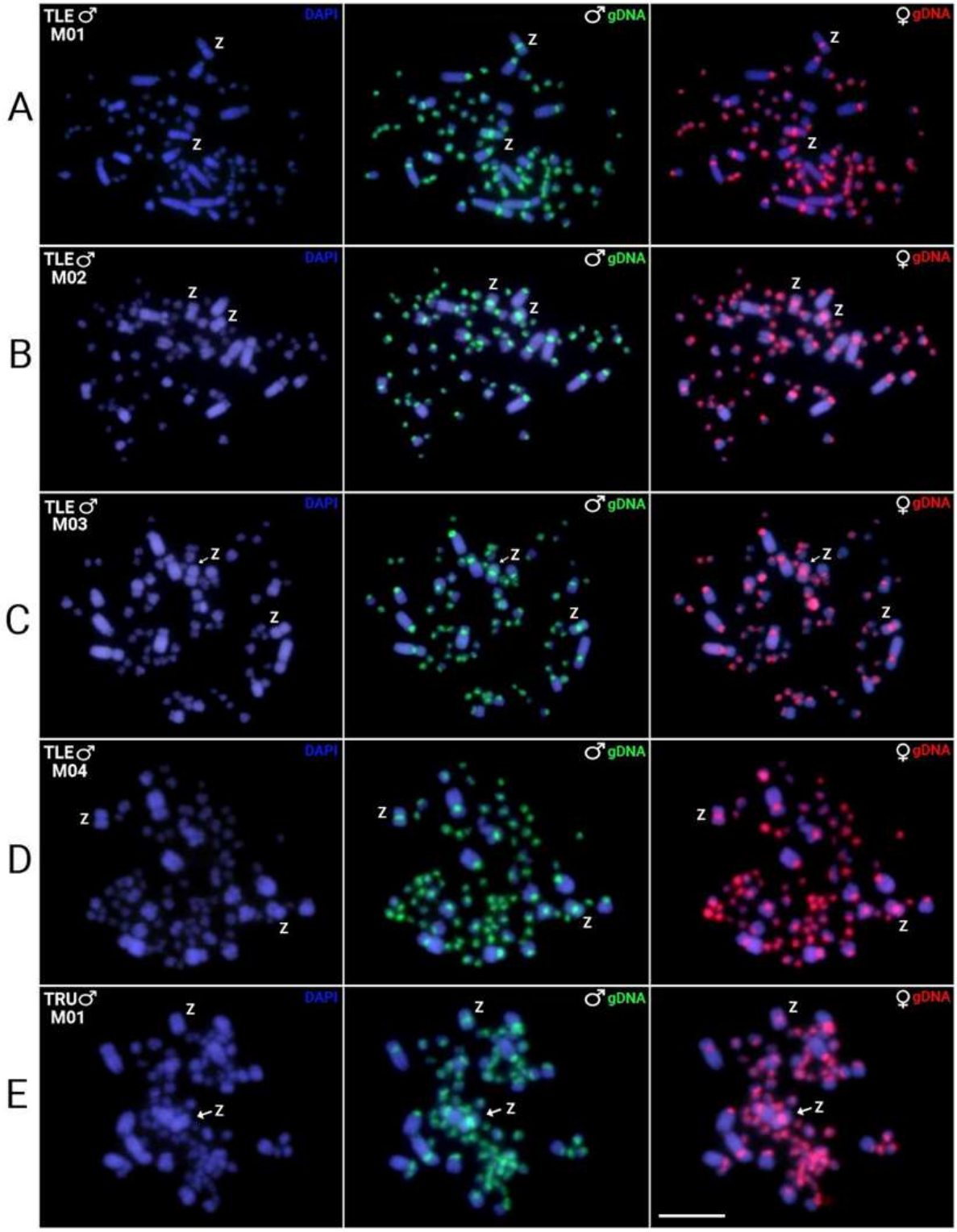
Microscopic Analysis and Image Processing

To corroborate the 2n, karyotype structure, FISH, and CGH results, at least 30 metaphase spreads per individual were examined. Images were captured using an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan), with CoolSNAP, and the images were processed using Image-Pro Plus software version 4.1 (https://mediacy.com/image-pro) (Media Cybernetics, Silver Spring, MD, USA). Chromosomes were classified according to (Levan et al. 1964).

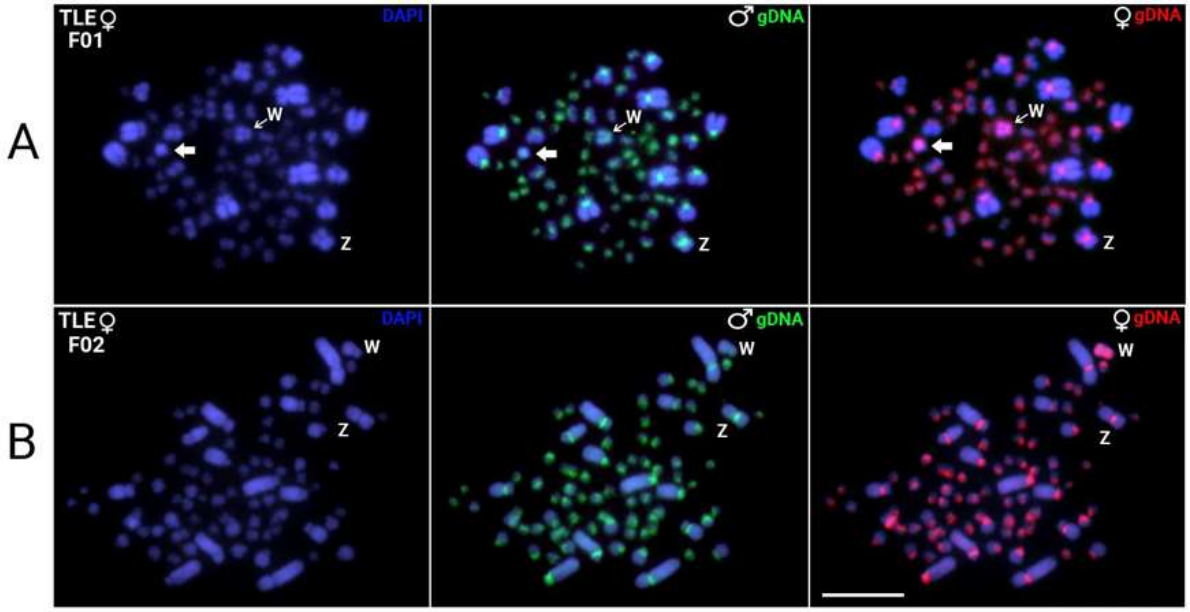
Supplementary Figures



Supplementary Figure 1. Repeat landscapes of the TleSatDNAs in female (A) and male (B) of TLE. In addition, repeat landscapes of females (C) and males (D) suppress TleSat01–1220. Image from Souza et al. (2024)



Supplementary Figure 2 Comparative genomic hybridization in all the four *T. leucomelas* and one *T. rufiventris* male individuals analyzed in the present work: TLE♂M01, TLE♂M03, TLE♂M04 (A, C, D) from Porto Vera Cruz (RS), TLE♂M02 from Belém (PA) (B), and TRU♂M01 (E). The first column corresponds to DAPI, the second to male gDNA, and the last one to female gDNA. The marged images are located in **Figure 6** and **7**. Both TLE♂M03 and TLE♂M04 showed similar patterns of intragenomic-CGH, for this reason only TLE♂M03 is represent on Fig. 6. Image from Souza et al. (2024)



Supplementary Figure 3 Comparative genomic hybridization in all the two *T. leucomelas* female individuals analyzed in the present work: TLE♀F01 and TLE♀F02. The first column corresponds to DAPI, the second to male gDNA, and the last one to female gDNA. The merged images are located in Fig. 7. Both TLE♀F01 and TLE♀F02 showed similar patterns of intragenomic-CGH, for this reason only TLE♀F01 is represented in Fig. 6. Image from Souza et al. (2024).

References

The reference list is compiled at the end of the document.

Chapter II

Cytogenomic analysis in Siriemas (Cariamidae): Insights into an atypical avian karyotype

Souza GM, Vidal JAD, Utsunomia R, Deon GA, Oliveira EHC, Frana RT, Porto-Foresti F, Liehr T, Souza FHS, Kretschmer R, Cioffi MB. Cytogenomic analysis in Seriemas (Cariamidae): Insights into an Atypical Avian Karyotype**. Journal of Heredity** (*under review*)

Abstract

Contrasting with most bird species that present an ancestral-like karyotype (with 2n = 80), the only extant Cariamidae birds, the Red-legged (*Cariama cristata*) and Black-legged (*Chunga burmeisteri*) Seriemas, have high 2n and atypically large Z chromosomes. We combined genomic, bioinformatic, and cytogenetic investigations to find out why *C. cristata*'s Z chromosome is so large compared to its autosomes. We started investigating the genomic organization of repetitive DNAs and their possible role in the Z expansion. Additionally, whole-genome alignments and chromosomal painting were performed against the emu (a species with an ancestral-like karyotype). The satellitomes of *C. cristata* and *C. burmeisteri* were composed of only four and 6 long satDNAs, respectively. These satDNAs showed similarity with other repetitive sequences, mostly transposable elements, and were mapped in the pericentromeric regions of several chromosome pairs. CcrSat02-1104 mostly covered the Z and W sex chromosomes, besides being spread throughout additional chromosomes. Interstitial telomeric sites were not detected, even in the Z chromosome, and none of the 16 microsatellites tested showed positive signals on the *C. cristata* chromosomes. The genome alignments showed that karyotype evolution occurred in *C. cristata* may have involved significant chromosomal reshuffling, particularly fission. Despite certain internal inversions, the Z chromosome remained homologous to the emu's. We suggested that the Z chromosome became the largest component in the karyotype due to fission events in the earliest ancestral macrochromosomes. Nonetheless, repetitive sequences also accumulated on the Z chromosome, resulting in its expansion relative to the pattern observed in ancestral avian groups.

Keywords: repetitive DNA, sex chromosome evolution, birds

Introduction

Birds are a very diverse group of vertebrates, consisting of around 11,000 living species classified into 44 orders, 253 families, and approximately 2,381 genera (Gill et al. 2024). Neoaves, which includes all modern birds except for Palaeognathae and Galloanserae, account for 95% of all diversity, but the evolutionary relationships of many taxa within this group are still uncertain due to their fast and recent diversification (Kretschmer et al. 2018a; dos Santos et al. 2019). Despite species richness, chromosomal data are available for just about 1,080 of them scattered throughout 140 genera (Degrandi et al. 2020). Although a wide range of chromosomal numbers can be observed among birds, ranging from 2n = 40 in *Falco columbarius* (Nishida-Umehara et al. 2008) and *Bycanistes bucinator* (Belterman and De Boer 1990) to 136–142 in *Corythaixoides concolor* (Les Christidis 1990), the majority of species have 2n = 80 chromosomes, which is considered the putative ancestral bird karyotype, as observed in basal groups as the emu (*Dromaius novaehollandiae*) (Nishida-Umehara et al. 2007) Besides, all birds present the same ZZ/ZW sex chromosome system (Gunski et al. 2017 describe an exception to this rule), where the Z chromosome is typically preserved in both morphology and gene content while the W is small and predominantly heterochromatic. Except for the Ratites (where the sex chromosomes are undifferentiated), birds in the Cariamidae family deviate from these standards by exhibiting unusually large Z chromosomes compared to its autosomes.

The *Cariama cristata* (Red-legged Seriema) and *Chunga burmeisteri* (Black-legged Seriema) are the only living representatives of the Cariamidae, the only family from the order Cariamiformes. The Seriemas are native to South America, with the Black-legged Seriema found only in a small central strip and the Red-legged Seriema in the west and central region, mostly in savannahs (Brooks 2014). The cytogenetic data of these species are restricted to some studies published more than 40 years ago, highlighting their significantly high diploid numbers (i.e., 2n = 108 in *C. cristata* and 2n = 106 for *C. burmeisteri*) compared to the putative ancestral bird karyotype (2n=80) (Takagi and Sasaki 1980; Sasaki and Takagi 1981; Belterman and De Boer 1984). Additionally, the Z chromosome in *C. cristata* (data for *C. burmeisteri* are lacking) represents the largest element in the karyotype (Belterman and De Boer 1984). Although previously classified in the order Gruiformes, the Cariamidae phylogeny is still debated. Currently, the two species are considered to belong to a different clade (Cariamiformes), which includes the extinct terror birds (Phorusrhacidae) and the Falconiformes as the closest living groups (Belterman and De Boer 1984; Prum et al. 2015).

The advance of sequencing technologies has allowed integrated genomic analysis with cytogenetics (i.e., cytogenomic), and recently, several investigations involving repetitive sequences have been used in different non-model groups, contributing to the understanding of their genome architecture (Cabral-de-Mello et al. 2021; Tunjić-Cvitanić et al. 2021; de Lima and Ruiz-Ruano 2022; Kretschmer et al. 2022; Peona et al. 2023; Cuadrado et al. 2023). In eukaryotic genomes, satellite DNAs (satDNAs) are tandemly repeated sequences that can reach up to megabases and a large portion of the whole genome (Plohl et al. 2012; Mora et al. 2023). Several analyses of the whole-set of satellite DNAs have been done in diverse invertebrate and vertebrate groups (Palacios-Gimenez et al. 2020; Cabral-de-Mello et al. 2021; de Lima and Ruiz-Ruano 2022; Montiel et al. 2022; Cabral-de-Mello et al. 2023; Rico-Porras et al. 2024; Crepaldi et al. 2021; Goes et al. 2022a; Kretschmer et al. 2022; de Oliveira et al. 2023; Deon et al. 2024), to understand the role of these sequences in karyotype evolution and sex chromosome´s differentiation. Nevertheless, satellitome investigations in birds are quite scarce, being limited to a few groups up to now (de Oliveira et al. 2023; Peona et al. 2023; Setti et al. 2024; Kretschmer et al. 2024), being absent for Cariamidae, Falconidae, and other families of birds of prey, groups that are recognized for having great karyotypic variability (O'Connor et al. 2024).

This study sought to address a fundamental question: why is *C. cristata's* Z chromosome so large compared to its autosomes? For that, we proposed some hypotheses and tested them combining cytogenetic, bioinformatic, and genomic analyses. We extensively examined the satellite DNA catalogs (satellitomes) of both Seriemas species and mapped satDNAs, rDNA, and a few microsatellite repeats in *C. cristata* using both in situ and in silico methods. Additionally, pairwise whole-genome alignments and chromosomal painting analysis with a Z-chromosome-specific probe were conducted using an ancestral avian karyotype. We showed that, as *C. cristata* macrochromosomes underwent several fissions, the Z chromosome has become the largest component of the karyotype. Nevertheless, repetitive sequences also accumulated on the Z chromosome, leading to its enlargement compared to the pattern in ancestral bird groups.

Materials and Methods

Sampling, DNA extraction, chromosomal obtainment, and C-banding

One female individual of *C. cristata* was collected in the municipality of Pelotas, Rio Grande do Sul State, Brazil, at the Núcleo de Reabilitação da Fauna Silvestre from the Universidade Federal de Pelotas. One male of the emu (*Dromaius novaehollandiae*) was obtained from an ex-situ individual in the municipality of Glorinha, Rio Grande do Sul State, Brazil. The sampling and all technical procedures were conducted according to the Brazilian Environmental Agency ICMBIO/SISBIO license (81564-1 and 68443-2). Mitotic chromosomes were acquired using the procedures outlined by Sasaki et al. (1968) utilizing skin biopsies for fibroblast cultivation. The genomic DNA (gDNA) of females of *C. cristata* was extracted using a phenol-chloroform procedure, according to Sambrook and Russell (2001). The C-positive heterochromatin was detected following Sumner et al. (1972). All experiments followed the guidelines and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Pelotas (113/2023).

Satellitome characterization and bioinformatic analyses

The female genomic DNA of *C. cristata* was sequenced using low-coverage short-read sequencing using the BGISEQ-500 platform at BGI (BGI Shenzhen Corporation, Shenzhen, China) with 150-bp paired-end sequences, that were used for satDNA characterization. The raw reads were deposited on the Sequence Read Archive (SRA-NCBI) under accession number (SRR30101299). Additionally, we used the genome available under NCBI access GCA_013396505.1 of *C. burmeisteri* to characterize its satellitome. The satellite DNA sequences of *C. cristata* and *C. burmeisteri* are available on Genbank-NCBI under the accession numbers PQ168284 - PQ168287 and PQ168288 - PQ168293 respectively.

The satellite DNA sequences for both species were analyzed using the RepeatExplorer and TAREAN package (Novák et al. 2010; Novák et al. 2013) available on the Galaxy server (https://galaxy-elixir.cerit-sc.cz/) and using the protocol performed by Ruiz-Ruano et al. (2016). We discarded the low-quality reads of raw reads with Q < 20, using the options LEADING:3 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN: 100 CROP: 101 and removed the adaptors with the Trimmomatic tool (Bolger et al. 2014). After this, we randomly selected 2 x 500.000 reads using SeqTK software (https://github.com/lh3/seqtk), and the selected reads were used for TAREAN analysis (https://repeatexplorer-elixir.cerit-sc.cz/galaxy). Afterward,

to prevent redundancy in analysis, the putative satDNA sequences were filtered from raw reads using the DeconSeq tool (Schmieder and Edwards 2011). This process was performed until the TAREAN tool found no putative satDNA sequences (after 2 iterations), to set the satDNA database to *C. cristata* and *C. burmeisteri*.

Subsequently, we removed sequences that had similarities with multigene families. After this, we searched for similarities among the isolated satDNAs using RepeatMasker (Smit et al. 2017). The sequences grouped together were aligned, and sequences with identity > 80% were classified as variants of the same satellite, and sequences between 50% and 79% were classified as belonging to the same superfamilies, as suggested by Ruiz-Ruano et al. (2016). To estimate the abundance for each satDNA family on the genome of *C. cristata* and *C. burmeisteri*, we aligned 2 x 5,000,000 randomly selected raw reads on the satDNA database using RepeatMasker with a publicly available script (https://github.com/fjruizruano/satminer/blob/master/repeat_masker_run_big.py) and to calculate the divergence for each satDNA family, we used calcDivergenceFromAlign.pl tool to obtain a histogram of the element genomic proportions plotted against the Kimura 2-Parameter divergence. The satDNA families were named in order of decreasing abundance in the genome, according to Ruiz-Ruano et al. (2016). Additionally, we searched the homology of the *C. cristata* and *C. burmeisteri´s* satDNAs (hereafter named CcrSatDNAs and CbuSatDNAs, respectively) with other repetitive sequences; for this, we used the database GIRI-Repbase (Kohany et al. 2006).

Primer design and satDNAs amplification using Polymerase Chain Reaction (PCR)

A total of four CcrSatDNAs were isolated (**Table 4**) and amplified by PCR using the set of primers described in the **Supplementary Table 4**. To amplify the satDNAs, we used the following amplification conditions: an initial denaturation step of 95 °C for 5 minutes, followed by 32 cycles with 95 °C for 20 seconds, 54 °C as annealing for 40 seconds, 72 °C for 50 seconds, and a final extension step of 72 °C for 5 minutes. To confirm the amplification of CcrSatDNAs, we used electrophoresis on 2% agarose gels, and the products obtained were quantified using the NanoDrop Spectrophotometer (ThermoFisher Scientific, Branchburg, NJ, USA).

Fluorescence in situ Hybridization (FISH)

All four CcrSatDNAs were labeled using a nick translation Kit from Jena Bioscience (Jena, Germany) incorporating the fluorophore Atto488-dUTP or Atto550-dUTP according to the instructions in the manufacturer's manual. The 18S rDNA fragments were obtained by PCR following the method described in Cioffi et al. (2009). According to the manufacturer's

recommendations, these fragments were labeled with Atto550-dUTP using nick translation (Jena Bioscience, Jena Germany).

The microsatellite repeats (A)n, (C)n, (GA)n, (CA)n, (GC)n, (TA)n, (GAA)n, (CAA)n, (TAA)n, (GAC)n, (CGG)n, (CAC)n, (CAG)n, (CAT)n, (GAG)n, and (TAC)n labeled directly with Cy3 at the 5' end during synthesis (VBC Biotech, Vienna, Austria) were also hybridized on *C. cristata* chromosomes. These sequences were selected from a pool of microsatellite repeats since they are commonly accumulated in several bird genomes (e.g., de Oliveira et al. 2017; Furo et al. 2017; Kretschmer et al. 2018b; Gunski et al. 2019; de Souza et al. 2020; Setti et al. 2024, among others). The FISH experiments were made following Kretschmer et al. (2023). The slides were counterstaining chromosomes with 4',6-diamidino-2-fenilindol (DAPI) Vectashield (Vector Laboratories, Burlingame, USA).

In-silico mapping

To analyze the distribution of CcrSat02-1104 on the first ten chromosomes and on the sex chromosomes of *C. cristata*, we used the pipeline CHRISMAPP (CHRromosome In Silico MAPPing) (https://github.com/LoriteLab/CHRISMAPP) described by Rico-Porras et al. (2024) with the genome assembly at the chromosome level available on NCBI (GCA_009819825.1).

Repetitive DNA landscape of the Z chromosome from Red-legged Seriema and the emu

We used RepeatModeler2 (an automated pipeline for de novo annotation of repetitive DNA from assembled genomes), and then to analyze the repeat composition of the Z chromosome of the Red-legged Seriema and the emu, we ran RepeatModeler2 (Flynn et al. 2020) with default parameters, using the genome assemblies available in GenBank NCBI (GCA_009819825.1 and GCA_036370855.1). After this, RepeatMasker was used to search for the abundance of this sequence in the Z chromosomes (Smit et al. 2017). The results were plotted in pie charts as a summary.

Pairwise whole-genome alignments

We employed NUCmer from the MUMmer software (Marçais et al. 2018) to carry out the pairwise whole genome alignment of two birds, including the emu (GCA_036370855.1) and the Red-legged Seriema (GCA_009819825.1) with minimum length of the exact match set as -l 50 and maximum gap size set as -b 400." The MUMmer package's delta-filter function was used to refine the alignments so that only the best one-to-one matches remained and only alignments longer than 400bp were kept. The filtered file was converted to the "coords" format with the show-coords function. The alignments were further processed with a customized

version of "nucmer_to_mcscan.sh"script obtained from " https://github.com/lurebgi/monkParakeet" (Huang et al. 2022). The resulting files were plotted with the MCscan (Tang et al. 2008) for synteny visualization.

Microdissection and chromosome painting

Fourteen copies of the Z chromosome from the Red-legged Seriema were isolated by glass-needle microdissection and amplified using the procedure described in Yang et al. (2009) to prepare whole chromosome probes, which were subsequently utilized in chromosome painting experiments. This species possesses an atypically large Z chromosome, easily identifiable after Giemsa staining, as microdissection requires. This probe (hereafter named CCR-Z) was then labeled with Spectrum Orange-dUTP (Vysis, Downers Grove, USA) in a secondary DOP PCR using 1 μl of the primarily amplified product as a template DNA, following Yang et al. (2009). This probe was hybridized metaphasic chromosomes of Red-legged Seriema (female) and of the emu (male) following Kretschmer et al. (2023).

Microscopy/image analysis

At least 30 metaphase spreads per individual were analyzed to confirm 2n and FISH results. Images were captured using CoolSNAP on an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan) and processed using Image-Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA).

Results

Karyotype description and C-banding

C. cristata presented a diploid number (2n) = 108 acrocentric chromosomes, with the Z corresponding to the largest element of the karyotype (**Figure 8**). C-banding indicated a few heterochromatic regions, being restricted to the centromere of some macrochromosomes and prominent in a few microchromosomes, including the W chromosome **(Supplementary Figure 4).**

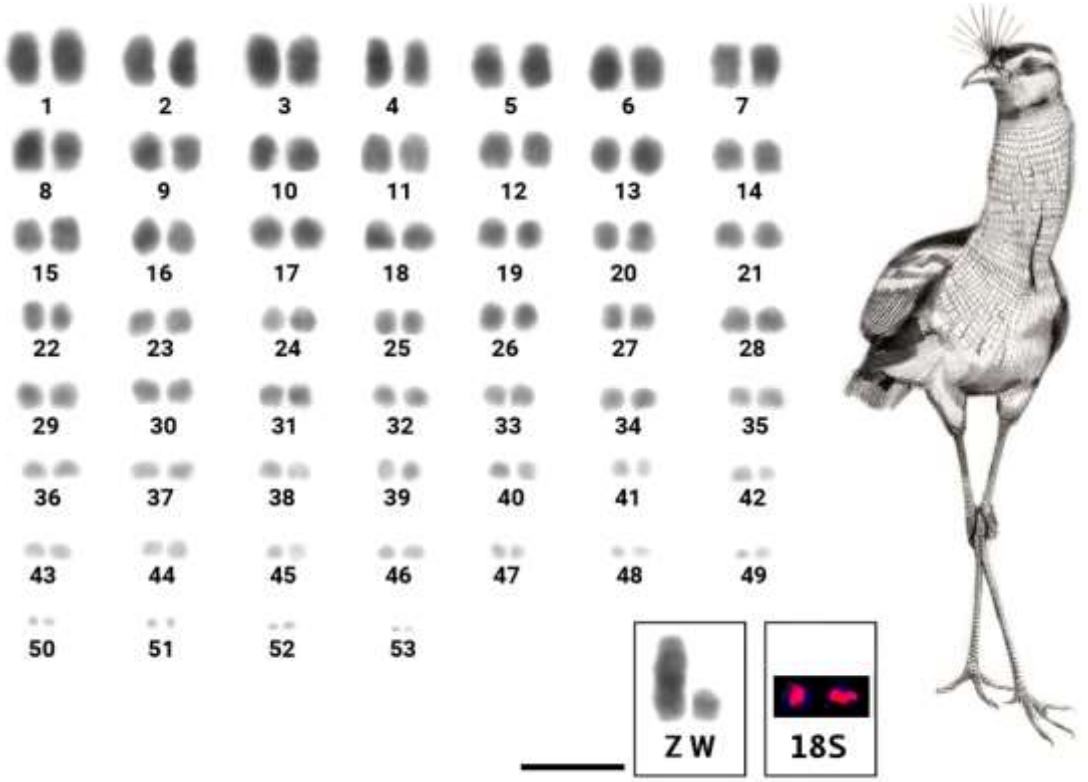


Figure 8. Giemsa-stained karyotype of the female Red-legged Seriema (*Cariama cristata*). The Z and W chromosomes and the pair of microchromosomes carrying the 18S rDNA site are highlighted. Bar = 20 μm.

Satellite composition of C. cristata and C. burmesteri

The satellitome of *C. cristata* was composed of four long satDNAs (>100 bp), with the repeat unit length (RUL) ranging from 179 to 5732 bp and A+T content ranging from 33% to 57% (**Table 4**). The satellitome of *C. burmeisteri* presented six satDNAs, with a predominance of long satDNAs. The RUL ranged from 14 to 599 bp and the A+T content from 23% to 54% (**Table 5**). In the comparative analysis between all satellites isolated from both species, we only identified correspondence between CcrSat01-179 and CbuSat01-179, with a similarity of 92% being considered variants of the same satDNA **(Supplementary Figure 5**). In the homology

search between other sequences in the database Repbase-GIRI, the satellites of *C. cristata* and *C. burmesteri* showed similarity with other repetitive sequences—some transposable elements and the satDNA CASAT2 of *Coragyps atratus* (**Supplementary Figure 6** and **Supplementary Table 2**).

Table 4. General features of the *C. cristata* satellitome. The repeat unit length (RUL), adenine plus thymine content (A+T), abundance and the K2P is Kimura Two-Parameter model divergence are indicated.

satDNA	RUL	A+T	Abundance	Divergence (K2P)
CcrSat01-179	179 bp	46%	0.02398%	6.62%
CcrSat02-1104	1104 bp	46%	0.01256%	19.07%
CcrSat03-5762	5762 bp	57%	0.00118%	1.31%
CcrSat04-209	209 bp	33%	0.00013%	5.34%

Table 5. General features of the *C. burmeisteri* satellitome. The repeat unit length (RUL), adenine plus thymine content (A+T), abundance, and the K2P is Kimura Two-Parameter model divergence are indicated.

satDNA	RUL	A+T	Abundance	Divergence (K2P)
CbuSat01-179	179 bp	49%	0.0333%	12.02%
CbuSat02-14	14 bp	36%	0.0066%	14.01%
CbuSat03-599	599 bp	38%	0.0021%	3.42%
CbuSat04-309	309 bp	23%	0.0013%	4.81%
CbuSat05-129	129 bp	47%	0.001%	13.05%
CbuSat06-185	185 bp	54%	0.0007%	9.23%

Chromosomal distribution of repetitive DNAs

All the CcrSatDNAs, except for CcrSat03-5762, showed positive signals on the female metaphasic chromosomes of *C. cristata* (**Figure 9**). CcrSat01-179 was mapped in the pericentromeric region of nine chromosome pairs, including macro and microchromosomes. CcrSat04-209, on the other hand, only showed positive signals in the pericentromeric region of four macrochromosome pairs. Conversely, CcrSat02-1104 was predominantly distributed throughout the whole W chromosome and the q arms of the Z sex chromosome. Despite being the third most abundant, CcrSat03-5762 did not produce visible FISH signals. This outcome may be attributed to their arrangement into clusters that are too small for effective cytological detection. The 18S rDNA cluster was found in one pair of microchromosomes (with an

approximate size between pairs 32 and 48) (**Figure 8**, boxed and **Supplementary Figure 7a**) and terminal signals in all chromosomes were detected using the telomeric probe. Interstitial telomeric sites (ITS) were not detected, even in the large Z chromosome (**Supplementary Figure 7b**). Positive hybridization signals were not detected for any of the sixteen examined microsatellites.

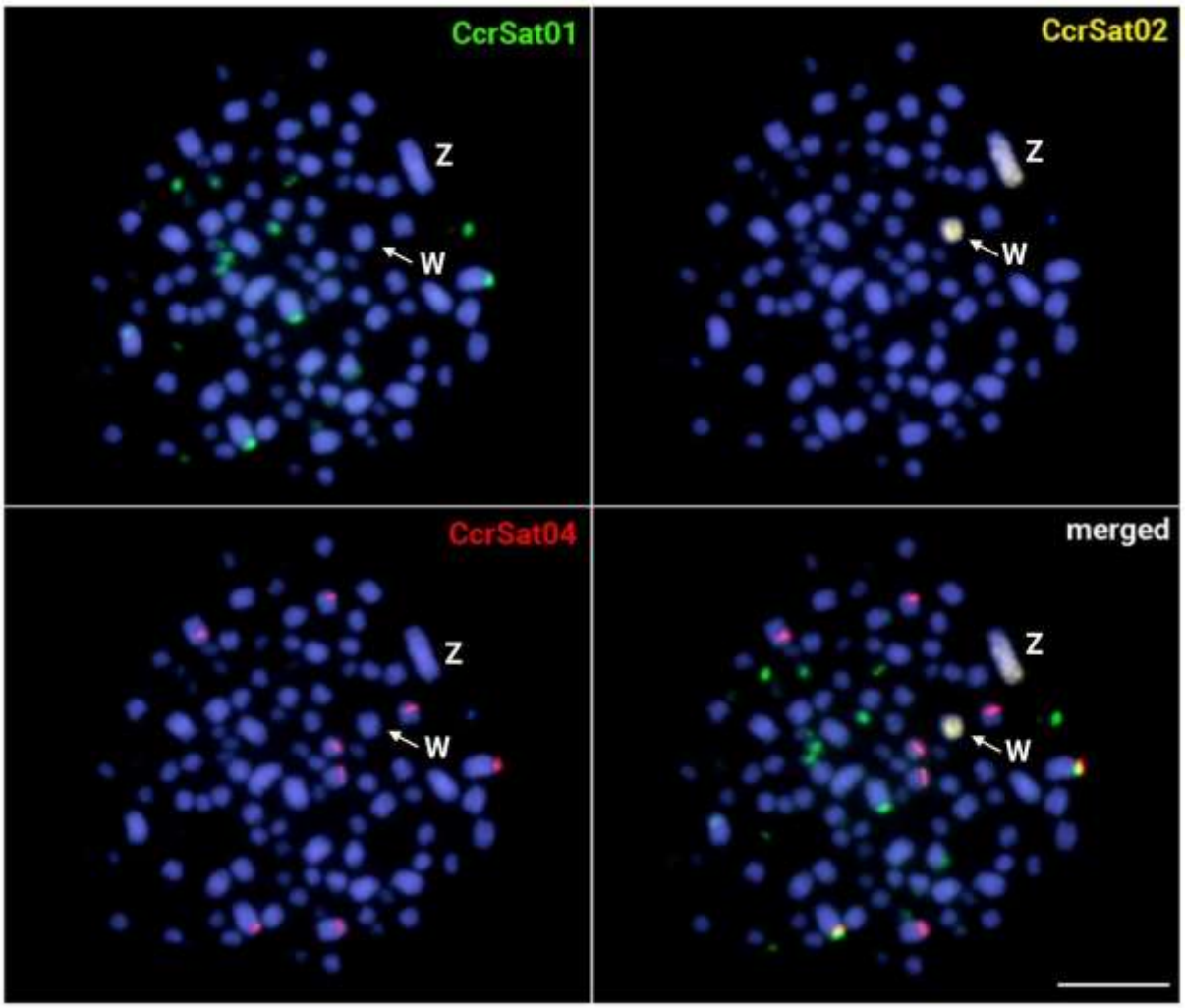


Figure 9. Mitotic chromosome spreads of *C. cristata* females with the isolated and labeled CcrSatDNAs. The name of each satellite family is indicated in the upper right corner: CcrSatDNA01-179 in green (Atto488-dUTP labeled), CcrSatDNA02-1104 pseudo colored in yellow (Atto425-dUTP labeled), and CcrSatDNA04-209 in red (Atto550-dUTP labeled). The Z and W sex chromosomes are highlighted. Bar = 10 μm.

In silico mapping of CcrSat02-1104

The results obtained using the *in silico* approach indicate the presence of CcrSat02-1104 dispersed in several chromosomes (**Figure 10**). Furthermore, we identified the presence of this satDNA in the sex chromosomes, being dispersed throughout the long extension of Z and present in almost all W.

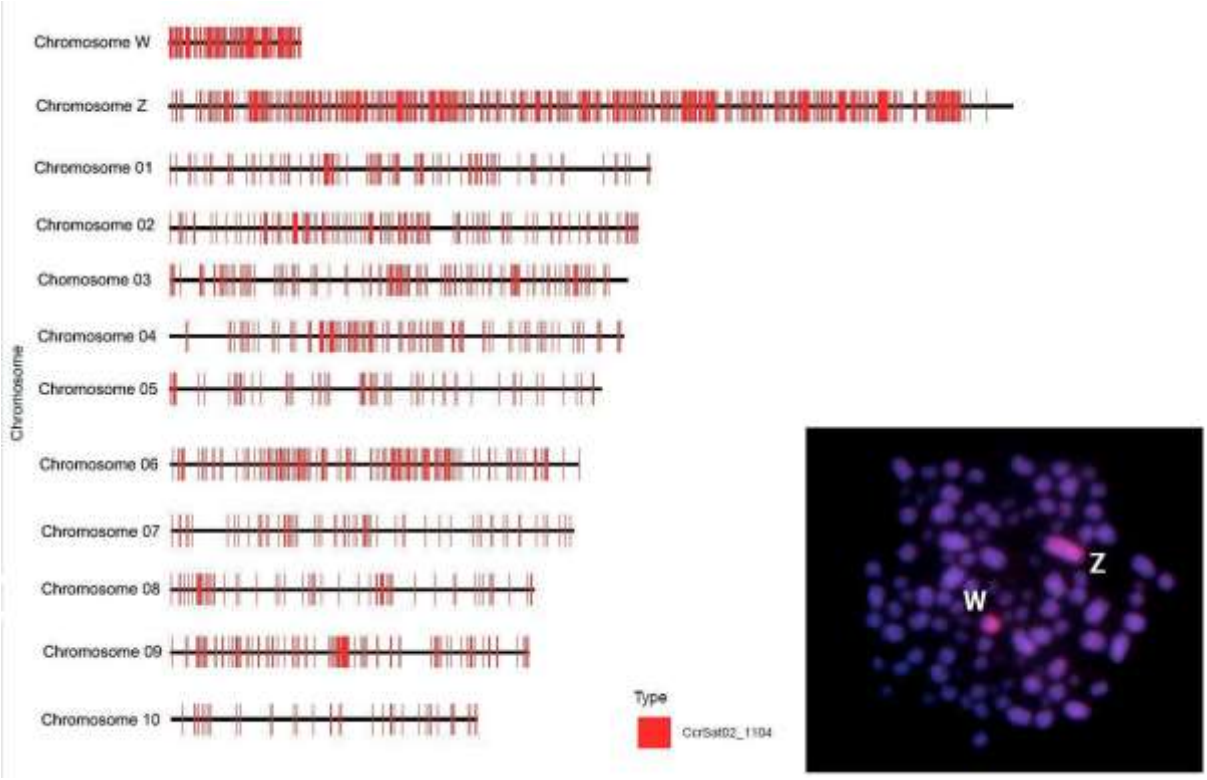


Figure 10. *In silico* mapping of CcrSat02-1104 in pseudo-chromosomes of *C.cristata* (1-10 autosomes) and ZW sex chromosomes. The *in situ* pattern of the satDNA is highlighted.

General repeat composition of the Z chromosome from Red-legged Seriema and the emu

The repetitive DNA composition of the Z chromosome of the Red-legged Seriema consisted of about 13.70%. The largest correctly annotated contributors are Transposable Elements (~11%), and satellite DNAs (0.58%). The Z chromosome of the emu included around 9.53% repetitive content consisting of about 4.6% transposable elements and 0.92% satellite DNAs **(Supplementary Figure 8)**.

Pairwise whole-genome alignments and whole chromosomal painting experiments

To reconstruct the evolutionary history of chromosomal changes that occurred in *C. cristata* (particularly those putatively involving the Z chromosome), we aligned its chromosomal-level assembly genome against the emu genome (a bird species with an ancestral-like karyotype). The results highlighted that extensive chromosomal reshuffling events,

particularly involving chromosomal fission, may have occurred during the chromosomal diversification of *C. cristata*. Nonetheless, none of the chromosomal changes involved the Z chromosome, which remained entirely homologous with the Z chromosome found in the emu, despite certain internal inversions detected (**Figure 11a)**. Likewise, the hybridization performed with the CCR-Z probe efficiently painted just the Z and W chromosomes of *C. cristata*, as well as both Z chromosomes of the emu, not pointing to any rearrangements involving this chromosome **(Figure 11b).**

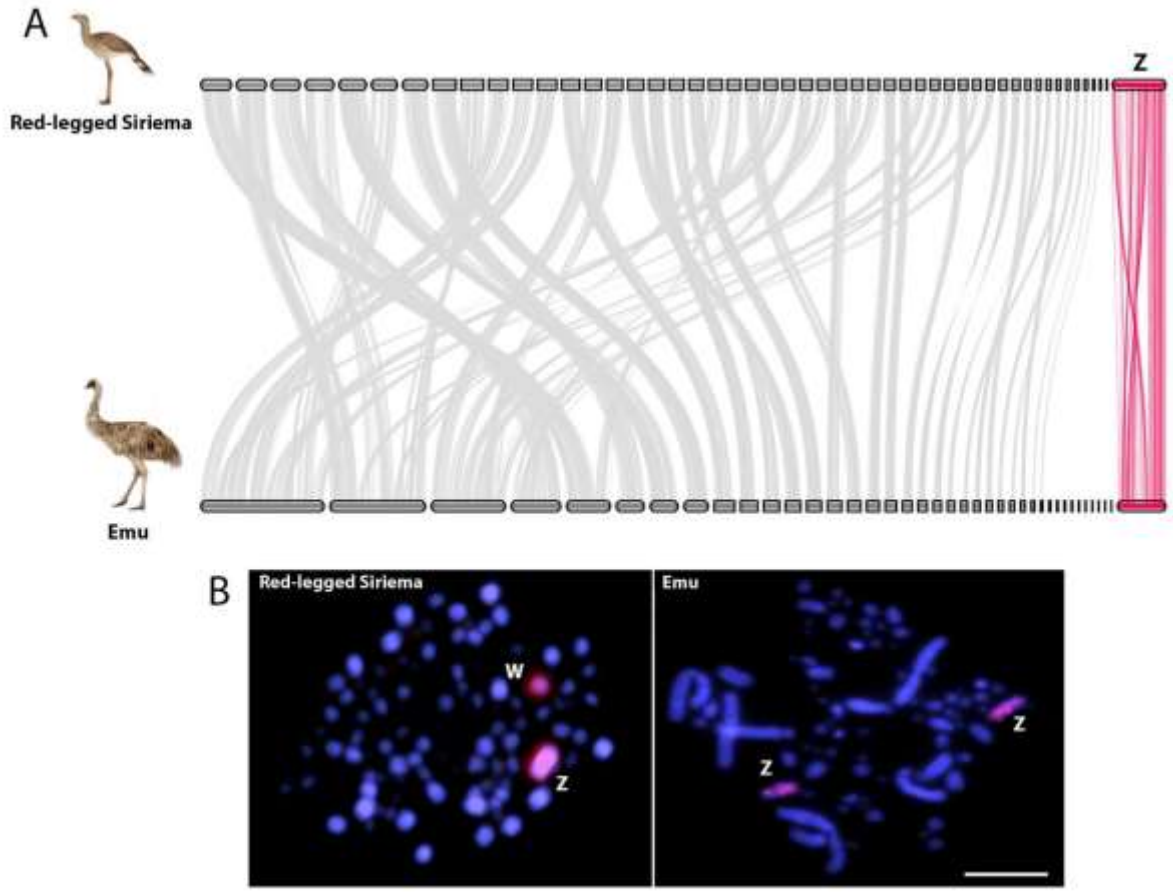


Figure 11. A) Pairwise whole-genome alignment between the chromosomal level-assembly of the Red-legged Siriema and the emu. Each bar represents one chromosome and the gray lines represent syntenic blocks between chromosomes. The Z chromosomes and their respective syntenic blocks are highlighted in red. B) Hybridization with the Z-specific probe of the Red-legged Siriema (CCR-Z) in metaphase plates of a female of the Red-legged Siriema and a male of the emu (B). The sex chromosomes are indicated. Bar = 20 μm.

Discussion

Revisiting the Karyotypes of the Red-legged Siriema (Cariama cristata)

Cytogenetic data in Seriemas are sporadic and restricted to the description of their diploid chromosome number. Nevertheless, due to the former methods, high-resolution preparations were unavailable, and the results were mostly inconclusive. Our data unambiguously established 2n = 108 for *C. cristata* in accordance with the findings of Belterman and De Boer (1984). Bird karyotypes typically have about 80 chromosomes, with the Z chromosome being the third or fourth largest in size (Griffin et al. 2007; Kretschmer et al. 2018a; O'Connor et al. 2024). *C. cristata* showed an atypical karyotype with a high diploid number that has probably evolved as a result of extensive chromosomal restructuring, mostly involving chromosomal fissions (**Figure 11a**). Besides, *C. cristata* presents an unusually large Z chromosome compared to autosomes, which represent the largest element of the karyotype **(Figure 8)**. The C-banding analysis did not indicate significant heterochromatin accumulation on the Z chromosome. In this species, heterochromatic regions were confined to the centromere of several macrochromosomes and were most prominent in certain microchromosomes, especially the W chromosome **(Supplementary Figure 4).**

From now on, we will initiate looking into the reasons for the significant size of the Z chromosome in comparison to its autosomes. We began with an examination of the genomic architecture of repetitive DNA sequences in both Siriemas and their potential contribution to the growth of the Z chromosome. Afterward, we proposed and discussed some hypotheses to explain this scenario.

Repetitive DNA landscape of Siriemas

In contrast to other vertebrates, the relatively short genomes of birds are a distinctive feature of the group, leading to the formulation of many theories to elucidate this phenomenon (Zhang and Edwards 2012; Zhang et al. 2014; Kapusta et al. 2017). One possible explanation is the scarcity of repetitive sequences in their genomes, although this feature has only been studied in a limited number of species (Kretschmer et al. 2018a). Our research showed that the genomes of *C. cristata* and *C. burmeisteri* only had four and six satDNAs, which were linked to larger sizes of repetition units **(Tables 1 and 2)**. These findings support the discoveries made by previous satellite investigations in birds (Peona et al. 2023; Kretschmer et al. 2024; de Oliveira et al. 2024; Setti et al. 2024). While satDNAs exhibit significant sequence variability

(Garrido-Ramos 2017; Ruiz-Ruano et al. 2016), recent research has provided support for the library hypothesis (Fry and Salser 1977), by revealing the conservation of some satDNAs across distinct species (Goes et al. 2022a; de Lima and Ruiz-Ruano 2022; Moraes et al. 2023, Goes et al. 2022b, Sales-Oliveira et al. 2024, Tunjić-Cvitanić et al. 2024). In this study, we have found that both Seriemas possess a common satellite DNA (CcrSat01 and CbuSat01) that has been conserved for approximately 7.6 million years (the proposed divergence time of these species according to Prum et al. 2015). These satellites exhibit a high level (92%) of similarity and are the most abundant ones in both species (**Supplementary Figure 5**). However, there is a significant degree of divergence in *C. burmeisteri* (**Table 5**), pointing out that it has undergone a higher rate of change. The investigation in REPBASE-Censor (Kohany et al. 2006) revealed that most of the satDNAs found in the genomes of both Seriemas are associated with other repeated sequences, most particularly with transposable elements (**Supplementary Figure 6 and Supplementary Table 1**). Transposable elements are sequences with high transposition capacity, making them important modulators of the genome and drivers of its evolutionary process. Investigations have demonstrated that these sequences participate in several processes, such as regulation of gene activation and suppression, centromere and telomere remodeling and even being responsible for the emergence and diversification of satDNAs (Li and Leung 2006; Kejnovsky et al. 2006; Gong et al. 2012; Garrido-Ramos 2015; Hayward and Gilbert 2022; Tunjić-Cvitanić et al. 2024). Except for CcrSat03-5762, all CcrSatDNAs displayed positive signals on *C. cristata* chromosomes **(Figure 9).** CcrSat01-179 was localized in the pericentromeric region of 9 macro and microchromosome pairs, whereas CcrSat04-209 only showed positive signals in 4 macrochromosome pairs **(Figure 9)**. In contrast, CcrSat02-1104 was hybridized throughout the whole Z and W chromosomes **(Figure 10**, **discussed below)**. Nevertheless, the *in silico* mapping of this satDNA to the *C. cristata* genome (NCBI, GCA_009819825.1) revealed that it is likewise distributed over many chromosomes, although at a lower density. This probably results in a non-clustered organization, thus hindering *in situ* experiments from producing detectable hybridization signals at the chromosomal level.

Notably, none of the satellite DNAs, despite their great abundance in the *C. cristata* genome, were found in all centromeres. SatDNAs are strongly linked to centromeric regions, as shown by their interaction with CENP-B proteins, which highlights their significant role in maintaining the structure of the centromere (Masumoto et al. 1989; Masumoto et al. 2004). Numerous investigations have shown that all centromeres have at least one main satDNA family, which is typically the most prevalent one in the genome. This scenario is supported by evidence found in insects (Palacios-Gimenez et al. 2017), fishes (Kretschmer et al. 2022; Toma

et al. 2023), amphibians (da Silva et al. 2020; da Silva et al. 2023), mammals (Gutiérrez et al. 2023) and even birds (Kretschmer et al. 2024). Nevertheless, in some species, the centromeric DNA is composed of different satDNA sequences (Gong et al. 2012; Plohl et al. 2014). In birds, despite a few exceptions (e.g., Takki et al. 2022), satellites with high centromeric abundance are not commonly identified. This characteristic may be the result of i) the rapid evolution of centromeric sequences (Zhang et al. 2014), ii) the lower prevalence of repetitive sequences (such as satellites) in bird genomes, or iii) as a result of the numerous fission processes that took place in *C. cristata* chromosomes, the new centromeres did not retain the primary centromeric repeat. Furthermore, the possibility that CcrSat01 arrays are too small to be identified on all chromosomes cannot be dismissed.

We did not find positive hybridization signals for any of the sixteen microsatellites tested. Similarly, a dove species (*Columbina picui*) also did not show any signal for the nine microsatellite sequences tested (Kretschmer et al. 2018b). These two cases contrast with most birds, in which some of the microsatellite sequences used in the experiments were found preferentially accumulated in heterochromatic regions, especially on the W chromosomes (Furo et al. 2017; Kretschmer et al. 2018b; Gunski et al. 2019; Kretschmer et al. 2020). A noteworthy finding is the absence of microsatellites in the Z chromosome of *C. cristata*. In contrast, in species from the Ramphastidae and Picidae families, both belonging to the Piciformes order and characterized by a large Z chromosome, the accumulation of several microsatellite sequences has been observed (de Oliveira et al. 2017; Kretschmer et al. 2020). These findings highlight the dynamic evolutionary pathway of sex chromosome differentiation in birds. Although many fission events likely took place during the evolution of *C. cristata*, the 18S rDNA cluster remained unchanged in just one pair of microchromosomes **(Figure 8** and **Supplementary Figure 6)**. This trait is regarded as an ancestral feature, as only a single pair of microchromosomes containing these sequences has been identified in Paleognath species (Nishida-Umehara et al. 2007; Degrandi et al. 2020). Except for *Harpia harpyja* and some Falconiformes species, most birds of prey typically have just two microchromosomes containing the 18S rDNA clusters (Degrandi et al. 2020).

The origin of the enlarged Z chromosome of C. cristata

Multiple studies have provided evidence for the association between repetitive DNAs and the evolution of sex chromosomes in various groups (Matsubara et al. 2015; Furo et al. 2017; Yurchenko et al. 2024). According to traditional evolutionary hypotheses, the gradual accumulation of repetitive sequences accounts for the differentiation of the specific sex chromosome and its subsequent reduction in size over time (Ohno 1967; Bull 1983;

Charlesworth et al. 2005). The ZZ/ZW sex system of birds is believed to have a common origin in all species and emerged around 60-100 million years ago (Fridolfsson et al. 1998). Most bird species (except for ratite birds where both Z and W have similar morphologies and are poorly differentiated) contain a small and heterochromatic W and a medium-sized Z chromosome (Kretschmer et al. 2018a; O'Connor et al. 2024). However, *C. cristata* largely deviates from this standard by presenting atypically large Z chromosomes in comparison with its autosomes. We, therefore, raised three main hypotheses to explain such a scenario: 1) The Z chromosome underwent fusion events with other macrochromosomes, becoming the larger chromosome of the karyotype; 2) The first macrochromosomes underwent several fissions, making Z the largest element; and/or 3) The accumulation of repetitive DNAs led to an increase in the Z chromosome.

The combination of whole-genome alignments and chromosomal painting against a species with an ancestral-like karyotype (emu) refuted the hypothesis that the Z chromosome originated by fusions involving other macrochromosomes. Instead, the Z chromosomes of both species remained completely homologous, despite the presence of certain internal inversions (**Figure 11**). These results align with prior findings indicating that chromosome Z typically displays a disproportionately higher number of inversions compared to other chromosomes in avian species (Hooper and Prince 2017). Accordingly, no interstitial telomeric sites (ITS) were detected in the Z chromosome **(Supplementary Figure 7).** This hypothesis was, in fact, highly unlikely, considering that this phenomenon is rare among birds, with only a limited number of studies documenting fusions between the Z (or W) and autosomes (Kretschmer et al. 2020; Huang et al. 2022; Souza et al. 2024). Additionally, fusion events would also result in a decline rather than an increase in diploid numbers, which is inconsistent with the current scenario.

The Z chromosome of *C. cristata* encompasses around 88 MB, slightly over the ancestral configuration observed in the emu (~ 86 MB). Nevertheless, the biggest macrochromosome (pair 1) of the emu contains around 220 MB, whereas pair 1 of *C. cristata* measures just about 50 MB. This information, along with whole-genome alignments (**Figure 11a**), indicates that, as *C. cristata* macrochromosomes underwent several fissions, the Z chromosome has notably become the largest component of the karyotype. However, the Z chromosome *C. cristata* still exceeds the size of the Z chromosome present in the emu by ~2 MB. Therefore, its current increased size could also be explained by additional enlargement triggered by a significant accumulation of repeats, since around 13.70% of the *C. cristata´* Z chromosome consists of repetitive DNA, compared to just 9.53% in the emu Z chromosome **(Supplementary Figure 8).** However, although largely accumulated on the Z chromosome, the

small proportion of SatDNAs (0.92%) seems insufficient to explain its enlargement. Instead, Transposable elements, as the predominant repetitive DNA class of the Z chromosome (**Supplementary Figure 8**) and significant contributors to the structure of avian sex chromosomes (Peona et al. 2021, 2023), may have substantially contributed to its expansion. *C. cristata*, like several woodpeckers (Picidae), also exhibits the Z chromosome as the largest component of the karyotype (de Oliveira et al. 2017). The Picidae are notable avian species due to their high abundance of transposable elements (Kapusta and Suh 2017). Furthermore, research has revealed the accumulation of microsatellites on the Z chromosome in these birds (de Oliveira et al. 2017).

Consequently, a combination of hypotheses 2 and 3 appears to be the most plausible scenario, as repetitive sequences accumulated on the Z chromosome, independent of the fission events that occurred in the ancestral macrochromosomes. The subsequent steps entail the establishment of oligonucleotide-based probes derived from a high-quality genome assembly to validate the chromosomal rearrangements indicated by the collinearity analysis to comprehensively elucidate the extensive genomic reorganization that occurred during the evolution of Seriema's karyotypes.

References

The reference list is compiled at the end of this document.

Supplementary Material

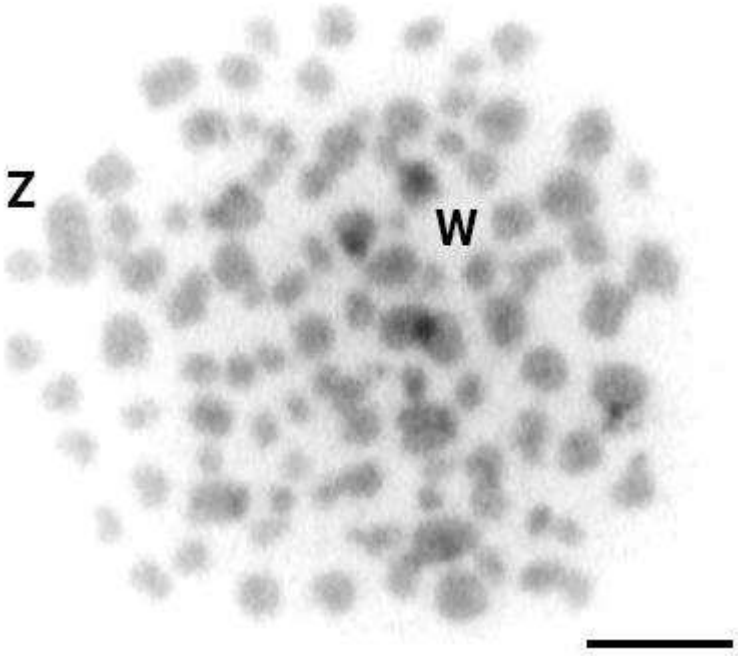
Supplementary Table 1: Primers designed for the CcrSatDNAs found in the genome of *Cariama cristata.*

Cariama cristata primers		
	Primer Forward	Primer Reverse
CcrSat01-179	TGGGAATGCAGATAGCCT	AAGCTCTAGCCTAGCAATA
CcrSat02-1104	ACGACTCTTACAGTGAAGA	TGAACATGAGCCAGCAGT
CcrSat03-5762	TCGTCTATGTTGAGTGC	TAGACAGCAGAGGAGAGA
CcrSat04-209	AGGGAGAAAGAGGGAGAG	TTCGCTGAAGTGACGGTTA

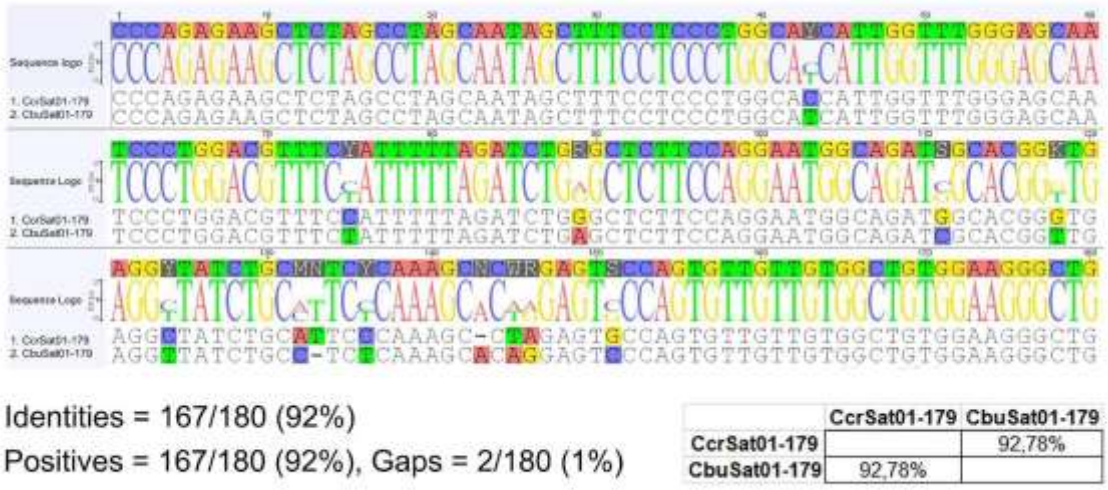
Supplementary Table 2: Summary of association between SatDNA of *Cariama cristata* and *Chunga burmeisteri* with other classes of repetitive DNA available by RepBase-CENSOR.

Name	**From**	**To**	**Name**	**From**	**To**	**Class**	**Dir**	**Sim**	**Pos/mn : TS**	**Score**
CcrSat02-1104	1	1104	CR1-J2_Pass	3170	4276	NonLTR/CR1	c	0.9140	1.4242	7877
CcrSat03-5762	1206	1248	MuDR-1N1_LSal	2765	2809	DNA/MuDR	c	0.7955	1.3333	216
	2164	2196	TurTru-6.27	845	876	NonLTR/L1	d	0.8788	3.0000	231
	2531	2562	Copia-51_ZM-I	2561	2592	LTR/Copia	d	0.8750	2.0000	212
	3190	3235	LINE1-6_SBi	2744	2790	NonLTR/L1	d	0.7872	1.8000	209
	3984	4046	Kolobok1-2_NV	2023	2084	DNA/Kolobok	d	0.7302	1.6667	206
	5374	5411	Gypsy-14_BD-I	575	610	LTR/Gypsy	d	0.8649	1.3333	201
CcrSat04-209	1	28	L1-53_LeLe	6435	6462	NonLTR/L1	d	0.9286	2.0000	215
	121	178	CR1-D	126	184	NonLTR/CR1	c	0.7966	2.2000	251
CbuSat03-599	166	227	Gypsy-668_AA-I	606	672	LTR/Gypsy	d	0.7879	1.1429	274
	337	405	TWIFBIG	3442	3512	DNA/hAT	c	0.7606	1.7143	262
	430	495	ATCOPIA9I	160	229	LTR/Copia	d	0.7246	1.6000	237
CbuSat04-309	1	308	OSTE1	237	542	DNA/MuDR	c	0.6667	1.9048	251
CbuSat05-129	4	68	BEL-54_CQ-I	1070	1130	LTR/BEL	c	0.7705	1.3750	231
CbuSat06-185	1	54	CASAT2	44	98	Simple/Sat/SAT	c	0.7818	2.2000	227

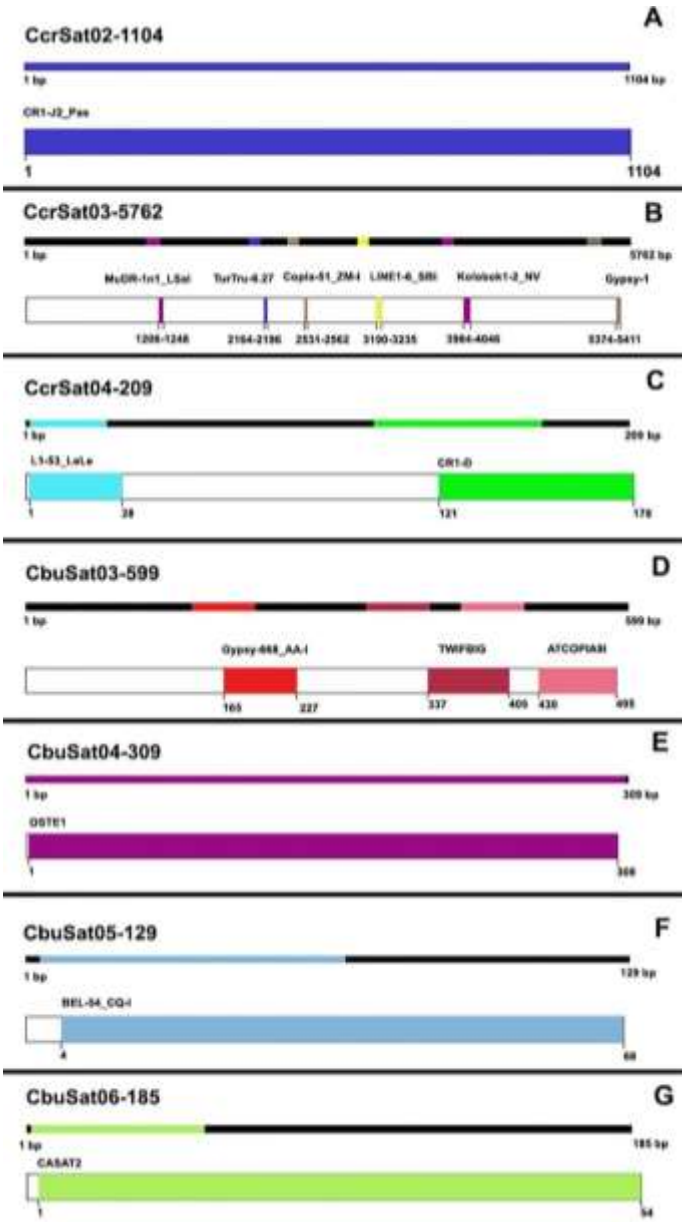
*__Class__: the class/subclass of repeat as specified in repeat annotation. **Dir:** indicate orientation. **Sim:** Direct (d) or complementary (c). **Pos:** similarity. **Mm:Ts:** ratio of positives to alignment length. ratio of mismatches to transitions in nucleotide alignment. **Score:** This column contains the alignment score obtained from blast.



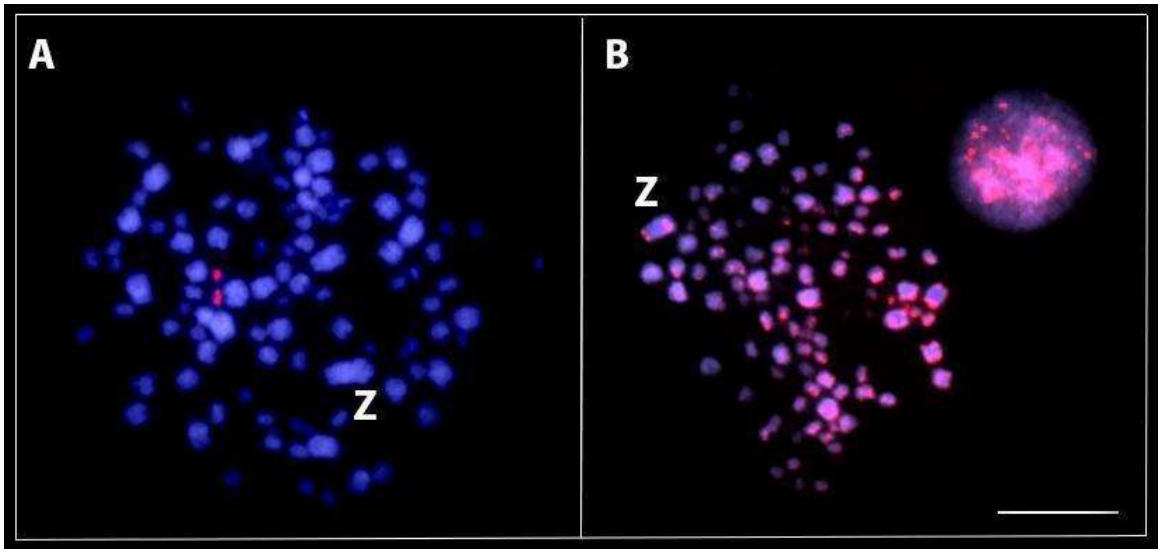
Supplementary Figure 4. Metaphase plate of a female from *C. cristata* highlighting the distribution of C-positive heterochromatin. Bar = 10 μm.



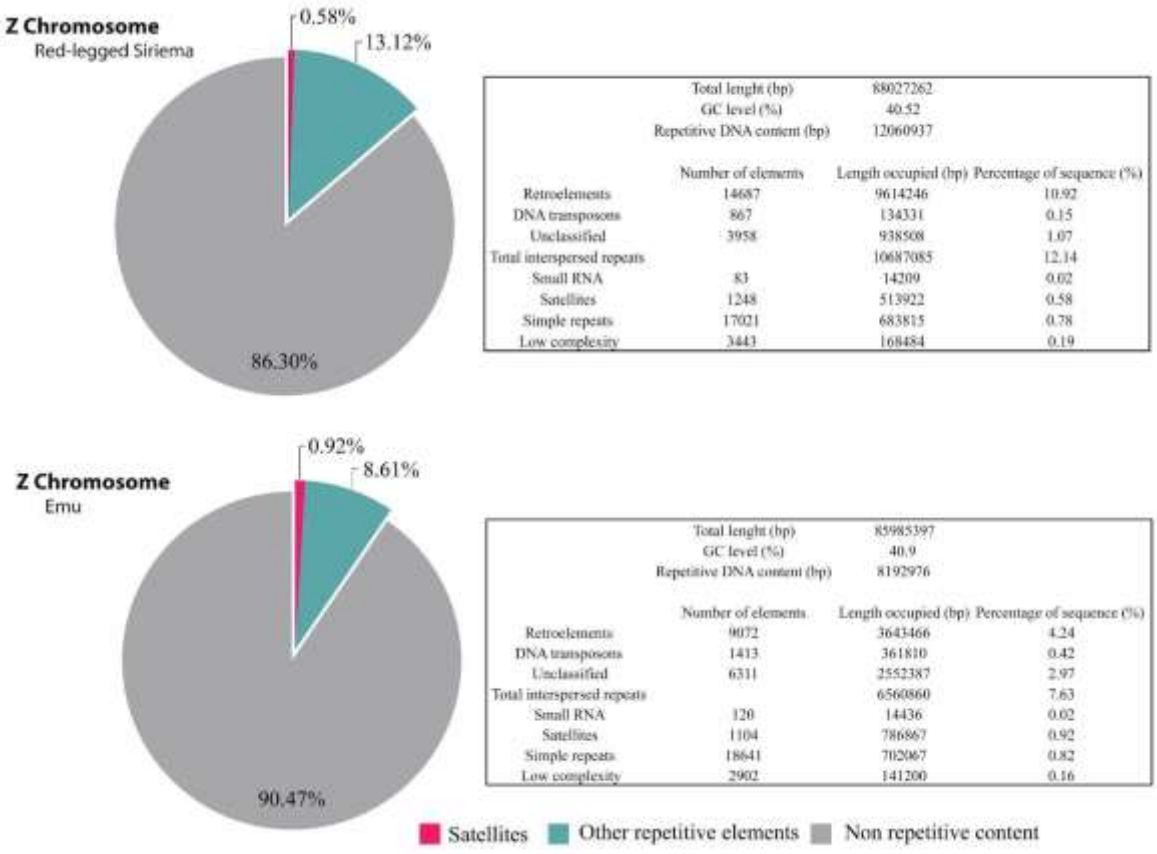
Supplementary Figure 5. Alignments of CcrSat01-179 and CbuSat01-179 satDNAs demonstrate the similarity of these sequences in both genomes.



Supplementary Figure 6: Association of satellite DNA of *C. cristata* (a-c) and *C. burmeisteri* (d-g) with repetitive elements. The association between satDNA and transposable elements (a-f) and the homology of CbuSat06-185 and CASAT2 in *Coragyps atratus* (g) are also demonstrated.



Supplementary Figure 7: Mitotic chromosome spreads of *C. cristata* females after the *in situ* mapping with 18S rDNA (a) and telomeric TTAGGGn (b) probes. The Z chromosomes are indicated. Bar = 20 μm.



Supplementary Figure 8: Summary of the overall repeat composition of the Z chromosome of the Red-legged Siriema (*C. cristata*) and the Emu (*D. novaehollandiae)*

Final Remarks

5. FINAL REMARKS

Based on the results obtained, we present new insights into the evolution of bird sex chromosomes. Given a group with a stable sex chromosome system and which stands out among vertebrates for its low proportion of repetitive DNAs in the genome, we analyzed two distinct models, which provided us with evidence of mechanisms that drive the evolution of the Z and W chromosomes and of karyotype rearrangements in general. In the first chapter, we analyzed species with 2n conserved for birds, however, for *T. leucomelas* we found an unusual scenario regarding its W chromosome. In addition to not presenting a large heterochromatic portion, we identified a polymorphism related to a satDNA that may be related to an exchange of material that occurred between the W chromosome and an autosome, an event that feeds the W chromosome with new sequences and drives its evolution in a scenario different from that dictated by traditional evolutionary models. In the second chapter, we found a scenario of species with highly rearranged karyotypes and a large Z chromosome. In this context, we identified that the high 2n and large size of the Z are probably related to successive fissions of the macrochromosomes, since we did not identify rearrangements involving autosomes and the Z. Whole-genome alignments and comparative chromosome painting revealed significant conservation across phylogenetically distant species; however, the atypical case of *C. cristata* demonstrates that, in addition to fissions, the accumulation of repetitive DNAs contributes to the enlargement of the Z chromosome. Our analyses of a relatively conserved group, specifically birds, revealed markedly distinct scenarios indicating that the evolution of karyotypes, sex chromosomes, and repetitive sequences follows diverse trajectories and complexities that increasingly challenge predictions and unidirectional narratives.

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