birds

MDPI

Article

Repeat Sequence Mapping Shows Different W Chromosome Evolutionary Pathways in Two Caprimulgiformes Families

Marcelo Santos de Souza [1], Rafael Kretschmer [2,*], Suziane Alves Barcellos [1], Alice Lemos Costa [1], Marcelo de Bello Cioffi [3], Edivaldo Herculano Corrêa de Oliveira [4,5], Analía Del Valle Garnero [1] and Ricardo José Gunski [1]

1 Programa de Pós-Graduação em Ciências Biológicas (PPGCB), Universidade Federal do Pampa, São Gabriel 97300-000, RS, Brazil; marcelodesouzabio@gmail.com (M.S.d.S.); suzianebarcellos@gmail.com (S.A.B.); alicelemoscosta14bio@gmail.com (A.L.C.); analiagarnero@unipampa.edu.br (A.D.V.G.); ricardogunski@unipampa.edu.br (R.J.G.)
2 Programa de Pós-Graduação em Genética e Biologia Molecular (PPGBM), Universidade Federal do Rio Grande do Sul, Porto Alegre 91509-900, RS, Brazil
3 Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos 13565-905, SP, Brazil; mbcioffi@ufscar.br
4 Instituto de Ciências Exatas e Naturais, Universidade Federal do Pará, Belém 66075-110, PA, Brazil; ehco@ufpa.br
5 Laboratório de Cultura de Tecidos e Citogenética, SAMAM, Instituto Evandro Chagas, Ananindeua 67013-000, PA, Brazil
* Correspondence: rafa.kretschmer@hotmail.com

Received: 16 November 2020; Accepted: 9 December 2020; Published: 11 December 2020

Simple Summary: Cytogenetic studies in Caprimulgiformes species are scarce; however, they have shown some interesting karyotype features, such as variation in the size of the W chromosome, which is usually small, like in the Scissor-tailed Nightjar (*Hydropsalis torquata*), but shows an uncommon larger size in the Common Potoo (*Nyctibius griseus*). Hence, in order to answer why two bird species from close families have different W chromosome sizes, and what caused the enlargement of the Common Potoo's W chromosome, we used classical and molecular cytogenetic approaches aiming to investigate their chromosome organization, with an emphasis on the sex chromosomes, and comparing the structural variation and repeat content in the karyotype using C-banding, G-banding and mapping of repetitive DNAs by fluorescent in situ hybridization (microsatellite repeats and 18S rDNA). Our finding revealed a much higher content of repetitive sequences in the W chromosome of the Common Potoo in comparison to the Scissor-tailed Nightjar, which can explain the difference in W chromosome size.

Abstract: Although birds belonging to order Caprimulgiformes show extensive karyotype variation, data concerning their genomic organization is still scarce, as most studies have presented only results obtained from conventional staining analyses. Nevertheless, some interesting findings have been observed, such as the W chromosome of the Common Potoo, *Nyctibius griseus* (2n = 86), which has the same morphology and size of the Z chromosome, a rare feature in Neognathae birds. Hence, we aimed to investigate the process by which the W chromosome of this species was enlarged. For that, we analyzed comparatively the chromosome organization of the Common Potoo and the Scissor-tailed Nightjar, *Hydropsalis torquata* (2n = 74), which presents the regular differentiated sex chromosomes, by applying C-banding, G-banding and mapping of repetitive DNAs (microsatellite repeats and 18S rDNA). Our results showed an accumulation of constitutive heterochromatin in the W chromosome of both species. However, 9 out of 11 microsatellite sequences hybridized in the large W chromosome in the Common Potoo, while none of them hybridized in the W chromosome of the

Scissor-tailed Nightjar. Therefore, we can conclude that the accumulation of microsatellite sequences, and consequent increase in constitutive heterochromatin, was responsible for the enlargement of the W chromosome in the Common Potoo. Based on these results, we conclude that even though these two species belong to the same order, their W chromosomes have gone through different evolutionary histories, with an extra step of accumulation of repetitive sequences in the Common Potoo.

Keywords: bird evolution; Caprimulgiformes; sex chromosome evolution; FISH

1. Introduction

In the last decades, a better understanding of the general steps involved in the evolution of sex chromosomes has been achieved, especially coupling cytogenetic and genomic tools [1,2]. Sex chromosomes are recognized by some peculiar aspects in their origin, including meiotic recombination suppression in the vicinities of the heterologous region of the proto-sex chromosomes, preserving the linkage of the sex-determining gene and sexually antagonistic genes throughout evolution [2,3]. This explains the lack of recombination between the X and Y sex chromosomes in species with male heterogamety, such as *Drosophila* and mammals, or between the Z and W in taxa with female heterogamety, such as birds and many fishes [4,5]. The mammalian XY and the bird ZW sex chromosome systems are apparently similar, with one partner (X and Z) larger and gene-richer than the other (Y and W), which is generally small, heterochromatic, and contains few single-copy genes. Nevertheless, because of their particular origins (different vertebrate ancestral autosomes), the gene content of the XY and ZW pairs are completely different [6]. The similarity of those chromosomes are the result of a genetic mechanism to differentiate the heterogametic element, probably caused by the lack of recombination [7], raising a heteromorphic sex chromosome pair.

The avian W chromosome is far less conserved and has run into shifting differentiation degrees, becoming highly differentiated from the homologous Z chromosome and broadly heterochromatic [8]. In contrast, the Z chromosome length is strongly conserved through most bird lineages [9,10]. Thus, the ancestors of all modern birds had a proto-Z chromosome, which was transmitted to the Paleognathae and Neognathae [1,10,11]. Although Paleognathae birds have less differentiated sex chromosomes, it has been proven they are homologous to those of the chicken [10–13].

Data concerning the evolution of sex chromosomes in birds indicate they have originated through the same mechanism—a gradual suppression of recombination around the sex-determining region and across the chromosome and subsequent loss of genetic material; however, different lineages went through different paths, as reviewed in Yazdi et al. [8]. Heteromorphic ZW chromosomes are observed in the karyotype of most bird species, from different lineages. However, species from basal groups show homomorphic karyotypes in both sexes [14–18]. For instance, it is believed that the sex chromosomes of non-ratite birds began to differentiate after the split of the ratites [2,19]. However, in birds belonging to more derived groups, such as Passeriformes or even Tinamiformes (Paleognathae order), the morphological difference between the Z and W sex chromosomes is contrasting, with the vast majority having the W sex chromosome smaller than the Z [18,20].

Cases in which the W chromosomes is similar size or even larger than the Z chromosome have been reported in some vertebrate groups: Squamata, Testudines, Anura, some fish orders, and a few bird species [21]. Among birds, some of these examples are the Surucua Trogon (*Trogon surrucura*, Trogoniformes, Trogonidae), which represents the only cytogenetically described Trogoniformes species, and presents a W chromosome with a similar size to the Z chromosome but morphologically different [22], and the Monk Parakeet (*Myiopsitta monachus*, Psittaciformes, Psittacidae), in which the Z and W sex chromosomes are homomorphic [23]. Furthermore, the Spot-flanked Gallinule (*Gallinula melanops*, Gruiformes, Rallidae) has a W chromosome larger than the Z [24].

Repetitive DNAs represent just a small fraction of a bird genome, comprising only about 4–10% of their total amount of DNA [25]. The possible explanation for the small percentage of repetitive DNAs and the small size of the bird genomes is the adaptation to the high rate of oxidative metabolism linked with the demands of flight for most species. Therefore, nonflying birds, such as the Common Ostrich (*Struthio camelus*), have bigger genomes than flying ones [26]. Thus, the reduced genome of birds is linked to the loss of repetitive elements [25,27–29], deletions of large segments of DNA, and loss of genes. Such a scenario may have had a significant effect on the emergence of many different birds' phenotypes, making possible a great diversity of species and adaptation to different environments [25,30,31].

Among the different classes of repetitive elements, microsatellites are represented by small sequences with 1 to 6 base pairs, repeated in tandem and scattered throughout the genome [32]. Although the amount of repetitive sequences is small in the avian genome, such sequences play an important role in the differentiation of sex chromosomes, as also observed in other vertebrate groups [3,33,34]. In birds, microsatellite accumulation has influenced the size and differentiation of the W chromosomes in some species belonging to the Gruiformes, Psittaciformes, and Passeriformes orders [20,23,24].

The order Caprimulgiformes includes birds with nocturnal habits, usually insectivorous, with a soft plumage and cryptic coloration, distributed in five extant families: Caprimulgidae, Nyctibiidae, Steatornithidae, Aegothelidae, and Podargidae [35] (Figure 1). From the cytogenetic and cytotaxonomic point of view, this order is one of the less-studied groups [36–39]. Nevertheless, the Common Potoo (Caprimulgiformes, Nyctibiidae) was one of the first examples of bird species with atypically large W chromosomes, based in conventional staining and C-banding analyses, and reported as an ancient ZW similar to those of ratites birds, except for heterochromatin accumulation in the W [39].

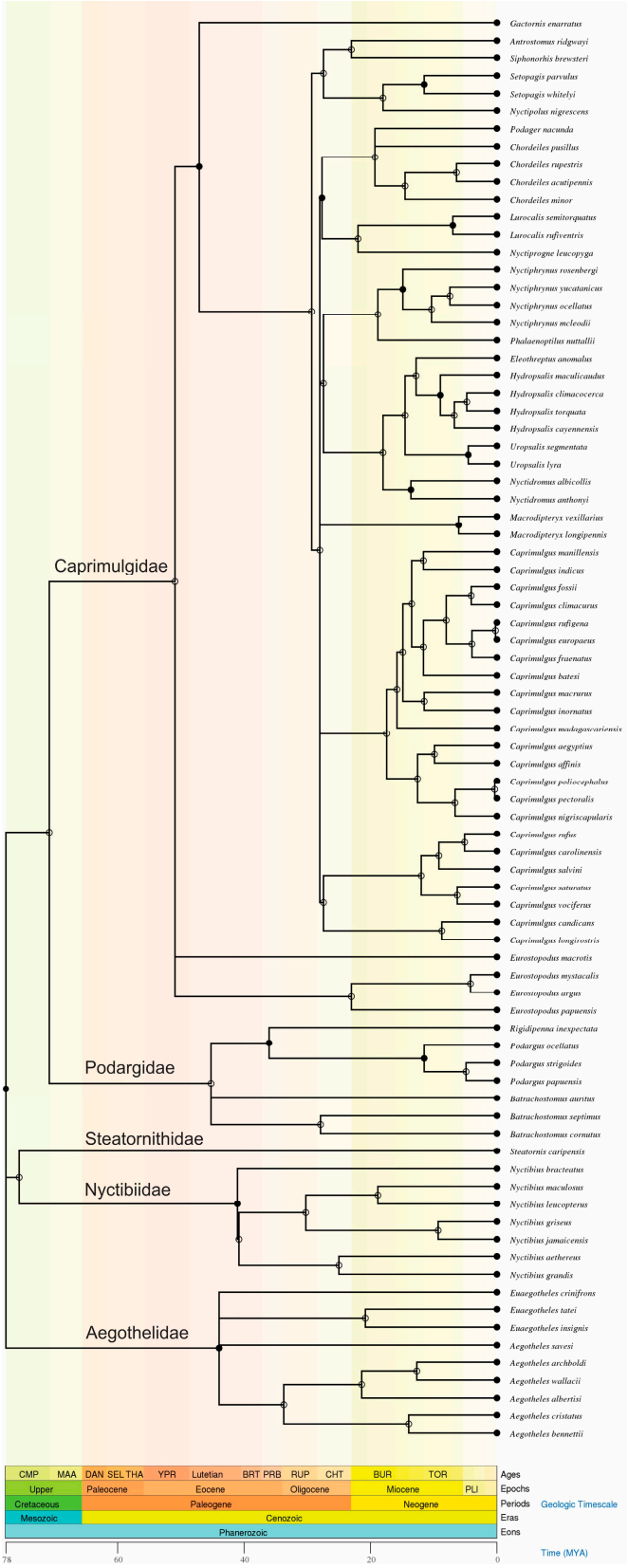

Figure 1. Phylogeny of the Caprimulgiformes order, indicating the five extant families (Caprimulgidae, Nyctibiidae, Steatornithidae, Aegothelidae, and Podargidae) in the respective branchs. The phylogenetic tree was sourced from TimeTree databases (http://www.timetree.org) [40].

Except for the uncommon W chromosome described in the Common Potoo, all Caprimulgidae species reported so far present typical avian sex chromosomes concerning their sizes, as observed in the Scissor-tailed Nightjar, the Least Nighthawk (*Nannochordeiles pusillus*) and the Little Nightjar (*Hydropsalis parvula*) [37], showing the W chromosome with significant differences in size and morphology when compared to the Z [39,41,42].

Thus, the present work was designed to analyze the microsatellite accumulation pattern in the genome of two Caprimulgiformes species, named the Common Potoo (Nyctibiidae) and the Scissor-tailed Nightjar (Caprimulgidae), in order to analyze the possible participation of these sequences in the differentiation and evolution of homomorphic and heteromorphic ZW chromosomes, respectively. These species have a median divergence time of 69 million years [40]. The results highlight the versatility throughout the evolutionary history of bird W chromosomes, despite their common origin.

2. Experimental Section

In this study, we used two females of the Common Potoo (Nyctibiidae) and one male and one female of the Scissor-tailed Nightjar (Caprimulgidae), collected using the method described by Nieto et al. [39]. Although we did not collect male individuals of the Common Potoo, it does not influence our results, since the aim was to compare the Z and W chromosomes. The animals were collected in Porto Vera Cruz, Rio Grande do Sul State (Brazil), following the authorization from "Sistema de Autorização e Informação em Biodiversidade" (SISBIO), permission numbers 44173-1 and 33860-4. The experiments followed protocols approved by the Ethics Committee on the Use of Animals (CEUA-Universidade Federal do Pampa, 018/2014).

Metaphases were obtained using bone marrow short-term culture [43] for the Common Potoo, and fibroblast cultures from tissue biopsies, according to Sasaki et al. [44], for the Scissor-tailed Nightjar. After incubation with colcemid, the process included a hypotonic treatment (0.075 M KCl) and fixation with methanol:acetic acid (3:1).

The distribution of the heterochromatic blocks was analyzed by C-banding [45], with modifications by Ledesma et al. [46]. The G-banding was performed according to Howe et al. [47]. Karyotypes were arranged by their arm ratios, and the chromosomes were classified as metacentric, submetacentric, acrocentric, and telocentric [48]. For composing the karyotype figures, Corel Draw® 12 was used.

18S rDNA fragments were amplified by PCR using primers NS1 5′-GTA GTC ATA TGC TTG TCT C-3′, NS8 5′-TCC GCA GGT TCA CCT ACG GA-3′, and nuclear DNA of Yellowtail Snapper (*Ocyurus chrysurus*, Perciformes, Lutjanidae) [49]. Afterward, fragments were labeled with digoxigenin by nick translation (Roche) and detected with Anti-Digoxigenin-Rhodamine, following the manufacturer's instructions. Preparation of slides, hybridization, and washes were performed according to Daniels and Delany [50].

For the FISH experiments, oligonucleotide probes containing the microsatellite sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, $(GC)_{15}$, and $(TA)_{15}$ were directly labeled with Streptavidin-Cy3 during synthesis (Sigma, St. Louis, MO, USA), as described by Kubat et al. [51].

For the analysis of conventional cytogenetic techniques, we used an optical microscope (OLYMPUS DP53). At least 20 metaphase spreads per individual were counted to determine the diploid chromosome numbers (2n) and to confirm the FISH results. Fluorescence in situ hybridization (FISH) images were captured using the 100× objective (UPlanFL N), using the software Axiovision® 4.8 (Zeiss, Germany), of an epifluorescent microscope (Imager Z2, Zeiss, Germany).

3. Results

3.1. Karyotype Description

The 2n previously reported for both species were confirmed, i.e., 2n = 86 for the Common Potoo and 2n = 74 for the Scissor-tailed Nightjar [37,39]. Both species have 12 macrochromosome pairs, including the ZW sex chromosomes. Submetacentric and acrocentric chromosomes are predominant among the macrochromosomes of both karyotypes and all microchromosomes present a telocentric morphology (Figure 2A–E). The W chromosome of the Common Potoo is submetacentric and similar in size and morphology to the Z chromosome, both with a size between the fourth and fifth autosomal pairs. On the other hand, the W chromosome of the Scissor-tailed Nightjar is metacentric, with a morphology and size between the 10th and 11th autosomal pairs, representing one of the smallest macrochromosomes. The Z chromosome of this species is an acrocentric chromosome, with a size between the third and fourth autosomal pairs (Figure 2C,F).

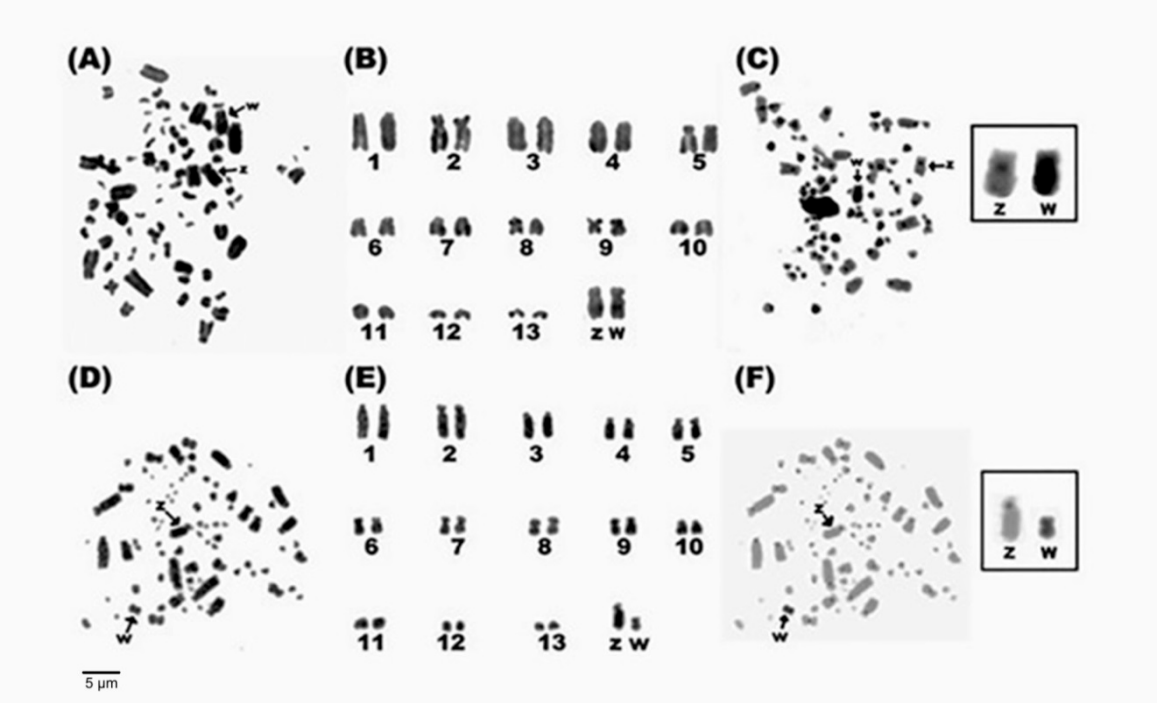

Figure 2. Metaphases, partial karyotypes, and C-banding in the Common Potoo (**A**–**C**) and in the Scissor-tailed Nightjar (**D**–**F**). Arrows indicate the Z and W sex chromosomes in the metaphases, and the C-banded sex chromosomes are also shown in boxes. Bar = 5 µm.

3.2. C-Banding

The C banding technique aims to stain specific differentially highly condensed DNA regions, which corresponds to constitutive heterochromatin. Our experiments revealed a concentration of constitutive heterochromatin mainly in the centromeric regions and the W sex chromosome in metaphases of both species. Although the Z and W chromosomes of the Common Potoo look very similar in conventional staining, a remarkable difference emerges between them after the C-banding procedure. Hence, while the Z has only one block of heterochromatin in the pericentromeric region, the W chromosome is almost completely heterochromatic. The same pattern was observed in the Scissor-tailed Nightjar (Figure 2D,F). The presence of some microchromosomes pairs with extensive constitutive heterochromatin accumulation was also found in the Common Potoo, but not in the Scissor-tailed Nightjar (Figure 2C,F).

3.3. G-Banding in the ZW Sex Chromosomes of the Common Potoo

We have performed G-banding in the metaphases of the Common Potoo in order to better distinguish the Z and W chromosomes regarding their content. Furthermore, the Scissor-tailed Nightjar karyotype by itself shows the heteromorphic Z and W chromosomes and we presume it is not necessary to present its G-banding. This technique allows each chromosome to be identified by its banding pattern. The negative bands (light bands) correspond to the euchromatin regions in the DNA having the greatest transcriptional activity, while the highly condensed chromatin with little or no transcriptional activity corresponds to the positive bands (dark bands). G-banding experiments revealed a distinct pattern of positive and negative bands in both the Z and W chromosomes, with variation in the number and distribution of bands in each chromosome (totals: Z = 14 bands/W = 12 bands). Remarkably, the G-banding pattern are different on the Z and W chromosomes (Figure 3).

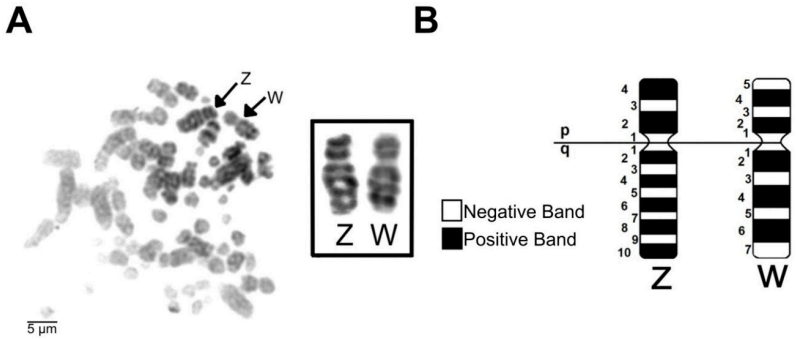

Figure 3. Metaphase of a female of the Common Potoo after the G-banding procedure. Arrows indicate the Z and W chromosomes and the G-banded sex chromosomes are also shown in boxes (**A**). G-banding scheme showing the distinct patterns on the Z and W chromosomes (**B**). Bar = 5 µm.

3.4. Microsatellite and 18S rDNA Hybridization

In both species, the 18S rDNA probes have hybridized on only one chromosome pair, co-localized with a large $(CGG)_{10}$ cluster in the Common Potoo (Figures 4A and 5F). Concerning the overall results of microsatellite repeats, different patterns of hybridization were observed when comparing the Common Potoo and the Scissor-tailed Nightjar, in different aspects such as signal presence, amount, and location. Hence, out of a total of 11 different microsatellite sequence probes—named $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, $(GC)_{15}$, and $(TA)_{15}$—seven exhibited signals exclusively in the Common Potoo ($(CAA)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, and $(GC)_{15}$) (Figure 5A–J).

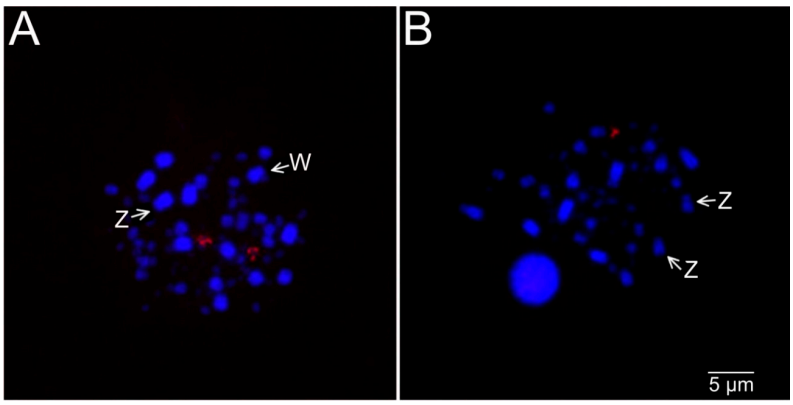

Figure 4. Metaphases of a female Common Potoo (**A**) and male Scissor-tailed Nightjar (**B**) hybridized with a 18S rDNA probe (red signals). Note that in the Scissor-tailed Nightjar (**B**) the NOR-bearing chromosomes are associated. Arrows indicate the sex chromosomes. Bar = 5 µm.

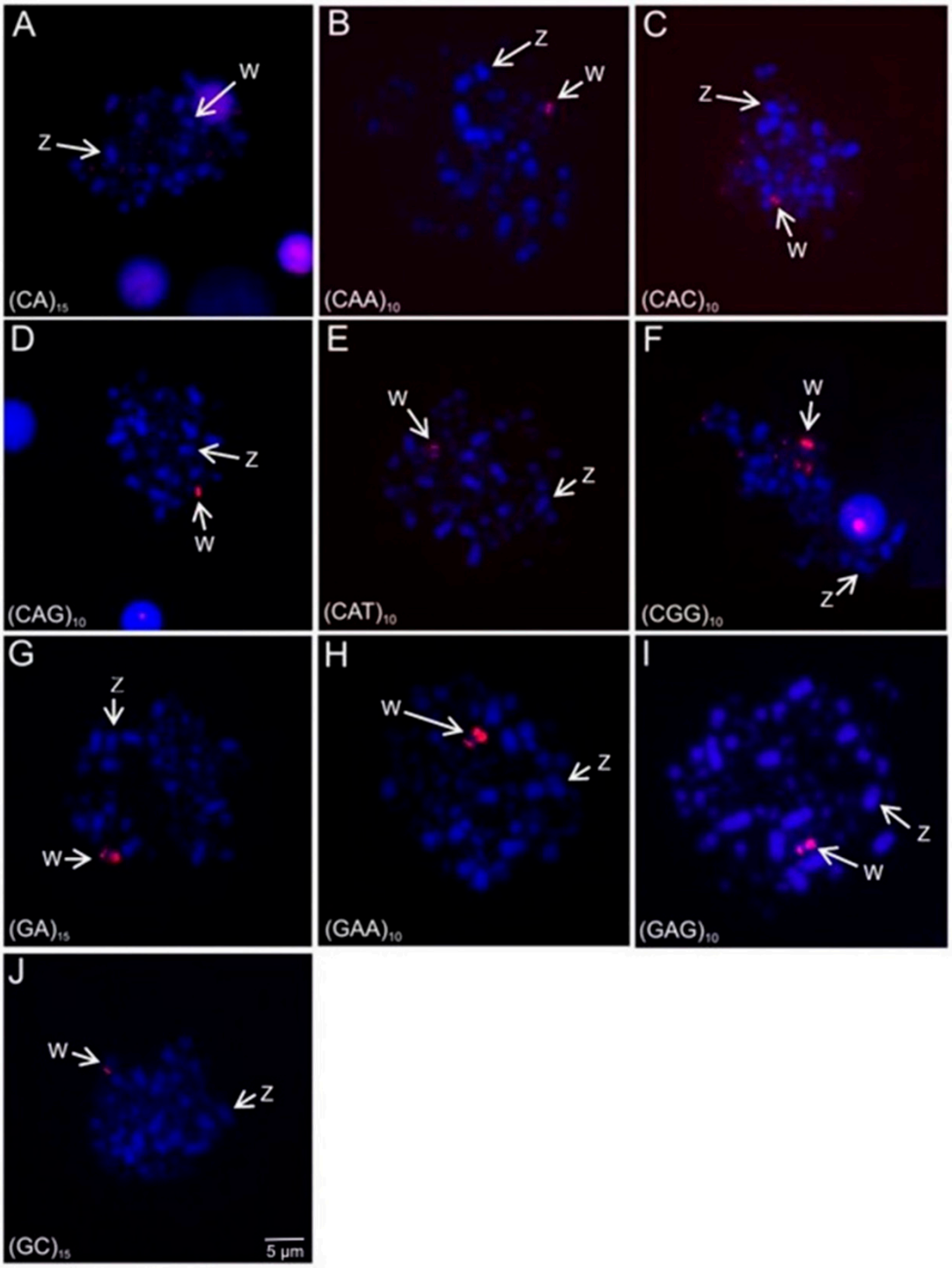

Figure 5. Metaphases of a female Common Potoo (**A**–**J**) highlighting the hybridization of distinct microsatellite sequences. The chromosome probes used are indicated on the bottom left. Arrows indicate the sex chromosomes. Bar = 5 µm.

The sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, and $(CGG)_{10}$ hybridized preferentially in the microchromosome of the Common Potoo. Surprisingly, none of the tested microsatellite motifs have been detected in the macrochromosomes of the Common Potoo, except in the W sex chromosome. From those 11 microsatellite sequences, only four have shown positive signals in the choromosome of the Scissor-tailed Nightjar. The sequences $(CA)_{15}$, $(CAC)_{10}$, $(CGG)_{10}$, and $(TA)_{15}$ hybridized in both the macro and micro autosomal chromosomes (Figure 6A–D). In the macrochromosomes, microsatellite sequence $(CA)_{15}$ is accumulated in the telomeric region of the short arm of pair 5, $(CAC)_{10}$ in the distal short arms of the chromosome pairs 1 and 2, and also in the distal area of the short arm of autosomal pair 6. The $(CGG)_{10}$ microsatellite probe has produced signals in the distal long arms of pair 1 as well in the NOR-bearer microchromosome pair. Finally, the sequence $(TA)_{15}$ produced distal telomeric signals in the short arm of pair 6. There were no microsatellite hybridizations in the Scissor-tailed Nightjar's W chromosome. Furthermore, the microsatellite motifs $(CA)_{15}$, $(CAC)_{10}$, and $(CGG)_{10}$ displayed signals in the chromosome complement of both species.

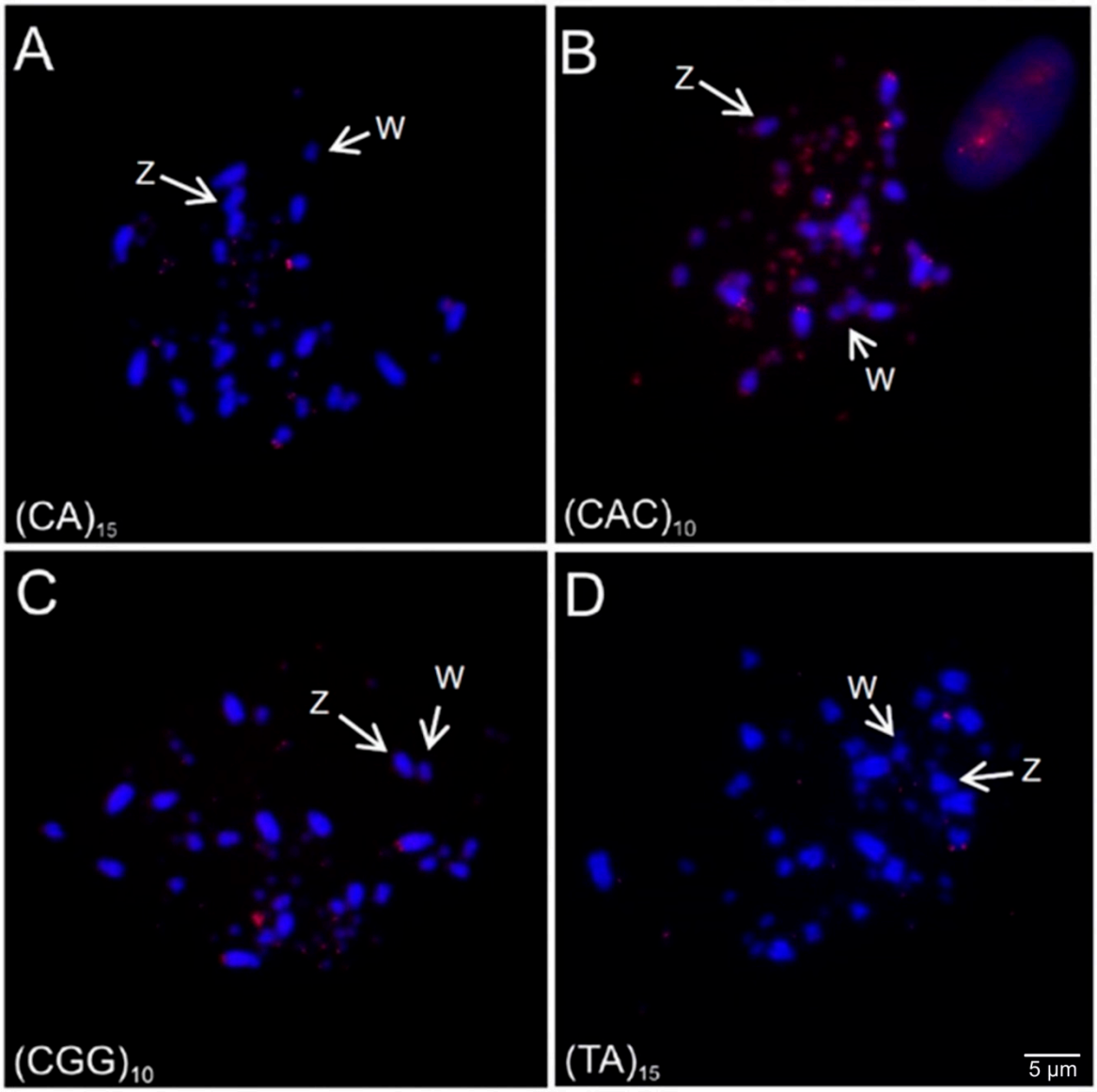

Figure 6. Metaphases of a female Scissor-tailed Nightjar (**A**–**D**) highlighting the hybridization of distinct microsatellite sequences. The chromosome probes used are indicated on the bottom left. Arrows indicate the sex chromosomes. Bar = 5 µm.

3.5. Common Potoo W Sex Chromosome

The W chromosome of the Common Potoo accumulated 9 out of the 11 microsatellite probes, with the only exception of the $(CA)_{15}$ and $(TA)_{15}$ sequences. Besides, several microsatellite sequences were observed exclusively in the W chromosome, such as $(CAG)_{10}$, $(CAT)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, and $(GC)_{15}$ (Figure 5D–J). The sequences and their distribution in the W sex chromosome of the Common Potoo are shown in Figure 7.

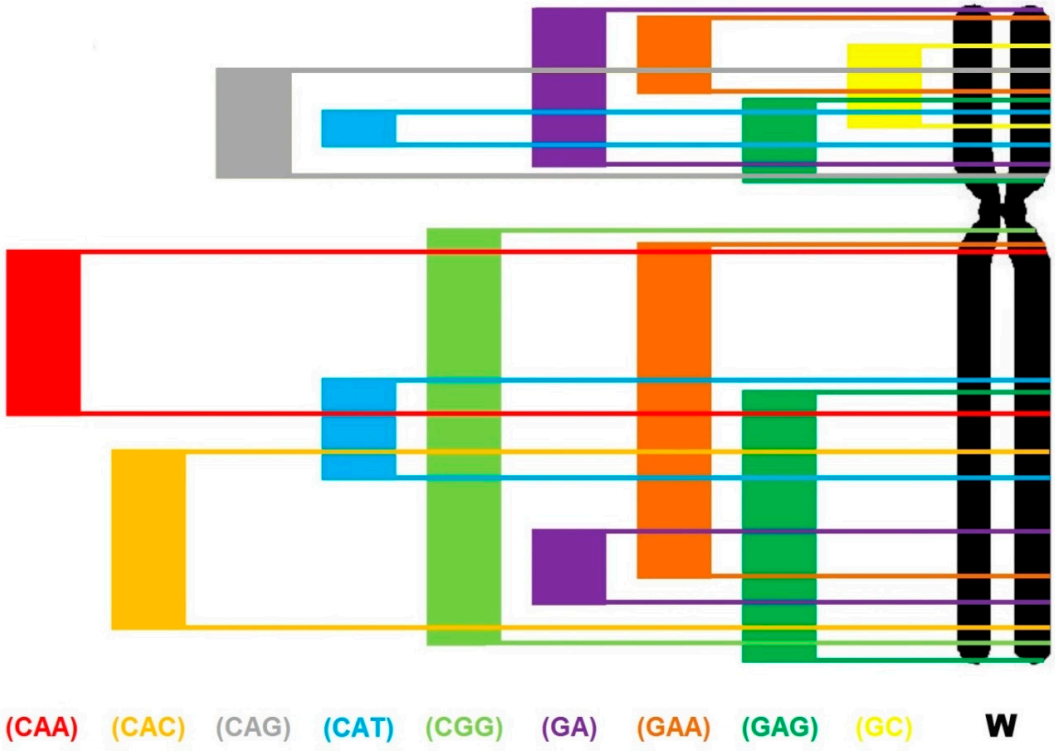

Figure 7. Distribution of the microsatellite probes in the W chromosome of the Common Potoo. Microsatellite sequences are depicted in different colors.

Conspicuous hybridization signals of the microsatellite motifs $(GA)_{15}$, $(GAA)_{10}$, and $(GAG)_{10}$ were observed both in the long and short arms of the W chromosome (Figure 5G–I). The sequences $(CAA)_{10}$, $(CAC)_{10}$, and $(CGG)_{10}$ hybridized on the W chromosome and also on some microchromosome pairs. No hybridization signals were observed in the Z sex chromosome of the Common Potoo for any of those microsatellite repeats.

4. Discussion

In this study, we used two species of birds representing homomorphic and heteromorphic ZW sex chromosomes, considering their length in terms of karyotyping, to investigate the role of repetitive DNA in the enlargement of the sex-specific (W) chromosome. We demonstrated that the W chromosome was subjected to particular evolutionary processes, as evidenced by their distinct heterochromatin composition, illustrated by the unequal distribution and accumulation of several microsatellite repeats. Thus, while in the W chromosome of the Scissor-tailed Nightjar no accumulation from those 11 microsatellite sequences tested in the experiments was observed, the W chromosome of the Common Potoo showed a large degree of accumulation of repetitive sequences.

4.1. Chromosomal Divergences between Common Potoo and Scissor-Tailed Nightjar

Despite the considerable variation in their diploid numbers (2n = 86 in the Common Potoo and 2n = 74 in the Scissor-tailed Nightjar), already demonstrated by previous reports [37,39], substantial

additional divergences could also be highlighted here. Concerning the number of microchromosomes in both species, we can assume that the reduction of these elements in the Scissor-tailed Nightjar probably resulted from interchromosomal rearrangements [52,53]. Although both species present specific constitutive heterochromatin-rich regions in their genomes, pairs of microchromosomes were almost completely heterochromatic only in the karyotype of the Common Potoo (Figure 2C,F).

Both species preserved a single pair of microchromosomes carrying ribosomal clusters, corroborating Degrandi et al. [54]. In fact, most birds species show only one microchromosomal pair bearing the 18S rDNA sequence, including the Palaeognathae, such as the Ostrich (Struthioniformes) and the Rhea (Rheiformes), considered more basal in avian phylogeny [17,55], and also most Neognathae birds, such as the Chicken (*Gallus gallus*, Galliformes), some Doves and Pigeons (Columbiformes), and the Great Kiskadee (*Pitangus sulphuratus*, Passeriformes) [54,56–58]. Interestingly, the 18S rDNA clusters are associated with the microsatellite sequence $(CGG)_{10}$ in the Common Potoo and the Scissor-tailed Nightjar (Figures 4B and 5C). Considering that similar results were described for three species of woodpeckers [33], and even in a species of fish, the Freshwater Sardine (*Triportheus trifurcatus*, Characiformes) [59], they indicate a certain affinity between the 18S rDNA cluster and the $(CGG)_{10}$ microsatellite sequence, which may be an interesting feature in evolutionary issues given its phylogenetic amplitude, which could indicate a conserved state of this character in distinct genomes.

The hybridization of microsatellite sequences in the chromosomes of the Common Potoo and the Scissor-tailed Nightjar presented differences regarding the amount, location, and signal intensity. Seven probes exhibited signals exclusively in the Common Potoo, $(CAA)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$, and $(GC)_{15}$, not showing any positive signals in the chromosomes of the Scissor-tailed Nightjar. This fact demonstrates a great difference in the diversity and accumulation of repetitive DNAs between these species. It should be noted that, among the autosomal chromosomes of the Common Potoo, the sequences $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, and $(CGG)_{10}$ produced signals in only a few microchromosomes with no visible hybridization signals in any of the autosomal macrochromosomes. In contrast, except for $(CAA)_{10}$, those oligonucleotide probes hybridized in the micro- and macrochromosomes of the Scissor-tailed Nightjar. However, there was also the hybridization of $(TA)_{15}$, which did not show signals in the Common Potoo, revealing a characteristic possibly intrinsic to the Scissor-tailed Nightjar genome [58]. The microsatellite probes $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, and $(CGG)_{10}$ produced positive signals in the genome of the Columbiformes and Piciformes, both in autosomal macro- and microchromosomes [34,58], also located in the euchromatic regions. Therefore, we may consider $(CA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, and $(CGG)_{10}$ as important elements of functional regions in bird genomes whereupon they are not associated with constitutive heterochromatin.

Altogether, these results may indicate that throughout the evolutionary history of this group, the ancestor of the Common Potoo accumulated more microsatellite sequences than the ancestor of the Scissor-tailed Nightjar, differentiating these two lineages in the order Caprimulgiformes.

4.2. The Sex Chromosomes of Common Potoo and Scissor-Tailed Nightjar: Same Origin, Different Evolutionary Pathways

Despite the obvious common origin of the W chromosomes of both species [55], it is particularly puzzling that these chromosomes followed different evolutionary pathways since their morphology and heterochromatin composition are strikingly different in each species. In the Scissor-tailed Nightjar, the W chromosome is one of the smallest metacentric elements and largely heterochromatic [37]. On the other hand, the Z chromosome presented just a small block of heterochromatin in the pericentromeric region and has an acrocentric morphology located between the 3rd and 4th autosomal pairs (Figure 2D,F). These same patterns are also frequently found in the sex chromosomes of other Neognathae species [41,42,60]. However, in the Common Potoo, the W chromosome is submetacentric and similar to the Z, both with a size between the 4th and 5th autosomal pairs. Indeed, the Z and W chromosomes of the Common Potoo appear to be homomorphic when analyzed in conventional Giemsa staining (Figure 2B). However, after C-banding, it was possible to observe a remarkable difference

between them, since the Z presented only a heterochromatic marking in its pericentromeric region, while the W was notably heterochromatic (Figure 2A–C). The G-banding pattern was also distinct between these chromosomes. Further, the comparative G-banding pattern of the Monk Parakeet W chromosome is quite different from what was observed in the Common Potoo, which is different in number and position of bands [23], suggesting the W chromosome has undergone different processes of differentiation regarding the composition and sequences organization, which becomes more evident after the chromosomal mapping of the microsatellite repeats, as discussed below.

The amount and diversity of microsatellites in the W chromosome of the Common Potoo are remarkable (Figure 6). Therefore, we can infer that the genome organization of this species has placed the repetitive sequences on the W chromosome by a process of accumulation and amplification of repetitive sequences, which led to an increment in the size of the W, similarly to what was found in Monk Parakeet [23]. Although the accumulation of repetitive DNAs in the W chromosome (similar to or larger than Z) have occurred independently in different species, as reported in the Monk Parakeet and the Spot-flanked Gallinule [23,24], these sequences have the typical mutational behavior, higher than other parts of the genome, leading to several microsatellite sequence amplifications in the W chromosome of the Common Potoo [58,61]. Thus, it is possible that this chromosome is in a distinct state of differentiation by the accumulation of repetitive microsatellite sequences, and not an intermediary representative for the ZW sexual differentiation system in the avian class, as proposed by Nieto et al. [39]. Nevertheless, these sequences do not have the same origin and are probably silenced through the extensive heterochromatin in the W chromosome.

Despite the accumulation of several microsatellites repeats in the W chromosome of the Common Potoo, in the Scissor-tailed Nightjar this chromosome did not show any microsatellite hybridization, similarly to what was observed in a study analyzing Picidae species [34]. Therefore, it is plausible that the W chromosome of the Common Potoo reacted to evolutionary pressures, more likely genetic drift, leading to this highly differentiated W chromosome. Thus, it demonstrates the importance of accumulation and amplification of repetitive sequences in the morphological and sequence differentiation of sex chromosomes in birds [23,34], as well as in the evolutionary dynamics of the W chromosome [20]. Indeed, previous works have also highlighted the key role of microsatellite in young/non-differentiated sex chromosomes in contrast to highly differentiated ones [62,63].

5. Conclusions

In conclusion, we have found different results for each species, indicating that the diversity of repetitive motifs is an important chromosome marker in avian evolutionary and genomic research, particularly for sex chromosomes. The large blocks of microsatellites and the heterochromatin amount in the W chromosome of the Common Potoo may be the result of the response mechanisms to different evolutionary pressures, leading this genome to accumulate repetitive sequences and resulting in the morphological differentiation and enlargement of this sex chromosome, which is uncommon for most bird species [20,24,60]. We can speculate that such a difference in the degeneration progression of homologous sex chromosomes might result from the lineage-specific mechanisms that influenced the rate of W-chromosome differentiation [8,21]. These characteristics highlight the importance of the Common Potoo as a model species for studies concerning the differentiation of the ZW sex system.

Author Contributions: Conceptualization, M.S.d.S. and R.J.G.; methodology, M.S.d.S., R.K., M.d.B.C., E.H.C.d.O., A.D.V.G. and R.J.G.; validation, M.S.d.S., R.K., M.B.C., E.H.C.d.O., A.D.V.G. and R.J.G.; formal analysis, M.S.d.S., R.K., S.A.B., A.D.V.G. and R.J.G.; investigation, M.S.d.S., R.K., S.A.B. and A.L.C.; resources, M.S.d.S., R.K., M.d.B.C., E.H.C.d.O., A.D.V.G. and R.J.G.; data curation, M.S.d.S., A.D.V.G., and R.J.G.; writing—original draft preparation, M.S.d.S.; writing—review and editing, M.S.d.S., R.K., S.A.B., A.L.C., M.d.B.C., E.H.C.d.O. and R.J.G.; visualization, M.S.d.S., S.A.B. and A.L.C.; supervision, E.H.C.d.O., A.D.V.G. and R.J.G.; project administration, R.J.G.; funding acquisition, M.d.B.C., E.H.C.d.O., A.D.V.G. and R.J.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Pró-Reitoria de Pesquisa, Pós-Graduação e Inovação from Universidade Federal do Pampa. MBC was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (401962/2016-4 and 302449/2018-3) e Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2018/22033-1).

Acknowledgments: The authors are grateful to Diversidade Genética Animal Research Group (Universidade Federal do Pampa), PROPPI—Universidade Federal do Pampa, SISBIO and Instituto Evandro Chagas for the contributions and logistic support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mank, J.E.; Ellegren, H. Parallel divergence and degradation of the avian W sex chromosome. *Trends Ecol. Evol.* **2007**, *22*, 389–391. [CrossRef] [PubMed]
2. Abbott, J.K.; Nordén, A.K.; Hansson, B. Sex chromosome evolution: Historical insights and future perspectives. *Proc. R. Soc. B* **2017**, *284*. [CrossRef] [PubMed]
3. Matsubara, K.; O'Meally, D.; Azad, B.; Georges, A.; Sarre, S.D.; Graves, J.A.M.; Matsuda, Y.; Ezaz, T. Amplification of microsatellite repeat motifs is associated with the evolutionary differentiation and heterochromatinization of sex chromosomes in Sauropsida. *Chromosoma* **2016**, *125*, 111–123. [CrossRef] [PubMed]
4. Charlesworth, D.; Charlesworth, B.; Marais, G. Steps in the evolution of heteromorphic sex chromosomes. *Heredity* **2005**, *95*, 118. [CrossRef] [PubMed]
5. Cioffi, M.B.; Camacho, J.P.; Bertollo, L.A. Repetitive DNAs and differentiation of sex chromosomes in Neotropical fishes. *Cytogenet. Genome Res.* **2011**, *132*, 188–194. [CrossRef] [PubMed]
6. Nanda, I.; Shan, Z.; Schartl, M.; Burt, D.W.; Koehler, M.; Nothwang, H.; Grützner, F.; Paton, I.R.; Windsor, D.; Dunn, I.; et al. 300 million years of conserved synteny between chicken Z and human chromosome 9. *Nat. Genet.* **1999**, *21*, 258. [CrossRef] [PubMed]
7. Graves, J.A. Sex chromosome specialization and degeneration in mammals. *Cell* **2006**, *124*, 901–914. [CrossRef]
8. Yazdi, H.P.; Silva, W.T.; Suh, A. Why do some sex chromosomes degenerate more slowly than others? The odd case of ratite sex chromosomes. *Genes* **2020**, *11*, 1153. [CrossRef]
9. Nanda, I.; Schmid, M. Conservation of avian Z chromosomes as revealed by comparative mapping of the Z-linked aldolase B gene. *Cytogenet. Genome Res.* **2002**, *96*, 176–178. [CrossRef]
10. Xu, L.; Zhou, Q. The Female-Specific W Chromosomes of Birds Have Conserved Gene Contents but Are Not Feminized. *Genes* **2020**, *11*, 1126. [CrossRef]
11. Matsubara, K.; Tarui, H.; Toriba, M.; Yamada, K.; Nishida-Umehara, C.; Agata, K.; Matsuda, Y. Evidence for different origin of sex chromosomes in snakes, birds, and mammals and step-wise differentiation of snake sex chromosomes. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18190–18195. [CrossRef] [PubMed]
12. Ogawa, A.; Murata, K.; Mizuno, S. The location of Z-and W-linked marker genes and sequence on the homomorphic sex chromosomes of the ostrich and the emu. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 4415–4418. [CrossRef] [PubMed]
13. Shetty, S.; Griffin, D.K.; Graves, J.A.M. Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Res.* **1999**, *7*, 289–295. [CrossRef]
14. Takagi, N.; Itoh, M.; Sasaki, M. Chromosome studies in four species of Ratitae (Aves). *Chromosoma* **1972**, *36*, 281–291. [CrossRef] [PubMed]
15. Gunski, R.J.; Giannoni, M.L. Nucleolar organizer regions and a new chromosome number for *Rhea americana* (Aves: Rheiformes). *Genet. Mol. Biol.* **1998**, *21*, 207–210. [CrossRef]
16. Sumner, A.T. *Euchromatin and the Longitudinal Differentiation of Chromosomes. Chromosomes-Organization and Function*, 1st ed.; Blackwell: Oxford, UK, 2003; pp. 117–132.
17. Nishida-Umehara, C.; Tsuda, Y.; Ishijima, J.; Ando, J.; Fujiwara, A.; Matsuda, Y.; Griffin, D.K. The molecular basis of chromosome orthologies and sex chromosomal differentiation in palaeognathous birds. *Chromosome Res.* **2007**, *15*, 721–734. [CrossRef] [PubMed]
18. Pigozzi, M.I. Diverse stages of sex-chromosome differentiation in tinamid birds: Evidence from crossover analysis in *Eudromia elegans* and *Crypturellus tataupa*. *Genetica* **2011**, *139*, 771. [CrossRef]

19. Fridolfsson, A.K.; Cheng, H.; Copeland, N.G.; Jenkins, N.A.; Liu, H.C.; Raudsepp, T.; Woodage, T.; Chowdhary, B.; Halverson, J.; Ellegren, H. Evolution of the avian sex chromosomes from an ancestral pair of autosomes. *Proc. Nat. Acad. Sci. USA* **1998**, *95*, 8147–8152. [CrossRef]
20. Barcellos, S.A.; Kretschmer, R.; de Souza, M.S.; Costa, A.L.; Degrande, T.M.; dos Santos, M.S.; de Oliveira, E.H.; Cioffi, M.B.; Gunski, R.J.; Garnero, A.D.V. Karyotype evolution and distinct evolutionary history of the W chromosomes in swallows (Aves, Passeriformes). *Cytogenet. Genome Res.* **2019**, *158*, 98–105. [CrossRef]
21. Schartl, M.; Schmid, M.; Nanda, I. Dynamics of vertebrate sex chromosome evolution: From equal size to giants and dwarfs. *Chromosoma* **2016**, *125*, 553–571. [CrossRef]
22. Degrandi, T.M.; del Valle Garnero, A.; O'Brien, P.C.; Ferguson-Smith, M.A.; Kretschmer, R.; de Oliveira, E.H.; Gunski, R.J. Chromosome painting in *Trogon surrucura* (Aves, Trogoniformes) reveals a karyotype derived by chromosomal fissions, fusions, and inversions. *Cytogenet. Genome Res.* **2017**, *151*, 208–215. [CrossRef] [PubMed]
23. De Oliveira Furo, I.; Kretschmer, R.; dos Santos, M.S.; de Lima Carvalho, C.A.; Gunski, R.J.; O'Brien, P.C.; Ferguson-Smith, M.A.; Cioffi, M.B.; de Oliveira, E.H. Chromosomal mapping of repetitive DNAs in *Myiopsitta monachus* and *Amazona aestiva* (Psittaciformes, Psittacidae) with emphasis on the sex chromosomes. *Cytogenet. Genome Res.* **2017**, *151*, 151–160. [CrossRef]
24. Gunski, R.J.; Kretschmer, R.; de Souza, M.S.; de Oliveira Furo, I.; Barcellos, S.A.; Costa, A.L.; Cioffi, M.B.; de Oliveira, E.H.C.; del Valle Garnero, A. Evolution of bird sex chromosomes narrated by repetitive sequences: Unusual W chromosome enlargement in *Gallinula melanops* (Aves: Gruiformes: Rallidae). *Cytogenet. Genome Res.* **2019**, *158*, 152–159. [CrossRef] [PubMed]
25. Zhang, G.; Li, C.; Li, Q.; Li, B.; Xu, L.; Pan, H.; Wang, Z.; Jin, L.; Zhang, P.; Larkin, D.M.; et al. Comparative genomics reveals insights into avian genome evolution and adaptation. *Science* **2014**, *346*, 1311–1320. [CrossRef]
26. Hughes, A.L.; Hughes, M.K. Small genomes for better flyers. *Nature* **1995**, *377*, 391. [CrossRef]
27. Kapusta, A.; Suh, A.; Feschotte, C. Dynamics of genome size evolution in birds and mammals. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E1460–E1469. [CrossRef] [PubMed]
28. Schmid, M.; Nanda, I.; Guttenbach, M.; Steinlein, C.; Hoehn, M.; Schartl, M.; Haaf, T.; Weigend, S.; Fries, R.; Buerstedde, J.M.; et al. First report on chicken genes and chromosomes 2000. *Cytogenet. Cell Genet.* **2000**, *90*, 169–218. [CrossRef] [PubMed]
29. Hughes, A.L.; Piontkivska, H. DNA repeat arrays in chicken and human genomes and the adaptive evolution of avian genome size. *BMC Evol. Biol.* **2005**, *5*, 12. [CrossRef]
30. Stevens, L. Sex chromosomes and sex determining mechanisms in birds. *Sci. Progress.* **1997**, *80*, 197–216.
31. Hughes, A.L.; Friedman, R. Genome size reduction in the chicken has involved massive loss of ancestral protein-coding genes. *Mol. Biol. Evol.* **2008**, *25*, 2681–2688. [CrossRef]
32. Ellegren, H. Microsatellites: Simple sequences with complex evolution. *Nat. Rev. Genet.* **2004**, *5*, 435. [CrossRef]
33. Pokorná, M.; Kratochvíl, L.; Kejnovský, E. Microsatellite distribution on sex chromosomes at different stages of heteromorphism and heterochromatinization in two lizard species (Squamata: Eublepharidae: *Coleonyx elegans* and Lacertidae: *Eremias velox*). *BMC Genet.* **2011**, *12*, 90. [CrossRef] [PubMed]
34. De Oliveira, T.D.; Kretschmer, R.; Bertocchi, N.A.; Degrandi, T.M.; de Oliveira, E.H.C.; de Bello Cioffi, M.; del Valle Garnero, A.; Gunski, R.J. Genomic organization of repetitive DNA in woodpeckers (Aves, Piciformes): Implications for karyotype and ZW sex chromosome differentiation. *PLoS ONE* **2017**, *12*, e0169987. [CrossRef]
35. Mayr, G. Osteological evidence for paraphyly of the avian order Caprimulgiformes (nightjars and allies). *J. Ornithol.* **2002**, *143*, 82–97. [CrossRef]
36. Belterman, R.H.R.; De Boer, L.E.M. A karyological study of 55 species of birds, including karyotypes of 39 species new to cytology. *Genetica* **1984**, *65*, 39–82. [CrossRef]
37. Nieto, L.M.; Gunski, R.J. Estudios cromossomicos em Atajacaminos (Aves, Caprimulgidae). *Boll. Soc. Biol. Concepc.* **1998**, *69*, 161–169.
38. Francisco, M.R.; Lunardi, O.; Garcia, C.; Junior, P.M. Karyotype description of the Semicollared Nighthawk, *Lurocalis semitorquatus* (Caprimulgidae). *Revist. Bras. Ornit.* **2006**, *14*, 63–65.
39. Nieto, L.M.; Kretschmer, R.; Ledesma, M.A.; Garnero, A.D.V.; Gunski, R.J. Karyotype morphology suggests that the *Nyctibius griseus* (Gmelin, 1789) carries an ancestral ZW-chromosome pair to the order Caprimulgiformes (Aves). *Comp. Cytogenet.* **2012**, *6*, 379. [CrossRef]

40. Kumar, S.; Stecher, G.; Suleski, M.; Hedges, S.B. TimeTree: A Resource for Timelines, Timetrees, and Divergence Times. *Mol. Biol. Evol.* **2017**, *34*, 1812–1819. [CrossRef]
41. Kretschmer, R.; de Lima, V.L.C.; de Souza, M.S.; Costa, A.L.; O'Brien, P.C.; Ferguson-Smith, M.A.; de Oliveira, E.H.C.; Gunski, R.J.; Garnero, A.D.V. Multidirectional chromosome painting in *Synallaxis frontalis* (Passeriformes, Furnariidae) reveals high chromosomal reorganization, involving fissions and inversions. *Comp. Cytogenet.* **2018**, *12*, 97. [CrossRef]
42. Kretschmer, R.; Ferguson-Smith, M.A.; de Oliveira, E.H.C. Karyotype evolution in birds: From conventional staining to chromosome painting. *Genes* **2018**, *9*, 181. [CrossRef] [PubMed]
43. Garnero, A.V.; Gunski, R.J. Comparative analysis of the karyotypes of *Nothura maculosa* and *Rynchotus rufescens* (Aves: Tinamidae): A case of chromosomal polymorphism. *Nucleus* **2000**, *43*, 64–70.
44. Sasaki, M.; Ikeuchi, T.; Makino, S. A feather pulp culture for avian chromosomes with notes on the chromosomes of the peafowl and the ostrich. *Experientia* **1968**, *24*, 1923–1929. [CrossRef] [PubMed]
45. Sumner, A.T. A simple technique for demonstrating centromeric heterochromatin. *Exp. Cell Res.* **1972**, *75*, 304–306. [CrossRef]
46. Ledesma, M.A.; Garnero, A.D.V.; Gunski, R.J. Análise do Cariótipo de duas espécies da Família Formicariidae (Aves: Passeriformes). *Ararajuba* **2002**, *10*, 15–19.
47. Howe, B.; Umrigar, A.; Tsien, F. Chromosome preparation from cultured cells. *JoVE J. Vis. Exp.* **2014**, *83*. [CrossRef]
48. Guerra, M. *FISH: Conceitos e Aplicações na Citogenética*, 1st ed.; Sociedade Brasileira de Genética: Ribeirão Preto, Brazil, 2004; pp. 88–89.
49. White, T.J.; Bruns, T.; Lee, S.J.; Taylor, J.L. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications*; Academic Press: Cambridge, MA, USA, 1990; Volume 18, pp. 315–322.
50. Daniels, L.M.; Delany, M.E. Molecular and cytogenetic organization of the 5S ribosomal DNA array in chicken (*Gallus gallus*). *Chromosome Res.* **2003**, *11*, 305–317. [CrossRef]
51. Kubat, Z.; Hobza, R.; Vyskot, B.; Kejnovsky, E. Microsatellite accumulation on the Y chromosome in *Silene latifolia*. *Genome* **2008**, *51*, 350–356. [CrossRef]
52. Burt, D.W. Origin and evolution of avian microchromosomes. *Cytogenet. Genome Res.* **2002**, *96*, 97–112. [CrossRef]
53. Skinner, B.M.; Griffin, D.K. Intrachromosomal rearrangements in avian genome evolution: Evidence for regions prone to breakpoints. *Heredity* **2012**, *108*, 37–41. [CrossRef]
54. Degrandi, T.M.; Gunski, R.J.; Garnero, A.D.V.; de Oliveira, E.H.C.; Kretschmer, R.; de Souza, M.S.; Barcellos, S.A.; Hass, I. The distribution of 45S rDNA sites in bird chromosomes suggests multiple evolutionary histories. *Genet. Mol. Biol.* **2020**, *43*, e20180331. [CrossRef] [PubMed]
55. Jarvis, E.D.; Mirarab, S.; Aberer, A.J.; Li, B.; Houde, P.; Li, C.; Ho, S.Y.; Faircloth, B.C.; Nabholz, B.; Howard, J.T.; et al. Whole-genome analyses resolve early branches in the tree of life of modern birds. *Science* **2014**, *346*, 1320–1331. [CrossRef] [PubMed]
56. Dyomin, A.G.; Koshel, E.I.; Kiselev, A.M.; Saifitdinova, A.F.; Galkina, S.A.; Fukagawa, T.; Kostareva, A.A.; Gaginskaya, E.R. Chicken rRNA gene cluster structure. *PLoS ONE* **2016**, *11*, e0157464. [CrossRef] [PubMed]
57. Rodrigues, B.S.; Kretschmer, R.; Gunski, R.J.; Garnero, A.D.V.; O'Brien, P.C.M.; Ferguson-Smith, M.A.; de Oliveira, E.H.C. Chromosome painting in tyrant flycatchers confirms a set of inversions shared by Oscines and Suboscines (Aves, Passeriformes). *Cytogenet. Genome Res.* **2018**, *153*, 205–212. [CrossRef] [PubMed]
58. Kretschmer, R.; de Oliveira, T.D.; Furo, I.O.; Silva, F.A.O.; Gunski, R.J.; Garnero, A.D.V.; Cioffi, M.B.; de Oliveira, E.H.C.; de Freitas, T.R.O. Repetitive DNAs and shrink genomes: A chromosomal analysis in nine Columbidae species (Aves, Columbiformes). *Genet. Mol. Biol.* **2018**, *41*, 98–106. [CrossRef] [PubMed]
59. Yano, C.F.; Poltronieri, J.; Bertollo, L.A.C.; Artoni, R.F.; Liehr, T.; de Bello Cioffi, M. Chromosomal mapping of repetitive DNAs in Triportheus trifurcatus (Characidae, Characiformes): Insights into the differentiation of the Z and W chromosomes. *PLoS ONE* **2014**, *9*, e90946. [CrossRef]
60. Smith, C.A.; Roeszler, K.N.; Hudson, Q.J.; Sinclair, A.H. Avian sex determination: What, when and where? *Cytogenet. Genome Res.* **2007**, *117*, 165–173. [CrossRef]
61. López-Flores, I.; Garrido-Ramos, M.A. The repetitive DNA content of eukaryotic genomes. *Repetitive DNA* **2012**, *7*, 1–28. [CrossRef]

62. Kejnovsky, E.; Hobza, R.; Cermak, T.; Kubat, Z.; Vyskot, B. The role of repetitive DNA in structure and evolution of sex chromosomes in plants. *Heredity* **2009**, *102*, 533–541. [CrossRef]
63. Cioffi, M.B.; Moreira-Filho, O.; Almeida-Toledo, L.F.; Bertollo, L.A.C. The contrasting role of heterochromatin in the differentiation of sex chromosomes: An overview from Neotropical fishes. *J. Fish Biol.* **2012**, *80*, 2125–2139. [CrossRef]