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

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

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

INTRODUCTION

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

heterochromatic (Kretschmer et al. 2018a). In contrast, some Struthioniformes species show a completely euchromatic chromosome (Nishida-Umehara et al. 2007). In other Orders such as Tinamiformes, this chromosome exhibits an intermediate CBG-banding pattern, containing euchromatic and heterochromatic blocks (Garnero et al. 2006).

Some classical cytogenetic techniques provide patterns of positive and negative bands, exposing points of reference on the full length of the chromosome and enabling the creation of ideograms (Ladjali et al. 1999). Changes in these patterns suggest the possible types of rearrangements caused by chromosomal differences that may have occurred during the evolution of the genome (Griffin et al. 2007). Examples of this are the chromosomal rearrangements already reported by GTG and RBG bands in *Gallus gallus* (Galliformes), which identified a paracentric inversion in the long arm of chromosome 2 (Nanda et al. 1994). Chromosomal polymorphisms were identified by GTG bands in *Synallaxis frontalis* (Passeriformes), where pericentric inversion involving the first and third pairs was observed (de Souza et al. 2019), and in *Treron phoenicoptera* (Columbiformes) in the first and second pairs (Gupta and Kaul 2014).

Chromosomal rearrangements occur during the evolutionary process at the specimen level (Kretschmer et al. 2018b). Among these chromosomal changes are commonly observed translocations, duplications, inversions, deletions, fusion, and fissions (Stock and Bunch 1982; Nascimento et al. 1994; Nanda et al. 2011). This occurs in regions involving repetitive sequences and in the proximity of heterochromatic regions (Farre et al. 2016).

Less than 12% of the species of the Aves Class have been characterized by cytogenetic studies, where Passeriformes Order contains most of the species described (Griffin et al. 2007; Degrandi et al. 2020). Parulidae (Passeriformes) is strictly underrepresented in these studies, the family contains 119 species divided into 21 genera, but only 8% of all species have been investigated cytogenetically by Giemsa staining, shown diploid variation from 76 to 80 chromosomes (Carvalho 1989; Hobart 1991). This study aimed to detect independent or simultaneous chromosomal rearrangements, and it also analyzes chromosomal banding convergences and divergences of three species of the Parulidae family – *Myiothlypis leucoblephara, Basileuterus culicivorus*, and *Setophaga pitiayumi* – using techniques of classical cytogenetics such as CBG, GTG, and RBG bands.

MATERIAL AND METHODS

Sampling and Collecting

Five specimens of Wood-Warblers were analyzed in the present study: *Myiothlypis leucoblephara* (1 male and 1 female), *Basileuterus culicivorus* (1 male and 1 female), and *Setophaga pitiayumi* (1 female). All specimens were collected using a *mist net* in São Gabriel, Rio Grande do Sul state, Brazil (latitude - 30°20'38"S and longitude -54°20'31"W), under license SISBIO nº 61047-3, and CEUA/UNIPAMPA nº 010/2018.

Cell Culture and Chromosome Preparation

Mitotic cells were obtained using a short-term bone marrow extraction technique (Garnero and Gunski 2000). Initially, biological material was extracted from femurs in 10ml of RPMI 1640 medium and incubated with 0.01 ml of colchicine solution (0.05%) at 37°C for 1 h. Cells were subsequently centrifuged and incubated for 20 min in hypotonic solution (0,075 M KCL) at 37°C. Finally, the cells were fixed with methanol and acetic acid (3:1). We analyzed approximately 40 metaphases per specimen to determine the diploid number in an optical microscope (OLYMPUS DP53). For composing karyotype figures, it was used program Corel Draw12®, and the chromosomes were classified in decrescent order according to the long arm (p), short arm (q), arm radio (r) and centromeric index (i) (Guerra 1986).

CBG, GTG, and RBG Banding

Regions of heterochromatic blocks were analyzed by CBG-banding (Ledesma et al. 2006). After treatment in 0.2N HCl for 15 min, the slides were incubated in Barium Hydroxide (50%) for 17 min at 37°C. Structural investigations of the GTG-banding were done according to Schnedl (1971), with modifications to the immersion period in saline solution, which occurred for 1 min. To obtain the RBG-banding, the protocol by Popescu (2000) was replicated with a modification of the incubation period in Earl buffer (pH 5.1) saturated with Na2H-PO4, which occurred for 30 min at 87°C. Subsequently, a wash step with distilled water was performed followed by immersion for 30 min in Earl buffer (pH 6.4), without the addition of NaHCO3, at 87°C. In all banding protocols, metaphases were stained with Giemsa (5% in 0.07 M phosphate buffer, pH 6.8).

The GTG and RBG bands position were classified according to the International System of Standardized

Avian Karyotypes (ISSAK). Band patterns were interpreted by comparison among the three species of this study, and the types of rearrangements were detected with the inferences by homology in model species *Gallus gallus* (Ladjali et al. 1999).

<div align="center">RESULTS</div>

Wood-Warblers analyzed in this study showed differences in karyotypes. We identified a chromosome number of 2n=76 for *Myiothlypis leucoblephara*, concomitant with the described by Carvalho (1989) in a male specimen. *Basileuterus culicivorus* presented a diploid number of 2n=78, and *Setophaga pitiayumi* 2n=80 (Figure 1). In the three species, the karyotypes exhibited 14 pairs of autosomal macrochromosomes and 1 pair of sex chromosomes ZZ or ZW. The remaining pairs were composed of microchromosomes. Autosomal macrochromosomes and sex chromosomes were morphometrically described, presenting only morphological divergences occurring among chromosomes 5, 6, and 7 in the three species (Table 1).

CBG-banding analysis identified constitutive heterochromatin in the centromeric regions of the macrochromosomes and revealed the W chromosome. This chromosome was positioned between the 6th and 7th pair and showed chromatic heterogeneity in CBG-banding in the three species. It was formed by a block of heterochromatin in the short arm and partial in the long arm, containing a telomeric euchromatic region in the long arm (Figure 2). In all analyzed species, the Z chromosome was euchromatic, with positive staining observed near the centromere and morphometrically positioned between the 4th and 5th pair of macrochromosomes.

In this study, we describe by GTG-banding the first 10 autosomes macrochromosomes, and ZW sex chromosomes (Figure 3). *M. leucoblephara* presented 137 GTG-bands distributed along the chromosomes, where the negatives integrated into terminal regions of the short and long arms of chromosomes 2, 4, 5, 6 and 7. Other chromosomes contained positive bands in their terminal regions. *B. culicivorus* had a total set of 139 GTG-bands, of which the negatives were also distributed in the terminal regions of the short and long arms of chromosomes 1, 2, 4 and 5, and in the terminal region of the long arm of chromosomes 3, 6 and 7. Other terminal regions of chromosomes contained positive bands. *S. pitiayumi* showed 137 GTG-bands along their chromosomes, with negatives forming the terminal regions of the short and long arms of the chromosomes 1, 3 and 4, and the terminal region of the long arm of chromo-
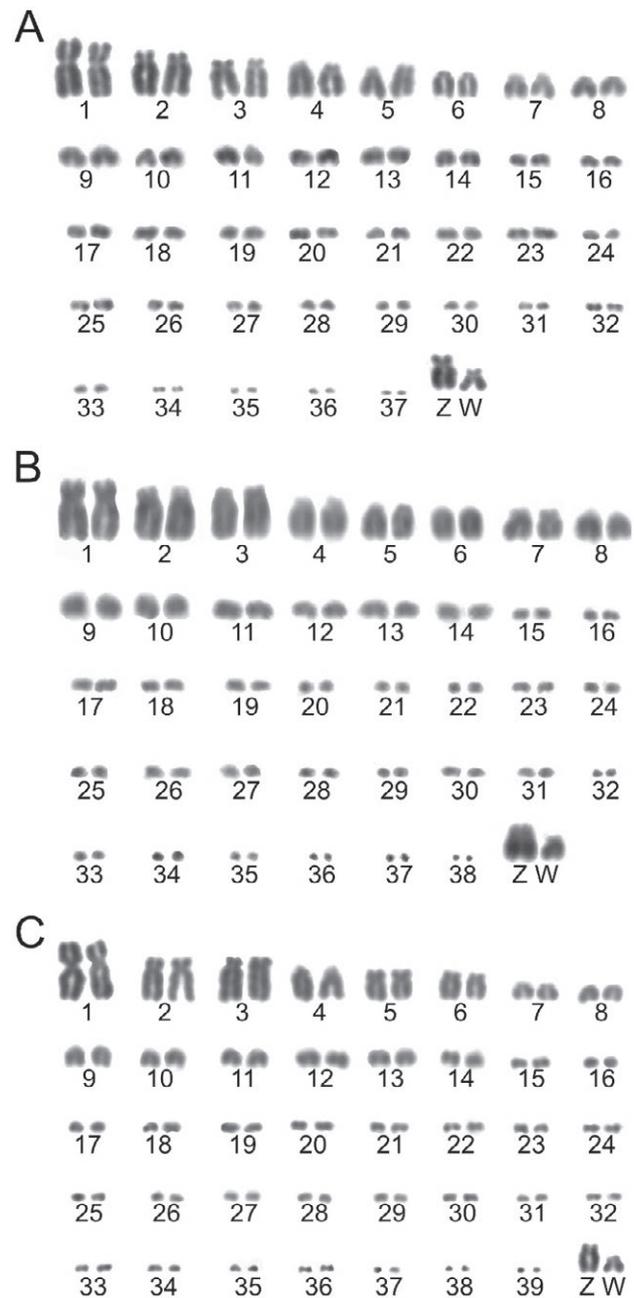

Figure 1. Species complete karyotype. Chromosomes arranged in descending order with Giemsa staining, followed by sexual chromosomes Z and W. *Myiothlypis leucoblephara* (A), *Basileuterus culicivorus* (B), and *Setophaga pitiayumi* (C).

somes 2, 5, 6, 7 and 8. The terminal regions of other chromosomes consisted of positive bands.

The reverse pattern was identified by RBG-banding, performed with the first 10 pairs of autosomal chromosomes and ZW. This data was shown to be compatible with the results obtained by GTG-banding (Figure

Table 1. Measurements and morphology of autosomal macrochromosomes and sex chromosomes of the species studied.

Chromo-some	Myiothlypis leucoblephara					Basileuterus culicivorus					Setophaga pitiayumi				
	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morpho-logy[d]	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morpho-logy[d]	Short arm[a]	Long arm[a]	R[b]	CI[c]	Morpho-logy[d]
1	6.3	10.6	1.68	37.28	SM	6.1	11.1	1.82	35.47	SM	6.2	10.9	1.76	36.26	SM
2	4.1	9.3	2.27	30.60	SM	4.3	9.5	2.21	31.16	SM	3.9	9.6	2.46	28.89	SM
3	2.3	8.5	3.70	21.30	A	2.2	9.8	4.45	18.33	A	2.8	9.6	3.43	22.58	A
4	2.1	8.2	3.90	20.39	A	2.1	8.9	4.24	19.09	A	2.1	8.7	4.14	19.44	A
5	1.9	7.3	3.84	20.65	A	1.7	7.4	4.35	18.68	A	3.1	6.3	2.03	32.98	SM
6	1.5	6.7	9.70	23.15	A	0	9.8	9.80	9.80	T	2.1	6.5	3.10	24.42	A
7	2.1	4.7	2.24	30.88	SM	2.1	6.2	2.95	25.30	SM	1.2	5.4	4.50	18.18	A
8	0	6.3	6.30	6.30	T	0	7.2	7.20	7.20	T	0	6.3	6.30	6,30	T
9	0	5.8	5.80	5.80	T	0	6.3	6.30	6.30	T	0	6.1	6.10	6.10	T
10	0	5.3	5.30	5.30	T	0	5.9	5.90	5.90	T	0	5.7	5.70	5.70	T
11	0	4.9	4.90	4.90	T	0	5.1	5.10	5.10	T	0	5.2	5.20	5.20	T
12	0	4.1	4.10	4.10	T	0	4.5	4.50	4.50	T	0	4.8	4.80	4.80	T
13	0	3.7	3.70	3.70	T	0	3.9	3.90	3.90	T	0	4.1	4.10	4.10	T
14	0	3.5	3.50	3.50	T	0	3.6	3.60	3.60	T	0	3.6	3.60	3.60	T
Z	3.2	7.1	2.22	31.07	SM	3.3	7.4	2.24	30.84	SM	3.1	7.2	2.32	30.10	SM
W	1.9	4.2	2.21	31.15	SM	1.8	4.3	2.39	29.51	SM	1.5	3.9	2.60	27.78	SM

[a]Length in micrometer (μm) q-long arm, p-short arm. [b]Relationship between p/q. [c]Centromeric index. [d]Chromosomal morphology: T-telocentric, A-acrocentric, SM-submetacentric.
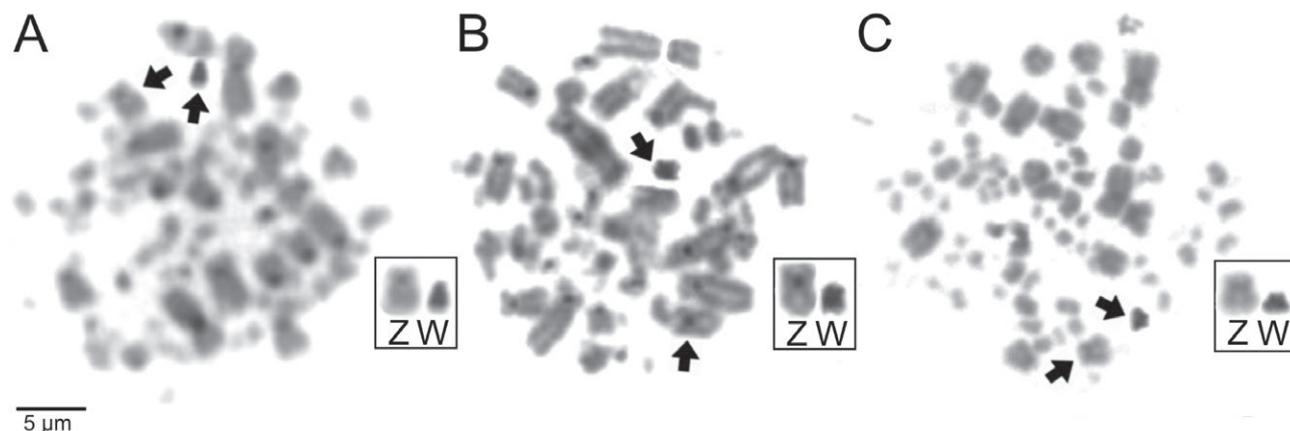

Figure 2. CBG-Banding metaphases with emphasis on the patterns of banding of sex chromosomes. *Myiothlypis leucoblephara* (A), *Basileuterus culicivorus* (B), and *Setophaga pitiayumi* (C).

3). Homologous and non-homologous regions among the three species were identified and compared with the homologous regions of model species *Gallus gallus* (Ladjali et al. 1999). In chromosome 1, a fission in region 2 of the short arm of *B. culicivorus*, and a paracentric inversion in region 1 of this same arm in *S. pitiayumi* were detected. In the long arm of this same chromosome, in region 4, a paracentric inversion was found in *B. culicivorus*. For chromosome 3, an inversion followed by deletion in region 1 of the long arm was detected in *B. culicivorus*. In the 5th pair, *B. culicivorus* also presented a fusion in region 1 of the short arm. A break followed by pericentric inversion was found in the 6 pair of the species *M. leucoblephara* and *S. pitiayumi*. In chromosome 7, *S. pitiayumi* also presented a fission in region 1 of the short arm. *M. leucoblephara* and *S. pitiayumi* showed a fusion in region 1 of the long arm in chromosome 8 (Figure 4).
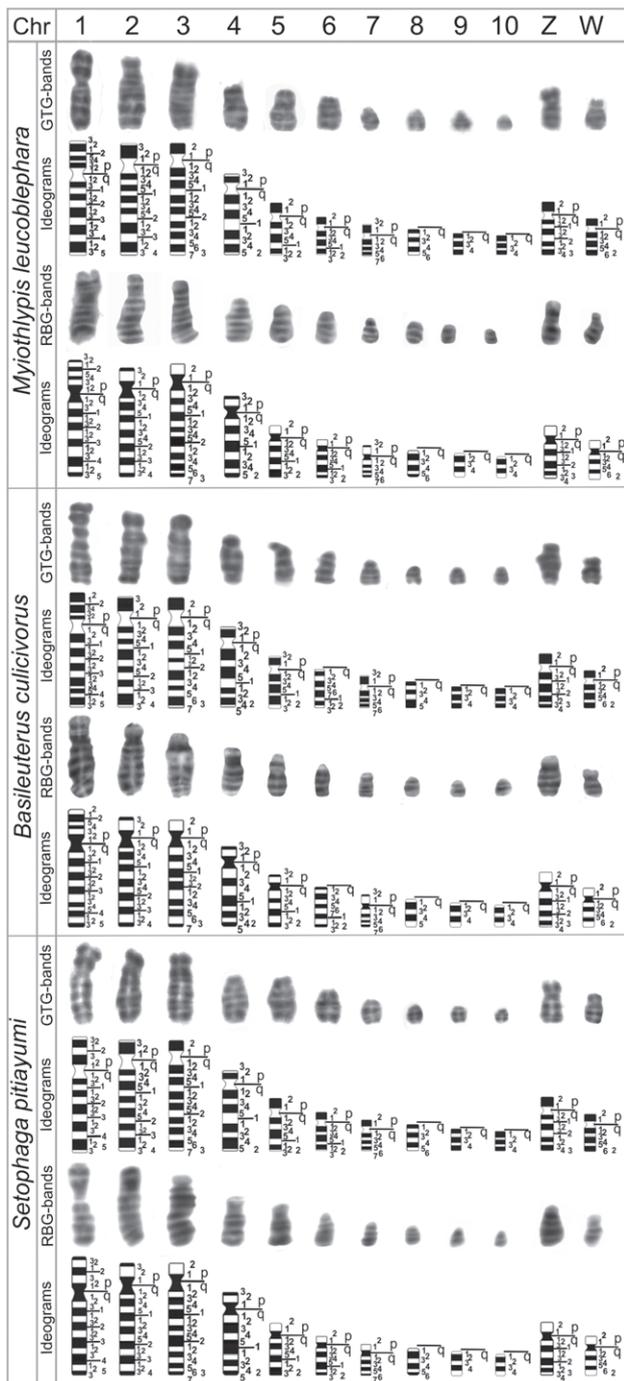
Figure 3. Description of GTG and RBG banding patterns and their respective ideograms. Light bands: negative GTG and RBG positive. Dark bands: positive GTG and Negative RBG.

DISCUSSION

The karyotypic structure of the three analyzed species in this study is similar to the typical avian karyotype (Figure 1 and Table 1), containing few pairs of macrochromosomes, many microchromosomes, a ZW heterogametic sexual system for females and ZZ homogametic for males (Christidis 1990). In the species of the family that has been previously studied, the frequency of the diploid number was within the standard, ranging from 76 to 80 chromosomes (Carvalho 1989; Hobart 1991).

Karyotypically, the three species presented the first pair of submetacentric chromosomes, supporting the theory that Passeriformes retain this morphology among its Oscines birds (Guttenbach et al. 2003). During the evolutionary changes of this chromosome, a break followed by fusion with a microchromosome forming this biarmed chromosome has been historically suggested in Galliformes (Stock and Bunch 1982). In Passeriformes, it was shown by fluorescent *in situ* hybridization (FISH) results that all species studied shared a fission of GGA1 (Kretschmer et al. 2018b).

CBG-banding identified a preferential accumulation of constitutive heterochromatin in the centromeric regions (Figure 2). The W chromosome showed a distinct banding pattern identified in Passeriformes, which is generally heterochromatic (Kretschmer et al. 2018a). In all three species, this chromosome has an euchromatic telomeric region in the long arm. We can infer that this chromosome has an intermediate CBG-banding pattern, it was seen in other Orders such as Tinamiformes in the *Crypturellus tataupa* species, where euchromatic and heterochromatic blocks occur simultaneously (Garnero et al. 2006). A similar pattern occurred in Charadriiformes in the *Burhinus oedicnemus* species, where a euchromatic band was found in the long arm of W chromosome (Nie et al. 2009).

Neognathae birds tend to have a reduction in the size of the W chromosome. Suggesting that this occurs due to loss of accumulated repetitive sequences and non-recombining regions. However, there are significant morphological differences in this chromosome, referring to loss and gain, followed by the accumulation of these sequences (Furo et al. 2017). In some species such as *Neochmia faeton* (Passeriformes), *Ardeola grayii* (Pelecaniformes), *Gallinula melanops* (Gruiformes), *Amazona aestiva* (Psittaciformes), and *Crotophaga ani* (Cuculiformes), this chromosome is considered the largest or one of the largest among chromosomal complement (Christidis 1989; Mohanty and Bhunya 1990; Furo et al. 2017; Gunski et al. 2019; Kretschmer et al. 2021).

The number of GTG and RBG bands obtained for the species was distinct (Figure 3), collaborating with the observed diploid number. It is possible to suggest the occurrence of interchromosomal and intrachromo-
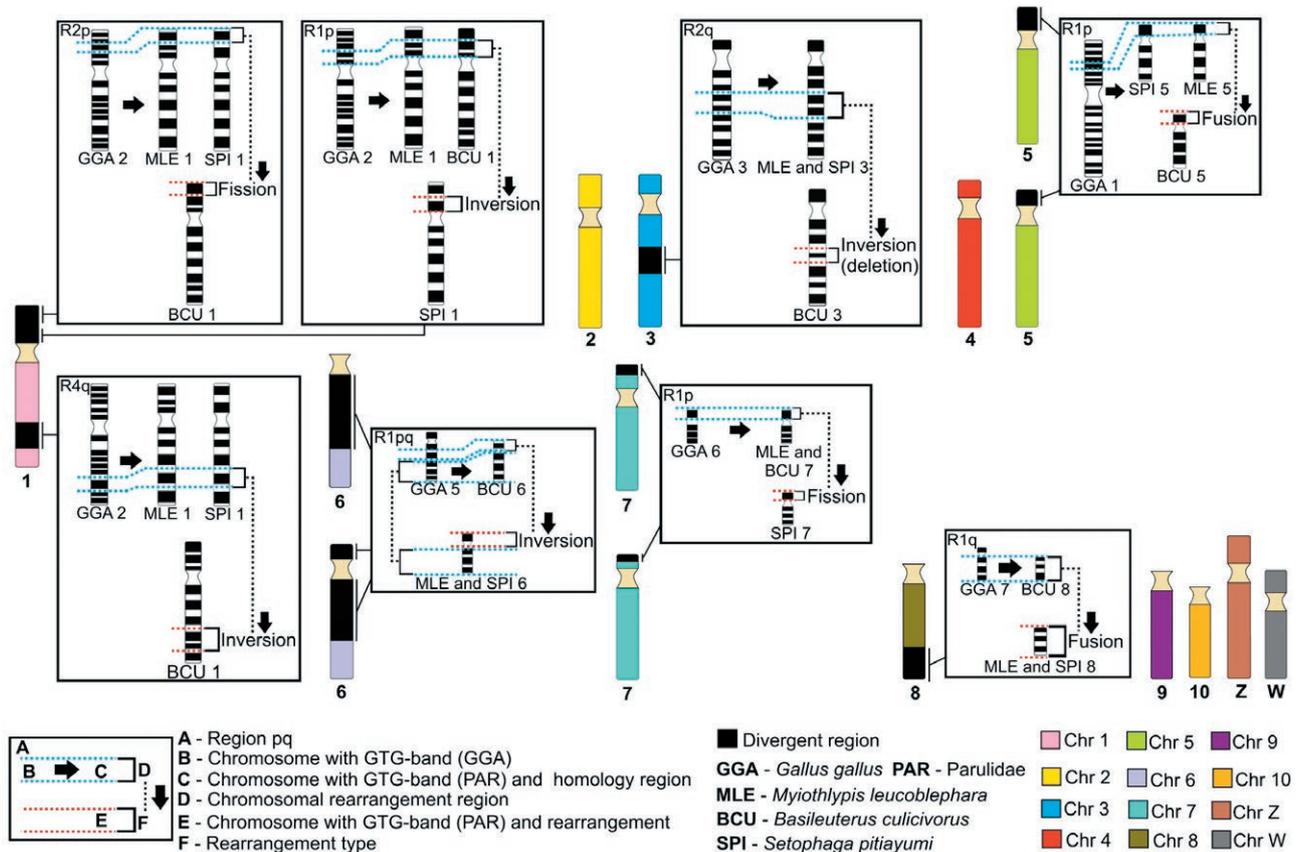
Figure 4. Ideograms of Parulidae with compiled data obtained by GTG and RBG bands, demonstrating the type of chromosomal rearrangements with divergences and convergences among bands pattern. Region of bands compared with *Gallus gallus* available in Ladjali et al. (1999). Chr-Chromosome.

somal rearrangements for these species, since fission, fusion, inversion, and deletion processes can be detected by banding patterns, which could be used as a reference point of the genomic organization (Ladjali et al. 1999; Nanda et al. 2011). However, we suggest that results should be analyzed in future studies by fluorescent *in situ* hybridization (FISH), giving additional information about this issue.

Comparisons of GTG and RBG band patterns among the three species showed distinct convergences and divergences (Figure 4). The macrochromosome pairs 1, 2, and 3 have similar morphology, but the banding patterns were not the same in these chromosomes for the parulids. In this context, Takagi (1974) found the same pattern in nine other Orders of the Aves Class, for example in Strigiformes, Columbiformes, and Gruiformes. Some studies have shown that chromosomes 1, 2, and 3 are actively linked to intrachromosomal rearrangement processes in the Passeriformes (Nanda et al. 1994). Our results indicate a sharing of number of chromosomal bands in *M. leucoblephara* and *B. culicivorus*

species, in region 1 of the first pair in short arm compared to the *G. gallus* (Ladjali et al. 1999). *S. pitiayumi* has a divergent pattern in this region, where possibly a paracentric inversion has caused this differentiation. *B. culicivorus* has rearrangement in region 2 of the short arm, where fission occurred in the telomeric region. Also, in region 4 of the long arm, there is a higher number of bands compared the other parulids, where a break followed by paracentric inversion could have caused this pattern.

B. culicivorus showed a reduction in the number of bands in region 2 of the long arm in the third pair compared to the other two parulids, which have similarities with *G. gallus* (Ladjali et al. 1999) in this region. For this differentiation, a possible paracentric inversion and deletion may have occurred. In *Synallaxis frontalis* species, there is a pericentric inversion involving the first and third pairs (de Souza et al. 2019). Nevertheless, this diversity of rearrangements involving chromosomes 1, 2, and 3 is not restricted to Passeriformes. In Columbidae, the *Treron phoenicoptera* species has a chromosomic

rearrangement of inversion in first and second pairs (Gupta and Kaul 2014).

In parulids, chromosomes 4 and 5 showed the same number of regions, containing differences only in morphology the 5 pair and number of bands. In region 1 of the short arm of chromosome 5, species *B. culicivorus* has three bands, while *M. leucoblephara* and *S. pitiayumi* contain two bands. The corresponding region of the *G. gallus* (Ladjali et al. 1999) is similar, inferring that a possible fusion is related to the increase in bands in *B. culicivorus*. Passeriformes have a unique evolutionary history for the 5th chromosome pair, where it is assumed to have occurred by fission of the short arm of chromosome 1 in the putative ancestral karyotype (PAK) (Kretschmer et al. 2018b). Using *G. gallus* (GGA) probes in Passeriformes, GGA1 usually hybridize two distinct chromosome pairs, for example, in *Saltator aurantiirostris* (Thraupidae) the second and fifth pairs (dos Santos et al. 2015).

The *B. culicivorus* chromosome 6 shows numerical conservation of positive and negative bands compared to the corresponding chromosome in *G. gallus* (Ladjali et al. 1999), which contains 7 bands in region 1 of the long arm. *M. leucoblepharus* and *S. pitiayumi* showed 5 bands for the same chromosome in this region. A possible break followed by pericentric inversion may have occurred, resulting in the changes found in biarmed chromosome, which has 2 bands in region 1 of the short arm in the two species. This is a rearrangement type that has been previously found in *Treron phoenicoptera* and *Synallaxis frontalis* by GTG-bands (Gupta and Kaul 2014; de Souza et al. 2019).

Morphology and number of bands in chromosome 7 found were similar in *M. leucoblephara* and *B. culicivorus*, which contained the same number of bands in the corresponding chromosome of *G. gallus* (Ladjali et al. 1999). However, *S. pitiayumi* shown a reduction in the number of bands in region 1 and morphological difference in this chromosome. Possibly, a fission in the terminal region of the short arm might have caused this reduction of bands and morphological differentiation in *S. pitiayumi*. In this perspective, multiple fragments of sites interstitial were found in non-telomeric regions in *Turdus merula* (Passeriformes), implying how active these regions are in relation to chromosomal rearrangements (Nanda et al. 2002).

In chromosome 8, region 1 of long arm, *B. culicivorus* showed similar patterns of bands number the *G. gallus* (Ladjali et al. 1999). *M. leucoblephara* and S. *pitiayumi* had an increase in bands, thus inferring the occurrence of a fusion in the telomeric region. Nevertheless, chromosomes 9 and 10 of the three species maintained morphological and numerical band similarities. The difference between *M. leucoblephara* and *S. pitiayumi* in chromosome 8 is a chromosomal rearrangement caused by fusion in the terminal region of the long arm, considering that *B. culicivorus* species has a similar pattern found in *G. gallus* (Ladjali et al. 1999) in the correspondent chromosome. The telomeric region is an area rich in repetitive sequences which have been reported as hotspots of chromosomal fusion and fission (Nanda et al. 2011). The similarities of chromosomes 9 and 10 of the three species suggest conservation.

In the three species, Z chromosome presented high evolutionary stability in terms of morphology and band patterns. In many Passeriformes, the Z chromosome has the same submetacentric morphology (Kretschmer et al. 2018b). Furthermore, some studies have shown that there is a high syntenic degree of this chromosome among several families of this group (Griffin et al. 2007). It is important to emphasize that GTG and RBG bands analyses have already identified a paracentric inversion in the terminal region of the long arm of the Z chromosome in *Alectoris chukar* (Galliformes) (Ouchia and Ladjali 2018). Signals of hybridization in this chromosome also demonstrated that the accumulation of the repetitive sequences are responsible for the main cause of its enlargement, as in *Myiopsitta monachus* (Psittaciformes) (Furo et al. 2017) and *Nyctibius griseus* (Caprimulgiformes) (de Souza et al. 2020).

In conclusion, the cytogenetic analyses performed in this study in the three parulids species provided an accurate description of the karyotypic structuring. Through CBG, GTG, and RBG bands, the information was obtained on chromatic patterns and chromosomal rearrangements which should be analyzed by molecular cytogenetic techniques in the future. Our results support the identification of speciation processes at the karyotypic of this group.

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STATEMENT OF ETHICS

The protocols used in this experiment were approved by the Ethics Committee on the use of animals (CEUA – Universidade Federal do Pampa, 010/2018).

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