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Chromosome spreading of the retrotransposable *Rex*-1 and *Rex*-3 elements and 5S rDNA clusters in the karyotype of *Eigenmannia catira* (Gymnotiformes: Sternopygidae)

Correspondence:
Carlos Alexandre Fernandes
cafernandes@uem.br

Lucas Pietro Ferrari Gianini[1], Ana Carolina Neiva de Oliveira[2], Wagner Correia[3] and Carlos Alexandre Fernandes[1,4]

Eigenmannia exhibits marked variability in its chromosome structure, including the presence or absence of sex chromosomes. In this study, we present the karyotype and chromosomal localization of four repetitive DNA classes in *E. catira* from the upper Paraná River basin. The observed diploid number for this population was 2n = 36, with a karyotype comprising 2 metacentric + 10 submetacentric + 8 subtelocentric + 16 acrocentric chromosomes, and a fundamental number (FN) of 56 for both sexes. Using silver nitrate staining and Fluorescence *in situ* Hybridization (FISH) with the 18S rDNA probe, we detected a single pair of nucleolar organizing regions (NORs) on the subtelocentric chromosome pair 10. Multiple 5S rDNA sites were identified by FISH, located on as many as 21 chromosomes. Additionally, we observed heterochromatic regions in the pericentromeric region of most chromosomes, and the presence of *Rex*-1 and *Rex*-3 retroelements in small clusters dispersed across the chromosomes. Our NOR data confirmed the characteristic tendency of this genus to display simple NORs and a significant amount of heterochromatin associated with transposable elements, which may account for the dispersion of 5S rDNA within the genome of this species. These findings provide valuable insights into the karyotypic evolution of *Eigenmannia*.

Keywords: FISH, Karyotype evolution, Ribosomal DNA, Species complex, Transposable elements.

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1 Programa de Pós-Graduação em Biologia Comparada, Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil. (LPFG) luquindia@gmail.com, (CAF) cafernandes@uem.br (corresponding author).

2 Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil. (ACNO) ra119066@uem.br.

3 Centro Educacional de Mundo Novo, Av. Filinto Muller, 1694, 79980-000, Mundo Novo, MS, Brazil. (WC) vavabiomsn@hotmail.com.

4 Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia) - Universidade Estadual de Maringá, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil.

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Eigenmannia exibe uma variabilidade marcante em sua estrutura cromossômica, incluindo a presença ou ausência de cromossomos sexuais. Neste estudo, apresentamos o cariótipo e a localização cromossômica de quatro classes de DNA repetitivas em *E. catira* da bacia do alto rio Paraná. O número diplóide observado para essa população foi de 2n = 36, com um cariótipo compreendendo 2 metacêntricos + 10 submetacêntricos + 8 subtelocêntricos + 16 cromossomos acrocêntricos e um número fundamental (NF) de 56 para ambos os sexos. Usando coloração de nitrato de prata e hibridação *in situ* por fluorescência (FISH) com a sonda de DNAr 18S, detectamos um único par de regiões organizadoras nucleolares (RONs) no par de cromossomos subtelocêntricos 10. Vários sítios de DNAr 5S foram identificados por FISH, localizados em até 21 cromossomos. Além disso, observamos regiões heterocromáticas na região pericentromérica da maioria dos cromossomos, e a presença de retroelementos *Rex*-1 e *Rex*-3 em pequenos clusters dispersados pelos cromossomos. Nossos dados de RON confirmaram a tendência característica desse gênero de exibir RON simples e uma quantidade significativa de heterocromatina associada a elementos transponíveis, o que pode ser responsável pela dispersão do DNAr 5S dentro do genoma desta espécie. Essas descobertas fornecem informações valiosas sobre a evolução cariotípica de *Eigenmannia*.

Palavras-chave: Complexo de espécies, DNA ribossômico, Elementos transponíveis, Evolução do cariótipo, FISH.

INTRODUCTION

Gymnotiformes is an order of fish found in the Neotropical region, consisting of approximately 273 valid species divided into five families: Apteronotidae, Sternopygidae, Gymnotidae, Hypopomidae, and Rhamphichthydae (Fricke *et al*., 2024). Gymnotiformes are popularly known as "electric fish," a term derived from their peculiar characteristic of continuous emission of weak electrical discharges. This unique feature serves the function of localization and communication, particularly in their nocturnal habits (Alves-Gomes, 2001). The Sternopygidae family consists of five genera: *Archolaemus* Korringa, 1970, *Distocyclus* Mago-Leccia, 1978, *Eigenmannia* Jordan & Evermann, 1896, *Rhabdolichops* Eigenmann & Allen, 1942, and *Sternopygus* Müller & Troschel, 1846. This family is found in all South American countries except Chile (Fricke *et al*., 2024). *Eigenmannia*, which is the most diverse genus in Sternopygidae, has 32 valid species (Fricke *et al*., 2024), with the highest diversity found in the Amazon basin (Dagosta, de Pinna, 2019).

In *Eigenmannia*, even though in recent decades there has been a considerable increase in taxonomic contributions, the genus continues with its taxonomy based on groups of species, in which the separation between individuals is made by the body morphological pattern, a condition found in several groups of fish with Neotropical distribution, as they have little phenotypic diversity and wide geographic distribution (Alves-Gomes *et al*., 1995; Albert, Campos, 1998; Albert, 2001; Albert, Crampton, 2003; Tagliacollo

et al., 2016). The differences in the morphological pattern alternate between having an evident upper medial dark stripe, which extends along the body axis (*E. trilineata* complex with 16 species), individuals measuring 300 mm or more, with a vast and opaque body in life, with absence of longitudinal stripes (*E. humboldtii* complex with three species) and translucent white/yellowish coloration in life, with absent longitudinal stripes, large eye and long caudal filament in *E. macrops* (Boulenger, 1897) (Peixoto *et al*., 2015; Waltz, Albert, 2017, 2018). *Eigenmannia catira* Cardoso & Dutra, 2023 is one of 16 species in the *E. trilineata* complex, distinguished from all its congeners by a unique combination of morphometrics, meristics, osteological characters, a significant COI genetic divergence, and its karyotype (Cardoso, Dutra, 2023).

The *Eigenmannia* genus, known for its rich diversity, exhibits marked variability in its chromosome structure. The diploid numbers range from 2n = 28 in *E. guairaca* Peixoto, Dutra & Wosiacki, 2015 (Sene *et al*., 2014) to 2n = 38 in species such as *E. virescens* (Velenciennes, 1836) (Fernandes *et al*., 2020), *E. dutrai* Peixoto, Pastana & Balen, 2021 (Sene *et al*., 2014), *E. limbata* (Shreiner & Miranda Ribeiro, 1903), and *E. microstomus* (Reinhardt, 1852) (Araya-Jaime *et al*., 2022). These variations, which may or may not include sex chromosomes, such as sex chromosome XX/XY (Almeida-Toledo *et al*., 2002) and ZZ/ZW (Almeida-Toledo *et al*., 2001; Silva *et al*., 2009; Fernandes *et al*., 2020) in *E. virescens*, and sex chromosome ZZ/Z0 type in *E*. aff. *trilineata* López & Castello, 1966 (Araya-Jaime *et al*., 2017), add to the diversity of this genus. The observation of multiple sex chromosome systems, such as $X_1X_1X_2X_2/X_1X_2Y$ in *E. guairaca* (Almeida-Toledo *et al*., 1988; Fernandes *et al*., 2010; Sene *et al*., 2014), and ZW_1W_2/ZZ in *E.* aff. *desantanai* Peixoto, Dutra & Wosiacki, 2015 (Araújo *et al*., 2023), further enriches our understanding of this diverse genus.

In this genus, the distribution pattern of constitutive heterochromatin appears to be conserved in populations from the same locality, primarily located in pericentromeric regions of most chromosomes (Almeida-Toledo *et al*., 1996; Silva *et al*., 2009; Sene *et al*., 2014; Fernandes *et al*., 2020; Araya-Jaime *et al*., 2022; Araújo *et al*., 2023). Nucleolar organizing regions have been identified in only one chromosome pair, characterized as a system of simple NORs (Almeida-Toledo *et al*., 1996; Sene *et al*., 2014; Fernandes *et al*., 2020; Araya-Jaime *et al*., 2022), except in *Eigenmannia* sp.1, which presented a system of multiple NORs (Almeida-Toledo *et al*., 1996). The presence of multiple 5S rDNA sites on different chromosomes is evident in *E. virescens* (Fernandes *et al*., 2020), *E. microstomus*, *E. limbata* (Araya-Jaime *et al*., 2022), *E. guairaca*, *E. catira*, and *E. dutrai* (Sene *et al*., 2014), suggesting a possible association with transposable elements (Sene *et al*., 2014). Conversely, the mapping of *Rex*-1 and *Rex*-3 retroelements was conducted in a few species of *Eigenmannia*, revealing their distribution in small clusters in both the euchromatic and heterochromatic portions of the studied genomes (Sene *et al*., 2015). The retrotransposable elements *Rex*-1 and *Rex*-3 were mapped in several fish species (review in Carducci *et al*., 2018). However, there still needs to be more information about the distribution pattern of these elements in Gymnotiformes, especially considering their potential role in the dispersion of other repetitive elements (Cioffi *et al*., 2009; Pansonato-Alves *et al*., 2013; Prizon *et al*., 2018).

In order to enhance our understanding of the chromosomal structure and the behavior of repetitive DNA sequences in the *Eigenmannia* genome, we are pleased to present the karyotype and chromosomal localization of four repetitive DNA classes (18S and 5S rDNA, retrotransposable Rex-1, and Rex-3 elements) in *E. catira* from the upper

Paraná River basin. Our findings reveal a new instance of chromosome spreading of the 5S rDNA clusters in Gymnotiformes, which could be attributed to the synteny of *Rex*-1 and *Rex*-3 elements with chromosomes carrying 5S rDNA.

MATERIAL AND METHODS

Study and sampling area. A total of twenty-five (7 females and 18 males) individuals of *E. catira* (Gymnotiformes, Sternopygidae) were obtained from the Iguatemi River (Fig. 1A), located in the municipality of Mundo Novo, Mato Grosso do Sul, Brazil (23°89'52.78"S 54°25'91.67"W). Voucher specimens were deposited in the fish collection at the Universidade Estadual de Maringá, Brazil, as *E. catira* (NUP 25154) (Fig. 1B).

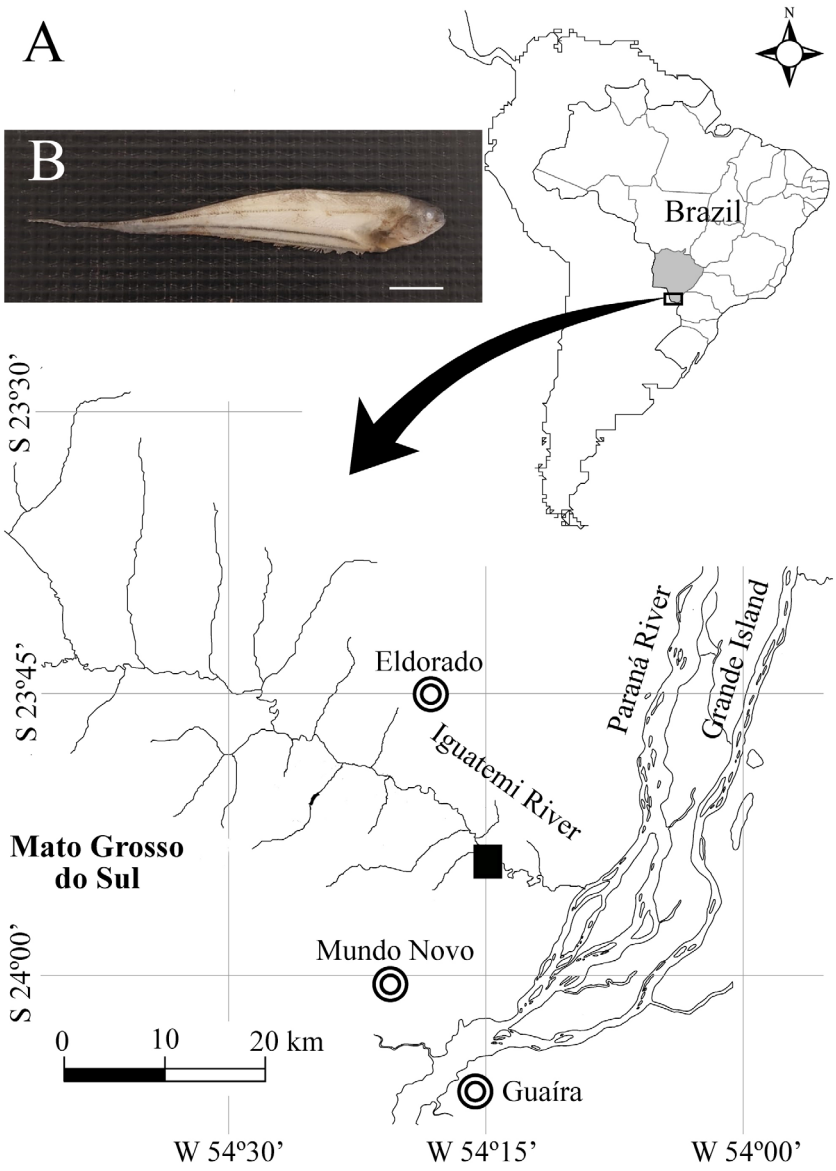

FIGURE 1 | The Iguatemi River (**A**) is located in the upper Paraná River basin, where individuals of *Eigenmannia catira* were captured in Mato Grosso do Sul, Brazil. The dark square indicates the sampling point. Specimen of *E. catira* collected (**B**), scale bar = 1 cm.

Cytogenetic analysis. Metaphase chromosomes were obtained from anterior kidney cells using the air-drying technique (Bertollo *et al*., 2015). The nucleolar organizer regions (NORs) were detected employing silver nitrate staining (Howell, Black, 1980). Heterochromatin was determined following the C-banding technique (Sumner, 1972) and stained with propidium iodide (Lui *et al*., 2012).

At least 30 metaphases were analyzed for each individual, and those with better chromosome morphology were used for the karyotype analysis. The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) according to (Levan *et al*., 1964). The fundamental number (FN) was calculated according to the chromosomal arm numbers (the chromosomes m, sm, and st were considered to contain two arms – *p* and *q* arms – and the a with one arm – only *q* arm).

The location of the 5S and 18S rDNA sites in the chromosomes was performed by fluorescence *in situ* hybridization (FISH) with modifications (Margarido, Moreira-filho, 2008; Pinkel *et al*., 1986) using probes from the genome of *Megaleporinus elongatus* (Valenciennes, 1850) (Martins, Galetti Jr., 1999) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti Jr., 2004), respectively. The probes were labeled through nick translation with digoxigenin-11-dUTP (5S rDNA) and biotin-16-dUTP (18S rDNA) (Roche). Detection and amplification of the hybridization signal were carried out using avidin-FITC and anti-avidin biotin (Sigma) for probes labeled with biotin and anti-digoxigenin rhodamine (Roche) for probes labeled with digoxigenin. Chromosomes were counterstained with DAPI (50 μg ml^{-1}).

Transposable element probes were produced using the primers Rex-3 [Foward (5'- CGGTGAYAAAGGGCAGCCCTG-3') and Reverse (5'-TGGCAGACNGGGGTGGTGGT-3') (Volff, 2006). REX-1 [Foward (5'- TTCTCCAGTGCCTTCAACACC-3') and Reverse (5' - TCCCTCAGCAGAAAGAGTCTGCTC-3') (Volff *et al*., 1999). Amplification was performed using PCR, and the probes were labeled according to the nick translation method using the Anti-digoxigenin-Rhodamine Kit (Roche). Chromosomes were counterstained with DAPI (50 μg ml-1).

Conventional and fluorescence chromosome preparations were analyzed under an epifluorescence microscope (Olympus BX51). The images were captured using the DP controller (Media Cybernetics) software and the image composition with Adobe Photoshop CS6.

RESULTS

All individuals of *Eigenmannia catira* had 36 chromosomes with a karyotype composed of 2 metacentric + 10 submetacentric + 8 subtelocentric + 16 acrocentric chromosomes, with a fundamental number (FN) equal to 56 for both sexes (Fig. 2A). No heteromorphic sex chromosomes were identified. Using silver nitrate impregnation, it was revealed that the Ag-NORs were located at the terminal region of the short arms in the last pair of subtelocentric chromosomes (pair 10), which coincided with the secondary constriction (Box in the Fig. 2A).

Heterochromatin was primarily found in the pericentromeric regions of most chromosomes, with a significant accumulation of heterochromatin at the sites of Ag-NORs and at the terminal regions of the long arms in pairs 9 and 11 (Fig. 2B).

The FISH technique using the 18S rDNA probe confirmed the labeling obtained by silver nitrate and did not detect any additional inactive major ribosomal clusters (Figs. 2C, 3). Multiple 5S rDNA sites were observed in the pericentromeric position in 21 chromosomes (Figs. 2C, 3), being that in pair 2 only one of the homologues presented marking. In the FISH experiments, small clusters of the *Rex*-1 and *Rex*-3 elements were observed dispersed throughout the chromosomes, spanning both euchromatic and heterochromatic regions (Fig. 3). However, despite employing identical experimental hybridization conditions in all assays, the *Rex*-1 element appears to be more abundant than *Rex*-3.

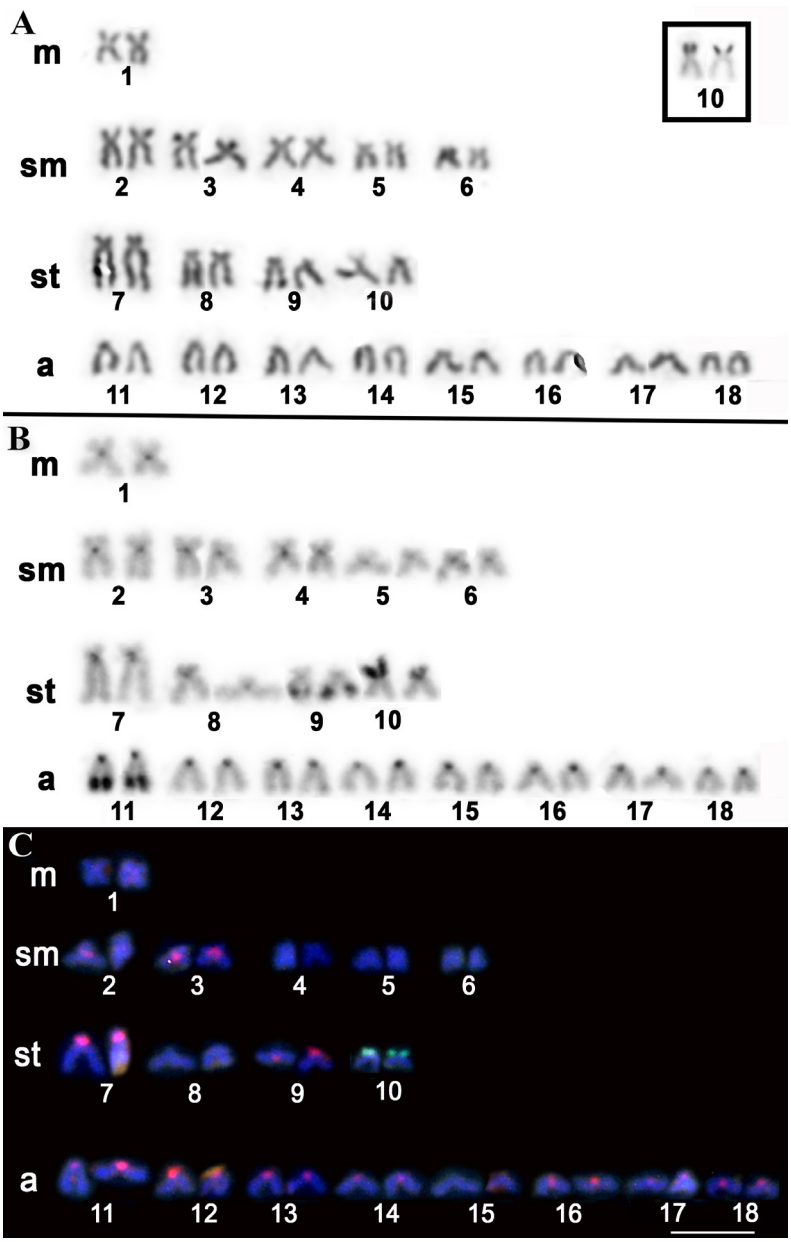

FIGURE 2 | *Eigenmannia catira* karyotypes stained with Giemsa (**A**), C-banding (**B**), and after double FISH with 18S rDNA (in green) and 5S rDNA (in red) probes (**C**). The highlighted box contains the pair carrying the nucleolar organizing region after impregnation with silver nitrate. Scale bar = 10 µm.

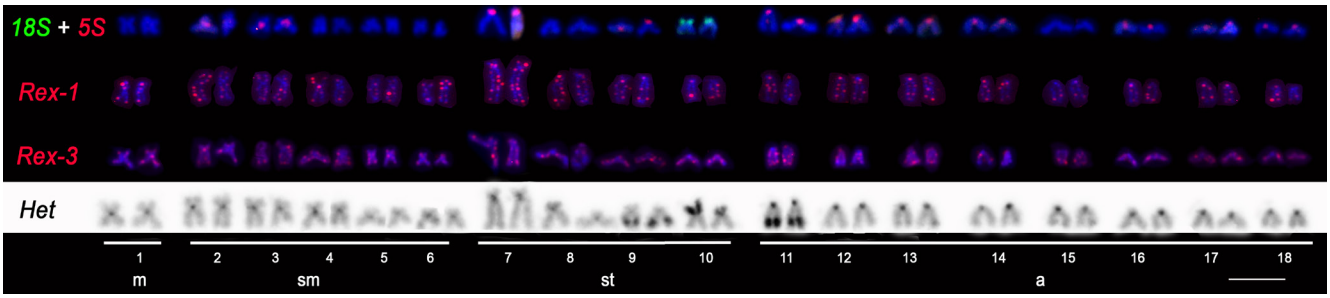

FIGURE 3 | *Eigenmannia catira* karyotypes comparing double FISH with 18S (in green) and 5S (in red) rDNA probes, FISH with *Rex*-1, *Rex*-3 probes, and C-banding (Het). Scale bar = 10 µm.

DISCUSSION

The karyotype of *Eigenmannia catira* analyzed in the present study showed that the morphology pattern of the chromosomes, C-banding, 5S and 18S rDNA differed from the patterns previously described for the species (Sene *et al*., 2014). The patterns of chromosomal morphology found in *E. catira* containing 2m + 10sm + 8st + 16a obtained here have never been observed in other populations of this species (Tab. 1). According to Cardoso, Dutra (2023), only one karyotypic analysis of the species, previously identified as *Eigenmannia* sp., has been carried out to date (Sene *et al*., 2014). In this study, the individuals from Hortelã River (upper Paraná River basin) had same diploid number (2n = 36), but 8m/sm + 28st/a (Sene *et al*., 2014), while in the present study 12m/sm +24st/a. It is important to note that despite the maintenance of the diploid number, rearrangements modifying the chromosomal morphology, such as pericentric inversions, have played a significant role in the karyotypic evolution of *E. catira*.

The terminal location of NORs on the short arm of the last pair of subtelocentric chromosomes, as revealed by our unique research using silver impregnation and FISH with the 18S rDNA probe, is a significant discovery. This finding, similar to that previously described in *E. catira* (Sene *et al*., 2014), confirms a simple NORs for this species. Simple NORs, a shared feature among *Eigenmannia* species, are predominantly located in the terminal portion of short arms (p) of subtelo/acrocentric chromosomes (Tab. 1). This fact, described for *Eigenmannia*, seems to also occur in another genus of Gymnotiformes; Milhomem *et al*. (2013) showed that despite the occurrence of high karyotypic variability in *Gymnotus* species, the NOR-bearing chromosomes are homeologous in different species (*G. inaequilabiatus* (Valenciennes, 1839), *G. pantherinus* (Steindachner, 1908) and *G.* cf. *carapo* Linnaeus, 1758), showing to be conserved in most species of this order. On the other hand, *E. guairaca* (previously cited as *Eigenmannia* sp.1 (2n = 28) and *Eigenmannia* sp.2 (2n = 31/32) by Sene *et al*., 2014 and as *E. trilineata* by Fernandes *et al*., 2010) and *E.* aff. *desantanai* (Araújo *et al*., 2023) are the only two species that presents NORs located in the interstitial position, possibly indicating that pair 10 of these species may have arisen through fusion events involving ancestral chromosomes carrying ribosomal sequences.

The research findings indicate that the heterochromatin regions are present in all chromosomal pairs, with the majority being located in the pericentromeric regions. Only

TABLE 1 | Cytogenetic data of species in *Eigenmannia*, species name following Cardoso, Dutra (2023). [1]Previously mentioned as *Eigenmannia* sp.1, [2]previously mentioned as *E. trilineata*, [3]previously mentioned as *Eigenmannia* sp.2, [4]previously mentioned as *E. virescens*, [5]previously mentioned as *E. virescens*-XY, p = short arm. q = long arm. T = terminal region. I = interstitial region. 5S rDNA = number of carrier chromosomes, Ag-NORs/18S rDNA = carrier pair.

Species	2n	Sex chromosomes	5S rDNA	Ag-NORs/18S rDNA	References
E. guairaca[1]	28	undifferentiated	6	3q (T)	Sene *et al.* (2014)
E. aff. *desatanai*	30♂ 31♀	ZZ/ZW$_1$W$_2$	-	10q (I)	Araújo *et al.*(2023)
E. guairaca[2]	31♂ 32♀	X$_1$X$_1$X$_2$X$_2$/X$_1$X$_2$Y	-	10q (I)	Fernandes *et al.* (2010)
E. guairaca[3]	31♀ 32♂	X$_1$X$_1$X$_2$X$_2$/X$_1$X$_2$Y	2	10q (I)	Sene *et al.* (2014)
E. aff. *trilineata*	31♂ 32♀	ZZ/Z0	11	11q(I)	Araya-Jaime *et al.* (2017)
E. catira	36	undifferentiated	6	12p (T)	Sene *et al.* (2014)
E. catira	36	undifferentiated	21	10p (T)	This study
E. virescens	38	ZZ/ZW	11	15p (T)	Fernandes *et al.* (2020)
E. limbata	38	undifferentiated	4	10p (T)	Araya-Jaime *et al.* (2022)
E. microstoma	38	undifferentiated	22	14p (T)	Araya-Jaime *et al.* (2022)
E. dutrai[4]	38	undifferentiated	10	15p (T)	Sene *et al.* (2014)
E. dutrai[5]	38	XX/XY	10	15p (T)	Sene *et al.* (2014)

two pairs exhibit terminal markings on the long arms of subtelocentric and acrocentric chromosomes. A prior study of *E. catira* also observed similar heterochromatin patterns, with at least four chromosomal pairs showing terminal markings on the long arms of subtelocentric and acrocentric chromosomes (Sene *et al.*, 2014). Furthermore, in the current study, the heterochromatic regions aligned with the 5S rDNA sites in most chromosomes, with the exception of pair 9, resembling the pattern observed in *E. catira* (Sene *et al.*, 2014), as well as in other *Eigenmannia* species such as *E. virescens* (Fernandes *et al.*, 2020) and *E. microstomus* (Araya-Jaime *et al.*, 2022). Conversely, other species such as *E. limbata*, *E. guairaca*, and *E.* cf. *trilineata* showed a lower number of coinciding chromosomes with heterochromatic regions (Sene *et al.*, 2014; Araya-Jaime *et al.*, 2022).

The physical mapping of 5S rDNA in the *E. catira* genome showed these sites located on 21 chromosomes and non-syntenic with the 18S rDNA sites, a characteristic also previously observed in *E. catira*, but with only six chromosomes carrying 5S rDNA (Sene *et al.*, 2014). Multiple 5S rDNA sites appear to be a characteristic among other *Eigenmannia* species (Tab. 1), except in *E. guairaca* with a sexual system of the X$_1$X$_1$X$_2$X$_2$/X$_1$X$_2$Y type, which presented only two chromosomes carrying these cistrons (Sene *et al.*, 2014).

One possible hypothesis to account for the increased dispersion of copies of the 5S rDNA genes in *E. catira* is the potential use of a mechanism involving the spread of retrotransposons by the ribosomal DNA. This hypothesis finds support in the synteny of *Rex*-1 and *Rex*-3 elements present in pairs carrying the 5S rDNA described here. In the *E. catira* population described by Sene *et al.* (2015), the discrete presence of blocks

marked with *Rex*-1 and *Rex*-3 on the chromosomes may explain the lower number of chromosomes carrying 5S rDNA (6) compared to the *E. catira* population under study here (21). As a result, these elements could potentially be a contributing factor to the numerical variation in 5S rDNA sites between these two populations.

The *Rex*-1 and *Rex*-3 retrotransposons analyzed here showed a dispersed pattern throughout all chromosomes, and synteny with heterochromatin, euchromatin, and 5S rDNA regions, with *Rex*-1 having more abundant than *Rex*-3. Similarly, Sene *et al*. (2015) showed that the *Rex*-1 and *Rex*-3 transposable elements in *E. guairaca*, *E. dutrai*, *E.* cf. *trilineata*, and *E. catira*, are also organized into small clusters, including euchromatic and heterochromatic regions. However, in this study, *Rex*-3 elements are more abundant than *Rex*-1 elements, as well as more concentrated in the sexual chromosomes X_1 and X_2 of *E. guairaca* and in the X chromosome of *E. dutrai*, suggesting that *Rex*-3 played an essential role in the process of differentiation of the sex chromosomes of these species.

The available data is instrumental in gaining insight into the karyotypic evolution of Eigenmannia. Pericentromeric inversions are chromosomal rearrangements that are crucial in distinguishing karyotypes of *E. catira*. The data on NORs in *E. catira* confirm *Eigenmannia* tendency to have simple NOR sites. Multiple 5S rDNA sites across various *E. catira* chromosomes can be attributed to the synteny of *Rex*-1 and *Rex*-3 elements with chromosomes carrying 5S rDNA. These transposable elements may have played a vital role in spreading this ribosomal DNA within the genome of this species.

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AUTHORS' CONTRIBUTION

Lucas Pietro Ferrari Gianini: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing-original draft, Writing-review and editing.

Ana Carolina Neiva de Oliveira: Formal analysis, Methodology, Writing-review and editing.

Wagner Correia: Methodology, Writing-review and editing.

Carlos Alexandre Fernandes: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Visualization, Writing-original draft, Writing-review and editing.

ETHICAL STATEMENT

This study was carried out strictly following the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Animal Experimentation Ethics Committee of the Universidade Estadual de Maringá (License Number: nº 6792170120 – CEUA/UEM). The experiments followed ethical conduct, and before euthanasia, the fish were anesthetized with an overdose of clove oil (Griffiths, 2000). The animals were captured with authorization from the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio, number 73763–42).

COMPETING INTERESTS

The author declares no competing interests.

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