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Cytogenetic studies in *Eigenmannia virescens* (Sternopygidae, Gymnotiformes) and new inferences on the origin of sex chromosomes in the *Eigenmannia* genus

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Published: 21 November 2009

Received: 24 June 2009

BMC Genetics 2009, 10:74 doi:10.1186/1471-2156-10-74

Accepted: 21 November 2009

This article is available from: <http://www.biomedcentral.com/1471-2156/10/74>

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Abstract

Background: Cytogenetic studies were carried out on samples of *Eigenmannia virescens* (Sternopygidae, Gymnotiformes) obtained from four river systems of the Eastern Amazon region (Para, Brazil).

Results: All four populations had $2n = 38$, with ZZ/ZW sex chromosomes (Z, acrocentric; W, submetacentric). Constitutive heterochromatin (CH) was found at the centromeric regions of all chromosomes. The W chromosome had a heterochromatic block in the proximal region of the short arm; this CH was positive for DAPI staining, indicating that it is rich in A-T base pairs. The nucleolar organizer region (NOR) was localized to the short arm of chromosome pair 15; this result was confirmed by fluorescent *in situ* hybridization (FISH) with human 45S rDNA, and CMA₃ staining indicated that the region is G-C rich. FISH with telomeric probes did not show any evidence of interstitial telomeric sequences (ITS).

Conclusion: Previous studies have shown that the species *Eigenmannia* sp. 2 and *E. virescens* have differentiated sex chromosomes, and diverse sex chromosome systems have been described for *E. virescens* specimens obtained from different Brazilian rivers. A comparative analysis of the present data and prior reports suggests that the sex chromosomes of *Eigenmannia* may have arisen independently in the different populations.

Background

Eigenmannia Jordan & Evermann (1896) is a genus of electric fishes of the family Sternopygidae (Gymnotiformes). This genus is endemic to the main hydrographic basins of the neotropical region (e.g., Madalena, Orinoco, Amazonas and Paraná rivers), and includes species possessing an electric organ that generates wavelike electrical discharges

that are used for communication and electrolocation [1-6]. *Eigenmannia* is presently classified into eight valid species (Table 1) that may be divided in two groups [7,8]: *Eigenmannia microstoma* contains members that have large, dark bodies at sexual maturity, such as *E. microstoma*, *E. humboldtii*, *E. limbata* and *E. nigra*; while *Eigenmannia virescens* contains members that have two or three

Table 1: Geographic distribution of the nine valid species of the genus *Eigenmannia* [1,6,7,9].

Genus	Species	Author	Typical locality	Geographic distribution	Countries
<i>Eigenmannia</i>	<i>Eigenmannia humboldtii</i>	Steindachner, 1978	Madalena river-Colombia	North portion of South America	Brazil, Colombia and Venezuela
	<i>Eigenmannia limbata</i>	Schreiner & Miranda Ribeiro, 1903	Amazonas river-Brazil	Guianas and the Amazonas river basin	Brazil and Venezuela
	<i>Eigenmannia macrops</i>	Boulenger, 1897	Guiana	Guianas and the Amazonas river basin	Brazil and Guiana
	<i>Eigenmannia microstama</i>	Reinhardt, 1852	Santana lake-Brazil	High basin of the São Francisco river	Brazil
	<i>Eigenmannia nigra</i>	Mago-Leccia, 1994	Negro river, Amazonas river, at Venezuela	Negro river basin and Casiquiare river at Venezuela and Colombian Amazon	Brazil, Colombia, Guiana and Venezuela
	<i>Eigenmannia trilineata</i>	Lopes & Castello, 1996	de La Plata river-Argentina, close to Buenos Aires	Paraná-Paraguay rivers basin	Argentina, Brazil, Paraguay and Uruguay
	<i>Eigenmannia vicentespelaea</i>	Triques, 1996	Tocantins river-Brazil	São Vicente river, Tocantins river basin, São Domingos, Goiás state, Brazil	Brazil
	<i>Eigenmannia virescens</i>	Valenciennes, 1842	South America	From the west Andes at Orenoco basin until the de La Plata river	Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guiana, Paraguay, Peru, Suriname, Uruguay and Venezuela
	<i>Eigenmannia</i> sp. D	Albert, 2001	Salí river, Tucumán, Argentina	Salí and Hondo rivers	Argentina

longitudinal lines on their bodies at sexual maturity, such as *E. virescens*, *E. trilineata*, *E. vicentespelaea* and *E. sp. D* (undescribed species). The position of *Eigenmannia macrops* in these groups is still undefined [8]. Despite this classification, however, *Eigenmannia* is considered a taxonomically confusing genus because the relative lack of morphological variation among the species makes it difficult to define diagnostic traits [7,9,10]. In recent years, many cytogenetic studies have been carried out on *Eigenmannia* samples obtained from different Brazilian basins. The diploid numbers of these samples have ranged from $2n = 28$ in *Eigenmannia* sp. 1 [11] to $2n = 46$ in *Eigenmannia* sp. [12]. Both ZZ/ZW and XX/XY sex chromosome systems have been described for *E. virescens* [13,14], and a multiple system, $X_1X_1X_2X_2/X_1X_2Y$, was reported for *Eigenmannia* sp. 2 [15]. All these data are in Table 2.

Eigenmannia virescens Valenciennes (1842) appears to have one of the largest geographic distributions among the Gymnotiformes (Table 1). In the present study, we examined the karyotypes of *E. virescens* samples from four rivers of the Eastern Amazon region. We herein report our results, compare them with those described in the literature for species of *Eigenmannia* from other localities, and discuss the differences in the sex chromosomes of samples from different hydrographic basins.

Methods

Fifteen fishes of species *Eigenmannia virescens* (Figure 1) were collected from white-water rivers of the Eastern Amazon region during the rainy and dry seasons of 2005 to 2008 (Figure 2 and Table 3). The animals had body sizes ranging from 12 to 20 cm, and body masses ranging from 10 to 23 g. Before killing the fishes we used Benzocaine hydrochloride as an anaesthetic. Metaphase chromosomes were obtained following standard procedures [16]. The slides were analyzed using the following techniques: conventional Giemsa staining (Merck); C-banding [17]; Ag-NOR staining [18]; fluorochrome staining with CMA₃ [19] and DAPI [20]; and fluorescent *in situ* hybridization (FISH) with biotin-labeled human 45S rDNA [21] and telomeric probes (All Telomere Probes, Oncor). The chromosomes were classified according to a previously published strategy [22].

Results

All analyzed individuals of *E. virescens* (Figure 1) were found to have $2n = 38$, a fundamental number (FN) of 52 for males and 53 for females (Figures 3A and 3C), and a karyotypic formula (KF) of $14m/sm+24a$ for males and $15m/sm+23a$ for females. The species was found to have a simple sex chromosome system of ZZ/ZW, where the Z is acrocentric (a) and the W is submetacentric (sm). C-

Table 2: Cytogenetic studies in specimens of the genus *Eigenmannia*.

Species	2n	KF	CB	NOR	Sex chromosomes	Localities	References
<i>Eigenmannia</i> sp.	46	20m/sm+26st/a	-	4p (a)	Undifferentiated	Amazon basin, Jari river	[12]
<i>Eigenmannia</i> sp. ♀ and ♂	31/32	13m/sm+18st/a ♀ and 12m/sm+20st/a ♂	-	4p (a)	Undifferentiated	Amazon basin, Jari river	[12]
<i>Eigenmannia</i> sp.1 ♀ and ♂	28	14m/sm+14a	+	10q (a)	Undifferentiated	Mogi-Guaçu river	[16]
<i>Eigenmannia</i> sp.1 ♀ and ♂	28	14m/sm+14a	+	10q + 1 at 11q (a) + 1 at 3p (m)	Undifferentiated	Mogi-Guaçu river at Emas waterfall	[11]
<i>Eigenmannia</i> sp.1 ♀ and ♂	28	14m/sm+14a	+	3q (m)	Undifferentiated	Mogi-Guaçu river at Araras region	[11]
<i>Eigenmannia</i> sp.2 ♀ and ♂	31/32	8m+24a ♀ and 9m+22a ♂	+	10p (a)	X ₁ X ₂ Y/X ₁ X ₁ X ₂ X ₂	Tietê river	[15,23,24]
<i>Eigenmannia virescens</i> ♀ and ♂	38	16m/sm+22st/a	+	15p (st)	Undifferentiated	Mogi-Guaçu river	[13]
<i>Eigenmannia virescens</i> ♀	38	16m/sm+22st/a ♀ and 16m/sm+22st/a ♂	+	15p (st)	XX/XY	Tietê river	[13]
<i>Eigenmannia virescens</i> ♀ and ♂	38	23m/sm+15st/a ♀ and 22m/sm+16st/a ♂	+	17p (st-a)	ZZ/ZW	São Francisco river	[14]
<i>Eigenmannia virescens</i> ♀ and ♂	38	15m/sm+23st/a ♀ and 14m/sm+24st/a ♂	+	14p (a)	ZZ/ZW	Marajó Island	[14]
<i>Eigenmannia virescens</i> ♀	38	17m/sm+st/a	+	16p (a)	ZW	Middle Amazonas river	[14]
<i>Eigenmannia virescens</i> ♂	38	14m/sm+24st/a	+	15p (st)	ZZ	Murini river, eastern Amazonia	This work
<i>Eigenmannia virescens</i> ♀ and ♂	38	15m/sm+23st/a ♀ and 14m/sm+24st/a ♂	+	15p (st)	ZZ/ZW	Guamá river, eastern Amazonia	This work
<i>Eigenmannia virescens</i> ♀ and ♂	38	15m/sm+23st/a ♀ and 14m/sm+24st/a ♂	+	15p (st)	ZZ/ZW	Anequara river, eastern Amazonia	This work
<i>Eigenmannia virescens</i> ♀ and ♂	38	15m/sm+23st/a ♀ and 14m/sm+24st/a ♂	+	15p (st)	ZZ/ZW	Caripetuba river, eastern Amazonia	This work

*Abbreviation: 2n = diploid number; KF = Karyotypic Formula; CB = C-banding; NOR = Nucleolar Organizer Region; p = short arm; q = long arm; m = metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric. Symbols: (♀) = Female, (♂) = Male, (+) = technique used in the karyotype analysis, (-) = technique not used in the karyotype analysis.



Figure 1
***Eigenmannia virescens* collected from rivers in the Eastern Amazon region (bar: 1 cm).** Source: Laboratório de Citogenética-UFPA.

banding revealed the presence of constitutive heterochromatin (CH) in the centromeric regions of all chromosomes (Figures 3B and 3D), and a particularly notable heterochromatic block was found in the proximal region of the short arm of the W chromosome (Figure 3D). The Nucleolar Organizer Region (NOR) was localized to the short arm of pair 15, which is also the location of a secondary constriction that was found to have a size heteromorphism, being less evident in some cases (Figure 3A, box) and more evident in others (Figure 3C, box). DAPI fluorescence was found in the centromeric regions of all chromosome pairs. Consistent with the C-banding results (Figures 4A and 4C), the W chromosome had a strong signal at the proximal region of the short arm (Figure 4C). The CMA₃ results were consistent with our identification of the NOR (Figures 4B and 4D). Human 45S rDNA probes hybridized to the short arm of pair 15 (Figure 4E). Finally, FISH with (TTAGGG)_n telomeric probes did not

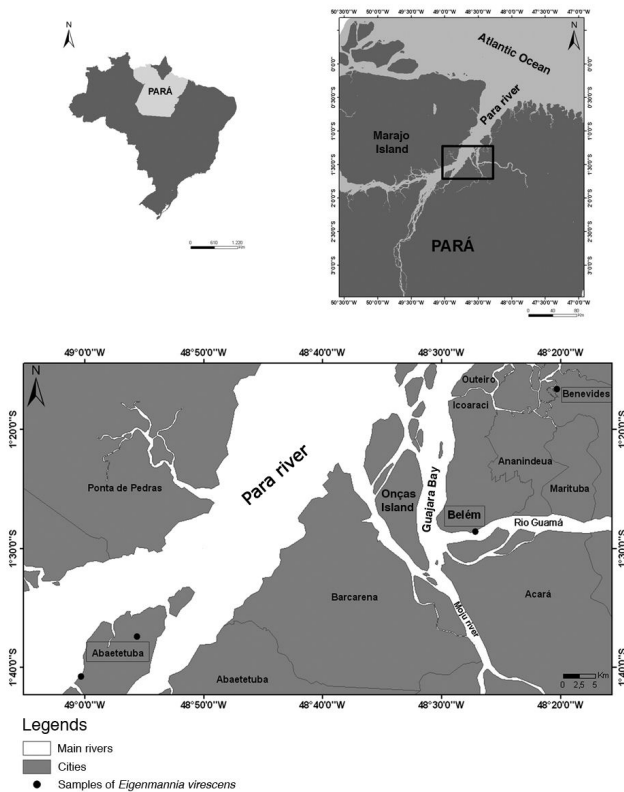


Figure 2
Map of the localities where the *Eigenmannia virescens* samples were collected. Source: Arcgis 9.1.

show any evidence of an interstitial telomeric sequence (ITS) (Figure 4F).

Discussion

We found that samples of *E. virescens* obtained from four localities in the Eastern Amazon region had the same karyotype ($2n = 38, ZZ/ZW$; KF 14m/sm+24a for males and 15m/sm+23a for females), suggesting the possibility

of genetic flow among these populations. This karyotype is similar to the one described for a sample from Marajo Island [14], with the exception that the W chromosome was described as being fully heterochromatic in the Marajo sample, whereas heterochromatin was found only at the proximal region in our sample.

Previous cytogenetic studies in *E. virescens* from many localities have consistently reported this same diploid number ($2n = 38$), but there are wide variations in terms of the reported KF, chromosome sex system, and characteristics of the W chromosome (Table 2). The differences in the KF (Table 2) can be explained by the occurrence of many pericentromeric inversions, which may suggest the presence of a postzygotic mechanism for reproductive isolation. As such, each of these populations can be accepted as a valid species, and *E. virescens* should potentially be considered a complex of morphologically similar species. Furthermore, the description of many different karyotypes for *E. virescens* may arise from the population structure of this genus, whose members typically live in small populations that have little vagility [25]. These characteristics may facilitate the fixation of chromosome rearrangements.

In terms of the chromosomes themselves, the distribution of CH in *E. virescens* is similar to that found in most Neotropical fishes [26]. The CH block in the W chromosome may have originated from the amplification of repetitive sequences, which is not an uncommon process [14,27]. In the Marajo Island, São Francisco River and Middle Amazonas samples, the NORs were localized to chromosome pairs 14, 17 and 16, respectively [14]. In a sample from Paraná River [13] and the present study, the NORs were localized to pair 15. These discrepancies may arise from differences in the classification and position of the chromosomes in the karyotype. Our DAPI and CMA₃ staining results, which are the first such reports for this species, agree with the findings from similar studies in other Neo-

Table 3: Samples of *Eigenmannia virescens* collected in different rivers from the Amazon basin.

Locality	River	Sample	GPS location	Voucher number at the MPEG
Benevides	Murini	2 males	01° 16' 34.8" S, 048° 20' 17.0" W	MPEG 15861, MPEG 15862
Belém	Guamá	4 (2 males e 2 females)	1°28'33.88"S 048°27'08.73"W	MPEG 15868, MPEG 15869*
Abaetetuba	Anequara	4 (2 males e 2 females)	01°40'42.6"S, 049°00'16.6"W	MPEG 15863, MPEG 15864, MPEG 15865, MPEG 15866
Abaetetuba	Caripetuba	10 (6 males e 4 females)	01°37'23.49"S 048°55'33"W	MPEG 15867, MPEG 15871 MPEG 15872 MPEG 15873 MPEG 15874 MPEG 15875 MPEG 15876 MPEG 15877 MPEG 15878 MPEG 15879

*The two numbers are related to the two lots deposited at the MPEG, each one with two *E. virescens* exemplars.

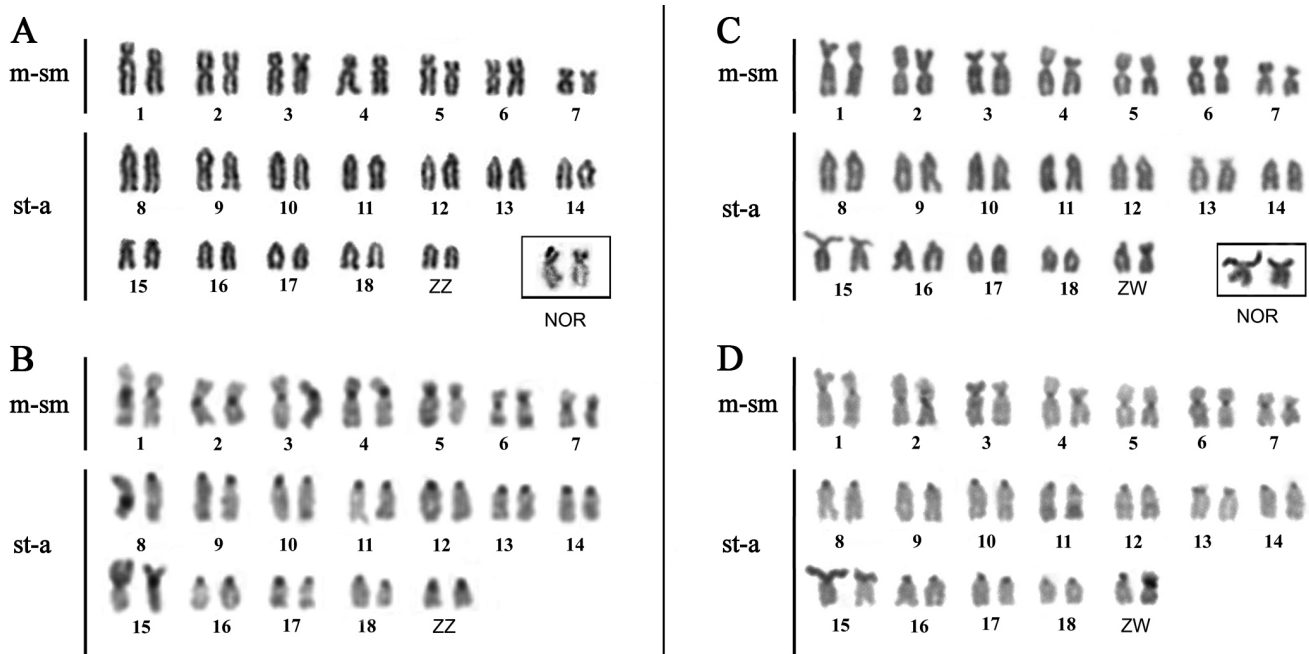


Figure 3

Karyotypes with $2n = 38$, ZZ/ZW, obtained from *Eigenmannia virescens* collected in Benfica, Abaetetuba and Belém. (A & B) Male karyotypes stained with Giemsa and C-banding, respectively. Box: pair 15, bearing the NOR. (C & D) Female karyotypes stained with Giemsa and C-banding, respectively. Box: pair 15, bearing the NOR. Abbreviations: m-sm = metacentric-submetacentric; st-a = subtelocentric-acrocentric.

tropical fishes [28-31]. More specifically, DAPI staining of the C-banding-positive regions showed that this CH is A-T rich, while CMA₃ staining of the NOR showed that the rDNA sequences are interspersed with G-C rich sequences. The rDNA probes hybridized to different-sized regions between homologs obtained from the different individuals sampled from the four localities. This heteromorphism may be the result of differences in the copy numbers of the ribosomal genes [12,32,33]. Finally, we found no evidence of ITS signals. This could be due to the modification of these sequences ((TTAGGG)*n*) after a fusion event, as a consequence of telomeric loss, or the absence of chromosome rearrangements involving the telomeres. A previous study compared the identification of ((TTAGGG)*n*) sequences in salmonids under different hybridizations stringencies [34], and found that lower stringency hybridizations identified more such sequences. This result suggests that such sequences may be modified after their inclusion as an ITS because of the progressive difficulty on the hybridization of the probe with the target DNA sequence. This may have happened on the karyotypes here studied.

Among the Neotropical fish karyotypes studied to date, 5.9% were found to have differentiated sex chromosome systems. However, the origin of these chromosomes is still unresolved [35]. Previous cytogenetic studies in *Eigen-*

mannia identified only two other species as having differentiated sex chromosomes: *Eigenmannia* sp.2 ($2n = 31/32$, $X_1X_1X_2X_2/X_1X_2Y$) and *E. virescens* ($2n = 38$, with undifferentiated, XX/XY, and ZZ/ZW sex chromosome systems variously described for samples collected from different localities) (Table 2). According to a previous hypothesis put forth to explain the evolution of the sex chromosomes in *E. virescens* [14], the differentiation of their sex chromosomes first arose in the Paraná basin, where some populations lack differentiated sex chromosomes. From these undifferentiated chromosomes, amplification of the CH on one homolog of an acrocentric pair led to the development of the XX/XY system found in other populations from that basin (Table 2). Thereafter, a pericentric inversion in one of these acrocentric chromosomes generated the ZZ/ZW system found in the São Francisco River. The authors suggested that the ZZ/ZW sex system found in the Amazon basin is a posterior situation, with heterochromatinization differentiating one of the homologs. In support of this hypothesis, the authors noted that differences in HC blocks could be used to distinguish the W chromosomes of the Marajo Island populations from those of the Middle Amazon River populations.

However, we believe that an alternative hypothesis could explain the evolution of the different sex chromosomes found in *Eigenmannia*. Based on careful consideration of

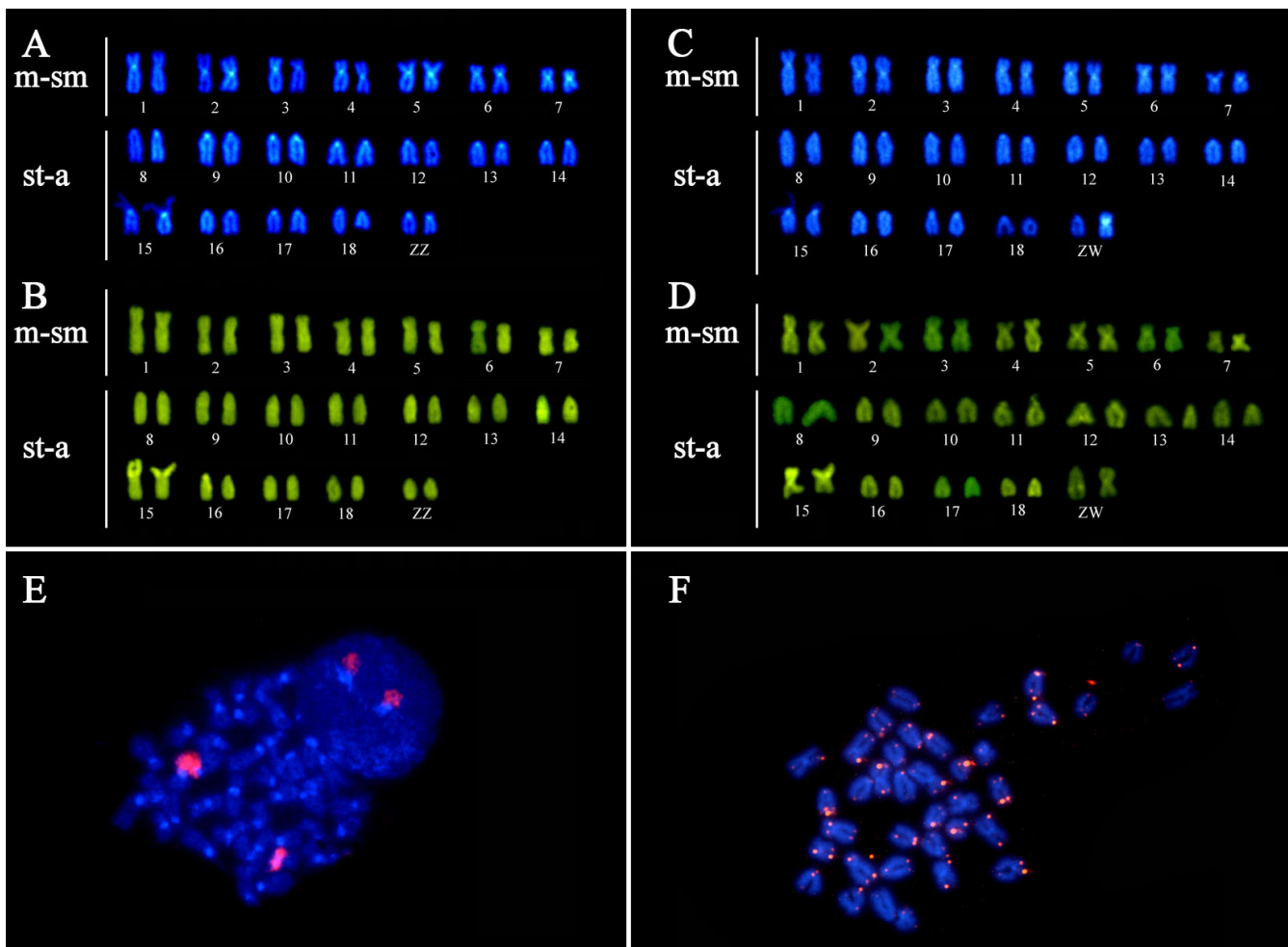


Figure 4
Karyotypes of *Eigenmannia virescens*: (A & C) DAPI banding consistent with the centromeric C-bands found in both male and female chromosomes. Note the bright staining of the W chromosome. (B & D) CMA₃ banding of pair 15, consistent with our localization of the NOR. (E) FISH with rDNA 45S probes in *E. virescens* metaphase spreads. (F) FISH with telomeric probes in *E. virescens* metaphase spreads. No evidence of ITS was observed.

our results and those from previous reports, we suggest that the ancestral karyotype was similar to that seen in modern populations lacking differentiated sex chromosomes (such as the Mogi-Guaçu sample; Figure 5B), and the systems found in the other populations arose independently from this ancestral system (Figure 5). In *Eigenmannia* sp.2 ($2n = 31/32$, $X_1X_1X_2X_2/X_1X_2Y$), a centric fusion between two acrocentrics (pairs 6 and 11) in a male karyotype would yield the metacentric Y (neo-Y), as previously suggested [[15,23,24] and Figure 5A]. The other populations could be generated as follows: a) the addition of heterochromatin to the distal region of the long arm in one of the homologs would yield the XX/XY system seen in the Tiete River sample (Figure 5C); b) a pericentric inversion in one of the homologs would yield the ZZ/ZW system found in the São Francisco River sample (Figure

5D); c) the addition of heterochromatin to the short arm of an acrocentric would yield the W chromosome seen in the sample from Marajo Island (Figure 5E); d) a pericentric inversion followed by the addition of heterochromatin to the pericentromeric region of the proximal long arm of the W would generate the pattern found in the Middle Amazon River sample (Figure 5F); and e) a pericentric inversion followed by heterochromatinization in the proximal region of the short arm of the W would differentiate this chromosome to that seen in the present work, assuming that there is genetic flow among the four populations of the Eastern Amazon region studied herein (Figure 5G).

Evidence supporting our proposal for the independent origin of sex chromosomes in different species of genus

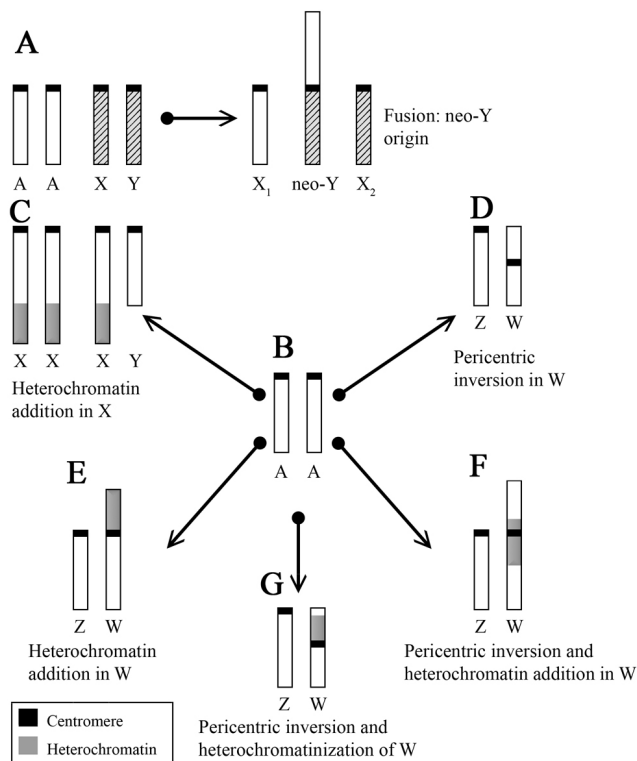


Figure 5
Sex chromosomes in various species of *Eigenmannia*.
 (A) Sex chromosome differentiation in *Eigenmannia* sp.2. (B-G) Independent sex chromosome differentiation mechanisms in populations of *E. virescens* from: (B) Mogi-Guaçu, (C) Tiete River, (D) São Francisco River, (E) Marajo Island, (F) Middle Amazonas River, and (G) sample from present work.

Eigenmannia can be found in two previous studies using sex chromosome probes produced by microdissection from two species: the Y of *Eigenmannia* sp.2 (E2Y) ($2n = 31/32$, $X_1X_1X_2X_2/X_1X_2Y$) and the X of *E. virescens* (EVX) ($2n = 38$, XX/XY) [36,37]. Cross-species experiments showed that these probes cross-hybridized to the autosomes but not the sex chromosomes, demonstrating that the sex chromosomes are not homologous between these two species (and therefore are likely to have arisen independently). The same thing could have happened among the ZZ/ZW system-utilizing species of *E. virescens*. Furthermore, the previous and present studies all found that the sex chromosomes of the various species have different characteristics (Figure 5). If we hypothesize that these forms evolved sequentially, we must assume that many evolutionary steps have occurred along the way. Since each studied taxon was found to have a different KF, we must also suppose that each karyotype gave rise to next, which does not appear likely. Collectively, these lines of evidence would seem to support our hypothesis that the sex chromosomes in different species of genus *Eigenmannia* may have independent origins.

Conclusion

We herein report that samples of *E. virescens* obtained from four localities in the Eastern Amazon region had the same karyotype and possessed differentiated sex chromosomes (ZZ/ZW). Previous studies showed that *Eigenmannia* sp. 2 and *E. virescens* have differentiated sex chromosomes, and diverse sex chromosome systems have been described for *E. virescens* from different Brazilian rivers. A comparative analysis of our present data and the previous reports led us to suggest that the sex chromosomes of different species of *Eigenmannia* may have arisen independently.

Authors' contributions

DSS collected the samples, collaborated on all cytogenetic procedures, undertook the bibliographic review, and coordinated the writing of this paper. JCP helped conceive the study and participated in developing the laboratory techniques, cytogenetic analyses and writing. SSRM participated in collecting the specimens and developing the laboratory techniques. CYN coordinated the study, helped develop the laboratory techniques and cytogenetic analyses, and reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was financially supported by CNPq, CAPES, FAPESPA and UFPA. JCP and CYN are CNPq Researchers, level I. DSS received a CAPES Masters Scholarship on Genetics and Molecular Biology. SSRM received a CNPq Doctors Scholarship on Genetics and Molecular Biology. We thank the Programa de Pós-Graduação em Genética e Biologia Molecular for supporting this study; researchers Ms. Jaime Ribeiro Jr. and Ms. Augusto Cesar Paes de Souza for their help with field work and species identification; and geologist João Marinho Milhomem Neto for help in mapping where the sample collections took place. The field collections were authorized by the IBAMA (Instituto Brasileiro de Meio Ambiente). Finally, we are grateful to Dr. Wolmar Benjamin Wosiacki (Curator of Fishes, Museu Paraense Emilio Goeldi) for vouchering the specimens and adding them to the museum's collection.

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