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Karyotypic and morphological divergence between two cryptic species of *Eigenmannia* in the Amazon basin with a new occurrence of XX/XY sex chromosomes (Gymnotiformes: Sternopygidae)

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Eigenmannia species are widely distributed in the Neotropics, with eight valid species currently recognized. Populations of *Eigenmannia* from three locations in the eastern Amazon were investigated using cytogenetic and morphological techniques, revealing two taxa designated here as *Eigenmannia* sp. "A" and *Eigenmannia* sp. "B". The species differ in three morphometric characters, two meristic characters, and one osteological character. *Eigenmannia* sp. "A" presents 2n = 34 (22 m/sm+12 st/a) and *Eigenmannia* sp. "B" presents 2n = 38 (14 m/sm+24st/a) and simple differentiated sex chromosomes of the type XX/XY. In both species the Constitutive Heterochromatin (CH) rich in A-T bases is distributed in the centromeric region of all chromosomes. *Eigenmannia* sp. "B" also presents CH blocks in the interstitial region of chromosome pairs 8, 9 and X which are positively stained with CMA_3, indicating G-C rich regions. The NOR is located on the short arm of chromosome pair 17 of *Eigenmannia* sp. "A" and on the short arm of pair 14 of *Eigenmannia* sp. "B". FISH with rDNA probes hybridized to different-sized regions between homologs, suggesting heteromorphism. The differentiation of the X chromosome in *Eigenmannia* sp. "B" could be the result of amplification of repetitive DNA sequences.

Espécies de *Eigenmannia* estão amplamente distribuídas na região Neotropical, com oito espécies válidas atualmente reconhecidas. Populações de *Eigenmannia* de três localidades do leste da Amazônia foram investigadas usando técnicas citogenéticas e morfológicas, revelando dois táxons designados aqui como *Eigenmannia* sp. "A" e *Eigenmannia* sp. "B". As espécies diferem em três caracteres morfométricos, dois merísticos e um osteológico. *Eigenmannia* sp. "A" apresenta 2n = 34 (22 m/sm+12st/a) e *Eigenmannia* sp. "B" apresenta 2n = 38 (14 m/sm+24st/a) e cromossomos sexuais de diferenciação simples, do tipo XX/XY. Em ambas espécies a Heterocromatina Constitutiva (HC) rica em bases A-T está distribuída na região centromérica de todos os cromossomos. *Eigenmannia* sp. "B" também apresenta blocos de HC na região intersticial dos pares cromossômicos 8, 9 e X que coraram positivamente para CMA_3, indicando regiões ricas em G-C. A NOR está localizada no braço curto do par 17 em *Eigenmannia* sp. "A" e no braço curto do par 14 em *Eigenmannia* sp. "B". FISH com sondas de rDNA hibridizaram em regiões de tamanhos diferentes entre os homólogos, sugerindo heteromorfismo. A diferenciação do cromossomo X em *Eigenmannia* sp. "B" pode ser o resultado de amplificação de sequências repetitivas de DNA.

Keywords: Cytogenetics, FISH, Knife fishes, Morphology, Sex chromosomes

Introduction

Neotropical electric fishes of the genus *Eigenmannia* Jordan & Evermann, 1896, popularly known as "knife fishes", "tuviras", "peixe-faca" or "sarapós", present an ample distribution from Panama to northern Argentina (Mago Leccia, 1978; Albert, 2001; Albert & Crampton, 2005). Eight valid species of *Eigenmannia* are recognized: *E. humboldtii* (Steindachner, 1878), *E. limbata* (Schreiner & Miranda Ribeiro, 1903), *E. macrops* (Boulenger, 1897), *E. microstoma* (Reinhardt, 1852), *E. nigra* Mago-Leccia, 1994, *E. trilineata* López & Castello, 1966, *E. vicentespelaea* Triques, 1996 and *E. virescens* (Valenciennes, 1842) (Albert, 2003).

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Albert (2001) proposed a phylogeny for Gymnotiformes, though did not identify synapomophies for *Eigenmannia*, classifying the genus in three groups of species (1) *Eigenmannia* gr. *microstoma*, containing *E. microstoma*, *E. humboldtii*, *E. limbata* and *E. nigra*; (2) *Eigenmannia* gr. *virescens*, containing *E. virescens*, *E. trilineata* and *E. vicentespelaea*; and (3) *Eigenmannia* gr. *macrops*, as monospecific. All groups are diagnosed by morphological characters except *Eigenmannia* gr. *macrops*, which was delimited without presenting any further information on diagnostic characters.

Cytogenetic studies of *Eigenmannia* demonstrate a high level of karyotypic diversity (Table 1). *Eigenmannia* gr. *virescens* is the most studied group, presenting karyotypic variation which ranges from 2n = 28 in *Eigenmannia* sp. 1 (Almeida-Toledo *et al.,* 1988), to 2n = 38 in *Eigenmannia virescens* (Almeida-Toledo *et al*., 2001, 2002; Silva *et al*., 2009). Previous results demonstrate the existence of differentiated sex chromosomes in the species of the *E.* gr. *virescens*, with two simple sex chromosome systems XX/XY (Almeida-Toledo *et al*., 2001) and ZZ/ZW (Almeida-Toledo *et al*., 2002; Silva *et al*., 2009), as well as the existence of multiple sex chromosome systems $X_1X_1X_2X_2/X_1X_2Y$, in *Eigenmannia* sp. "2" (Almeida-Toledo *et al*., 1984) and *E. trilineata* (Fernandes *et al*., 2010).

Although the taxa described in *Eigenmannia* are considered valid, various authors suggest that the species in this genus represent a complex, which is difficult to resolve (Shibatta, 1993; Campos-da-Paz, 1997). This is corroborated by cytogenetic studies that demonstrate karyotypic variation among samples both within and between hydrological basins (Moysés *et al*., 2005).

We investigated three populations of *Eigenmannia* from streams of eastern Amazonia, using cytogenetic, morphometric, meristic and osteological analyses. The results reveal the existence of two cryptic species, and the description of a new occurrence of sex chromosomes of the simple XX/XY type for *Eigenmannia* in the Amazon basin.

Table 1. Karyotypic studies in the genus *Eigenmannia*. Legend: 2n = diploid number; SC = Sex Chromosomes; ND = Not differentiated; KF = Karyotypic Formula; NOR = Nucleolar Organizing Region; CH = Constitutive Heterochromatin; CB = C band; p = short arm; q = long arm; m = metacentric; sm = submetacentric; st = subtelocentric; a = acrocentric. Symbols: (♀) = Female; (♂) = Male; (+) = technique performed on karyotype; (-) = technique not performed on karyotype.

Species	2n	KF	NOR	SC	Localities	Authors
Eigenmannia sp.	46	20m/sm+26st/a	4 p (a)	ND	Amazon basin, rio Jari-Pará, Brazil	Almeida-Toledo *et al*. (1985)
Eigenmannia sp.	31/32	13m/sm+18st/a♀; 12m/ms+20st/a♂	4 p (a)	ND	Amazon basin, rio Jari-Pará, Brazil	Almeida-Toledo *et al*. (1985)
Eigenmannia sp.1	28	14m/sm+14a	10 q (a)	ND	rio Mogi-Guaçu-São Paulo, Brazil	Almeida-Toledo *et al*. (1988)
Eigenmannia sp.1	28	14m/sm+14a	10q+1 at11q(a) +1 at 3p(m)	ND	Emas waterfalls rio Mogi-Guaçu-São Paulo, Brazil	Almeida-Toledo *et al.* (1996)
Eigenmannia sp.1	28	14m/sm+14a	3 q (m)	ND	Araras region rio Mogi-Guaçu-São Paulo, Brazil	Almeida-Toledo *et al.* (1996)
Eigenmannia sp.2	31/32	8m+24a♀; 9m+22a♂	10p (a)	$X_1X_1X_2X_2/X_1X_2Y$	rio Tietê-São Paulo, Brazil	Almeida-Toledo *et al.* (1984, 1988, 2000)
Eigenmannia sp. 3	36	7m/sm+29st/a♀; 6m/sm+30st/a♂	16p (a)	ZZ/ZW	rio Penápolis and Botucatu, rio Tietê-São Paulo, Brazil	Almeida-Toledo *et al.* (1996); Moysés *et al.* (2005)
E. virescens	38	16m/sm+22st/a	15 p (st)	ND	rio Mogi-Guaçu system-São Paulo, Brazil	Almeida-Toledo *et al.* (2001)
E. virescens	38	16m/sm+22st/a♀; 16m/sm+22st/a♂	15 p (st)	XX /XY	rio Tietê-São Paulo, Brazil	Almeida-Toledo *et al.* (2001)
E. virescens	38	23m/sm+15st/a♀; 22m/sm+16st/a♂	17p (st-a)	ZW/ZZ	rio São Francisco-Minas Gerais, Brazil	Almeida-Toledo *et al.* (2002)
E. virescens	38	15m/sm+23st/a♀; 14m/sm +22st/a♂	14p (a)	ZW/ZZ	Ilha de Marajó-Pará, Brazil	Almeida-Toledo *et al.* (2002)
E. virescens	38	17m/sm +21st/a	16p (a)	ZW	Middle rio Amazon-Amazonas, Brazil	Almeida-Toledo *et al.* (2002)
E. virescens	38	15m/sm+23st/a♀; 14m/sm+24st/a♂	15p (st)	ZZ/ZW	rios Murini, Anequara and Guamá, Pará, Brazil	Silva *et al*. (2009)
E. trilineata	31/32	8m+24a♀; 9m+22a♂	10p (a)	$X_1X_1X_2X_2/X_1X_2Y$	rio Iguatemi tributary (Mundo Novo)-Mato Grosso do Sul, Brazil	Fernandes *et al*. (2010)
Eigenmannia sp. "A"	34	22 m/sm+12 st/a	17p (a)	ND	Igarapé do Açaiteuazinho, Lago segredo and Balneário do Marapanim-Pará, Brazil	Present study
Eigenmannia sp. "B"	38	14 m/sm+24 st/a	14p (a)	XX/XY	Igarapé do Açaiteuzinho-Pará, Brazil	Present study

Material and Methods

Samples. Fifty three samples were collected from three localities in eastern Amazonia, Pará State, Brazil (Fig. 1): two localities in the municipality of Capanema, rio Quatipuru basin and one in the municipality of São Francisco, rio Marapanim basin. All specimens were deposited in the ichthyology collection of the Museu Paraense Emílio Goeldi, Pará State, Brazil (MPEG) (Table 2). Samples were collected using seine nets, and kept alive with portable aeration in thermally protected receptacles for transport to the laboratory. Sample collection was made under licence 020/2005 (ICMBio Registration: 207419).

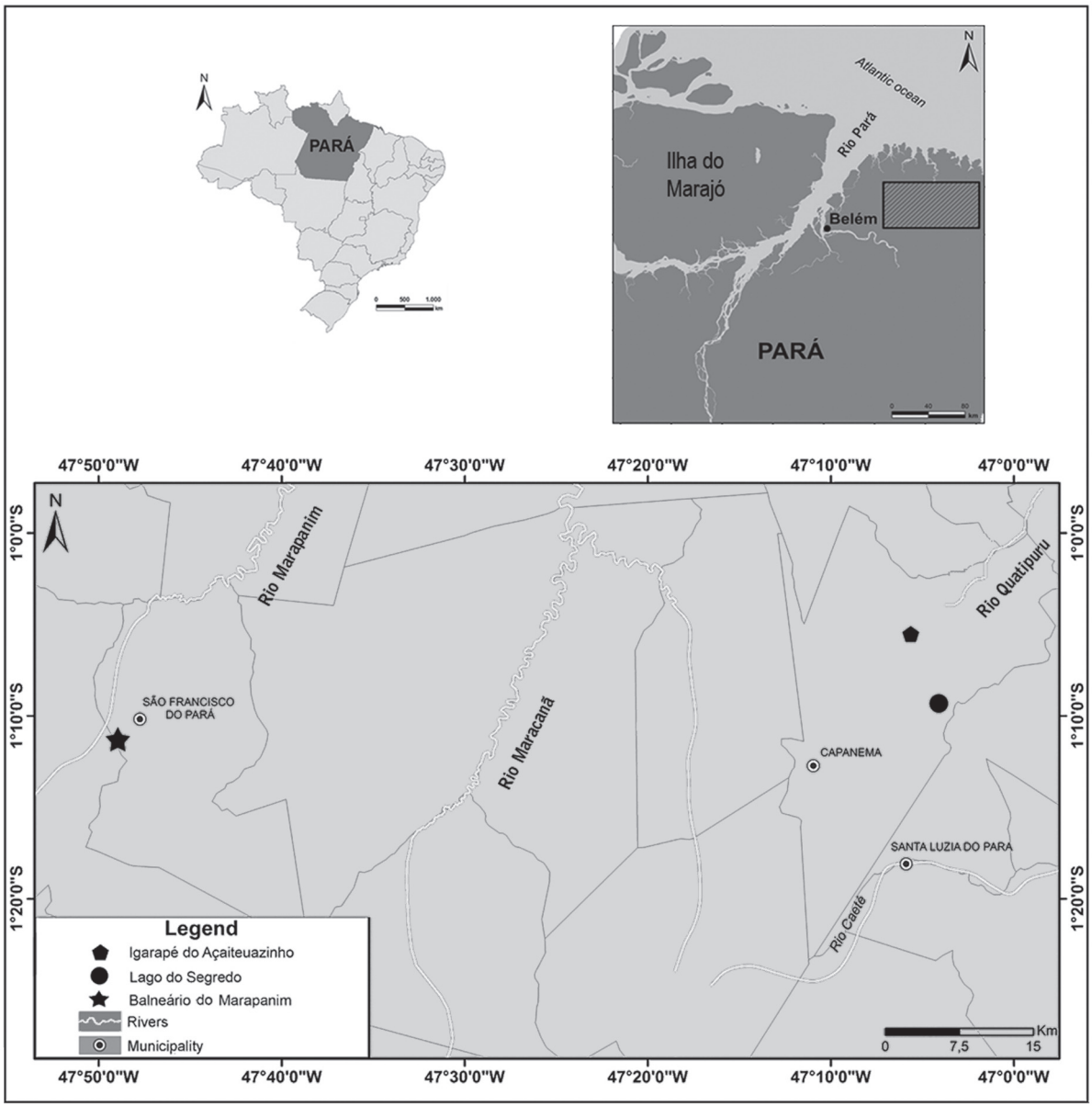

Fig. 1. Map of localities where *Eigenmannia* samples were collected.

Table 2. Localities of the samples of *Eigenmannia* in north-eastern Pará State. ? = undetermined sex.

Locality	River	Sample size (by gender)	Latitude and Longitude (GPS)	Vouchers
Igarapé do Açaiteuazinho	Quatipuru	24 (19♂; 5♀)	S=01°09'15'' W=47°04'06,4''	MPEG 27066, MPEG 27067
Lago do Segredo	Quatipuru	12 (2♂; 2♀; 8?)	S=01°05'31,2'' W= 47°5'37,8''	MPEG 27068
Balneário do Marapanim	Marapanim	17 (6 ♂; 7♀; 4?)	S=01°11'17,4'' W= 47°48'59,69''	MPEG 27061
		Total = 53		

Morphometry, meristics and osteology. Measurements were made on the left hand side of each specimen using a digital calliper to a precision of 0.1 mm while viewed under a stereomicroscope. Morphometric analyses were based on Mago Leccia (1978), Triques (1996) and Crampton *et al*. (2004). Taxonomic parameters for Gymnotiformes were followed where measures of the body are described in proportion to the length as measured from the tip of the snout to the end of the anal fin (LEA - Length to the end of the anal fin) and measures of parts of the head described in proportion to the Head Length (HL). Meristic analyses included: number of rays of the pectoral fin, number of lateral line scales (counting from the first scale with a lateral line tube to the end of the anal fin) and the number of scales above the lateral line (counted at the highest point along the body, approximately in line with the distal portion of the longest ray of the anal fin). Specimens were cleared and stained following Taylor & Van Dyke (1985). The osteological nomenclature of the maxilla follows Lundberg & Mago-Leccia (1986) and de Santana & Crampton (2011).

Cytogenetic methods. Metaphase chromosomes were obtained following the protocol of Bertollo *et al.* (1978). Conventional analyses were performed, including staining with Giemsa (Merck), C-banding (Sumner, 1972), impregnation with silver nitrate (Ag-NOR) (Howell & Black, 1980), staining with the fluorochrome DAPI (Pieczarka *et al*., 2006), and staining with Chromomycin A_3 (CMA_3) (Schweizer, 1980). The gonads were removed and prepared as a smear with a 32x20mm glass slide and observed under a stereomicroscope to determine the sex of the individuals.

Fluorescence *in situ* Hybridization (FISH) was performed with probes for 18S ribossomal DNA (rDNA 18S) obtained from the species *Prochilodus argenteus* Agassiz, 1829, marked with biotin or digoxigenin by nick translation (Hatanaka & Galetti Jr., 2004). The *in situ* hybridization was detected using avidin (Cy3 or FITC) or anti-digoxigenin (FITC). The chromosomes were classified and measured following Guerra (1986).

Abbreviations used in text are: HL – head length, LEA - length to the end of the anal fin, DAPI – 4',6-diamidino-2-phenylindole, Vector Laboratories, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO).

Results

Morphometric, meristic and osteological analyses. Morphometry, meristics and osteology revealed the existence of two distinct taxa, identified as belonging to the *Eigenmannia virescens* group, based on the characters proposed by Albert (2001). Forty-nine individuals from balneário Marapanim, lago do Segredo and Igarapé do Açaiteuazinho were designated as *Eigenmannia* sp. "A" (Fig. 2), and four individuals from do Açaiteuazinho were designated as *Eigenmannia* sp. "B" (Fig. 3). The populations here analyzed, although allocated in the species group *Eigenmannia virescens*, do not fit diagnoses of the species already described.

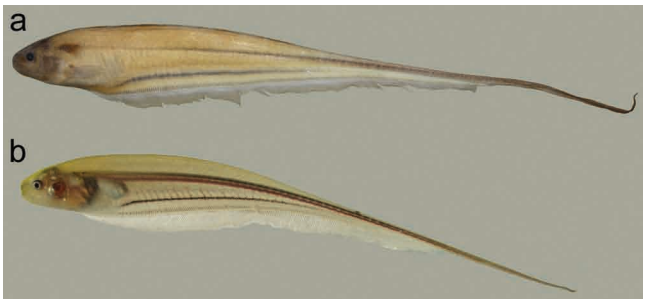

Fig. 2. Left lateral view of fixed specimen (a), MPEG 27068, 102.7 mm LEA, and live specimen (b) of *Eigenmannia* sp. "A", lago Segredo, rio Quatipuru, municipality of Capanema, Pará, Brazil.

Fig. 3. Left lateral view of fixed specimen (a) and live specimen (b) of *Eigenmannia* sp. "B", MPEG 27066, 131.8 mm LEA, from the igarapé do Açaiteuazinho, rio Quatipuru, municipality of Capanema, Pará, Brazil.

Eigenmannia sp. "A" presents a greater distance between the eye and naris compared to *Eigenmannia* sp. "B" (8.5-11.1% *vs*. 7.5% HL), a smaller orbital diameter (13.6-17.2% *vs*. 21.5-24.3% HL) and a shorter superior maxilla (16.3-19.0% *vs*. 22.3-24.0% HL), respectively. Two meristic characters also differ between the two species: the number of pectoral-fin rays, 15-16 in *Eigenmannia* sp. "A" *vs*. 17 in *Eigenmannia* sp. "B", and the number of lateral line scales, 122-129 in *Eigenmannia* sp. "A" *vs*. 115-119 in *Eigenmannia* sp. "B" (Table 3).

The morphology of the maxilla in *Eigenmannia* sp. "A" presents a small antero-dorsal process which is about half the size of the anterior naris, and the descending process is large, approximately the same width as the anterior naris (Fig. 4a). In *Eigenmannia* sp. "B", the antero-dorsal process is hypertrophied, being about the same size as the anterior naris, and the descending process is narrow (about half the width of the anterior naris) (Fig. 4b).

The two species present a yellow colouration when preserved, with three longitudinal stripes, one above the lateral line, one along the proximal portion of the pterygiophores of the anal fin and one along the base of the anal fin. They also present a dark band along the body localized between the lateral line and the stripe, which runs along the proximal portion of the pterygiophores of the anal fin.

Table 3. Morphometric and meristic data for *Eigenmannia* sp. "A" and *Eigenmannia* sp. "B". Legend: Min: Minimum; Max: Maximum; SD: Standard deviation; N: number of specimens analysed.

	Eigenmannia sp. "A"					*Eigenmannia* sp. "B"				
	Measurements									
	Min	Max	Mean	SD	N	Min	Max	Mean	SD	N
Total length (mm)	107.7	152.4	-	-	16	-	-	-	-	2
Length to end of anal fin (mm)	84.2	118.8	-	-	20	113.2	131,9	-	-	2
Head length (mm)	10.7	15.5	-	-	20	15.4	16.7	-	-	2
Percent of length to end of anal fin										
Head length	11.6	14.5	12.9	0.8	20	12.7	13.6	13.1	0.7	2
Preanal distance	14.2	16.3	15.2	0.6	20	16.0	17.2	16.6	0.9	2
Prepectoral distance	12.5	15.2	13.6	0.9	20	13.7	14.2	14.0	0.4	2
Preanus distance	6.8	8.7	7.6	0.6	20	7.4	7.5	7.4	0.1	2
Body depth	13.3	16.0	14.7	0.8	20	15.4	15.9	15.6	0.4	2
Body width	4.0	6.3	5.4	0.6	20	5.6	5.9	5.8	0.2	2
Anal-fin length	84.6	88.1	86.3	1.5	20	86.4	87.2	86.8	0.5	2
Pectoral-fin length	6.8	8.5	8.0	0.5	20	8.7	9.8	9.3	0.8	2
Caudal-filament length	27.6	33.8	31.0	2.2	16	-	-	-	-	-
Caudal-filament depth	1.2	1.5	1.4	0.1	16	-	-	-	-	-
Caudal-filament width	0.4	0.7	0.5	0.1	16	-	-	-	-	-
Percent of head length										
Snout length	21.2	28.1	25.1	1.9	20	23.9	27.0	25.5	2.2	2
Internasal distance	7.7	10.6	9.0	1.0	20	9.8	10.4	10.1	0.4	2
Snout-posterior naris distance	16.4	20.2	18.8	1.2	20	18.3	18.9	18.6	0.4	2
Oculo-nasal distance	8.5	11.1	10.0	0.9	20	7.5	7.5	7.5	0.1	2
Nasal width	13.2	18.4	15.3	1.7	20	16.1	16.5	16.3	0.3	2
Orbital diameter	13.6	17.2	15.5	1.1	20	21.5	24.3	22.9	2.0	2
Postorbital length	43.4	56.8	51.1	3.9	20	50.7	54.7	52.7	2.8	2
Opercular opening	20.9	28.3	24.7	2.0	20	21.8	31.9	26.9	7.2	2
Orbital depth	22.3	29.9	25.6	2.0	20	22.6	25.2	23.9	1.8	2
Interorbital distance	26.9	35.6	31.1	2.4	20	29.7	30.0	29.9	0.2	2
Head width at operculum	46.5	55.0	51.2	2.3	20	45.8	54.9	50.4	6.4	2
Head width at orbit	32.9	40.0	35.5	2.5	20	32.9	44.6	38.7	8.3	2
Head depth at supraoccipital	68.2	78.5	73.7	2.9	20	72.1	72.5	72.3	0.3	2
Head depth at orbit	47.0	58.4	52.6	3.3	20	52.9	57.0	55.0	2.9	2
Superior maxilla length	16.3	19.0	17.1	0.8	20	22.3	24.0	23.3	1.2	2
Gape width	14.8	19.8	17.4	1.4	20	14.8	18.1	16.4	2.3	2
Meristics										
Pectoral-fin rays	15-16					17				
Anal-fin rays	182-201					193-212				
Scale rows above lateral line	12-14					12				
Lateral line scales	122-129					115-119				

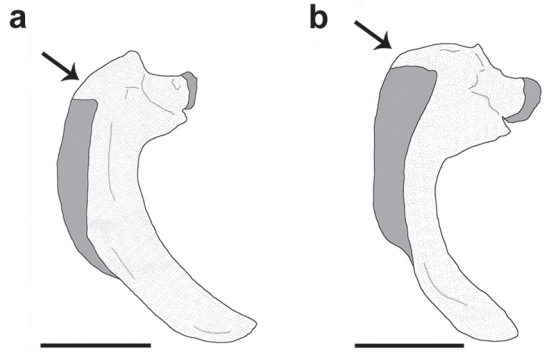

Fig. 4. Inverted lateral view, of the right maxilla of (a) *Eigenmannia* sp. "A" (MPEG 27061, 111.8 mm LEA) and (b) *Eigenmannia* sp. "B" (MPEG 27066, 117.6 mm LEA). Arrow indicates antero-dorsal process. Scale bar of 1 mm.

Cytogenetic analyses. Approximately 30 metaphase cells of each specimen were examined, revealing a difference in the karyotype of the two species. The species *Eigenmannia* sp. "A" presents 2n = 34 chromosomes and a karyotypic formula (KF) of 22 m/sm and 12 st/a without the presence of differentiated sex chromosomes (Fig. 5). *Eigenmannia* sp. "B" presents 2n = 38 and a KF of 14 m/sm and 24 st/a (Fig. 6). In males, a heteromorphic pair of acrocentric chromosomes was observed, with one of the homologous chromosomes distinctly larger than the other (Fig. 6b). This heteromorphism was not observed in the females, where the homologous chromosomes were both large (Fig. 6a).

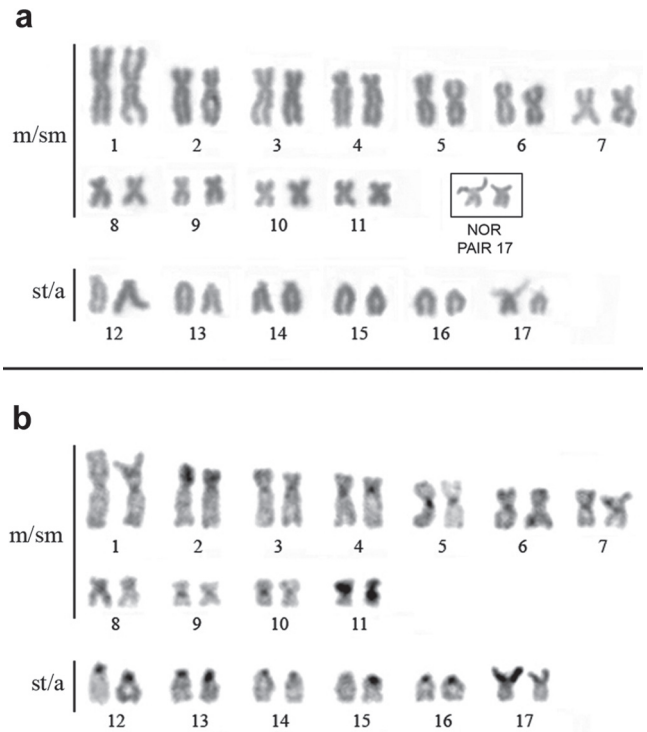

Fig. 5. Karyotype of *Eigenmannia* sp. "A" with 34 chromosomes. Conventional staining (a) and C banding (b). Box inset: chromosome 17 (p) with NOR. Legend: m/sm = metacentric/submetacentric and st/a = subtelocentric/acrocentric.

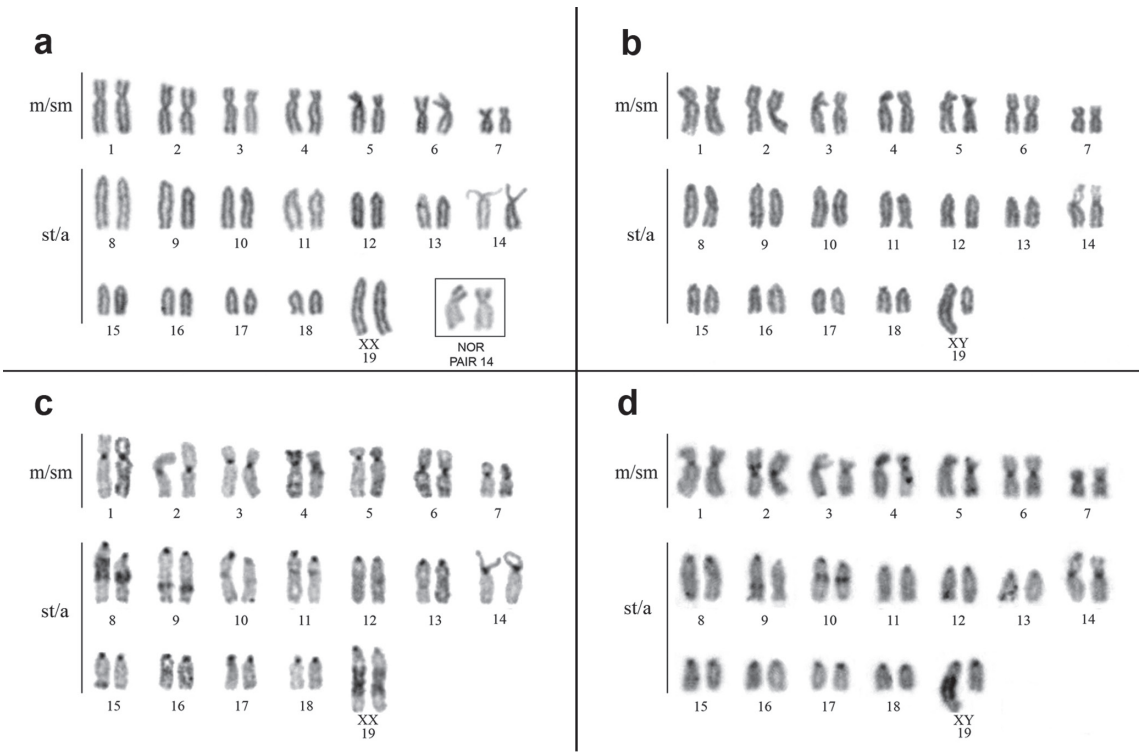

Fig. 6. Karyotype of *Eigenmania* sp. "B" with 2n = 38, XX/XY from the rio Quatipuru. Male karyotype stained with Giemsa (a) and C banding (c). Box inset: pair 14, with NOR. Female karyotype stained with Giemsa (b) and C banding (d). Legend: m/sm = metacentric/submetacentric and st/a = subtelocentric/acrocentric.

The Constitutive Heterochromatin (CH) is mainly distributed in the centromeric region of all chromosomes of both species (Figs. 5b, 6c-d). In *Eigenmannia* sp. "B" CH blocks were also observed in the interstitial regions of acrocentric chromosomes 8, 9 and 10. It was also possible to note a large CH block in the interstitial region of the X chromosome, which was absent in the Y (Figs. 6c-d).

The NOR was localized on the short arm of chromosome pair 17 in *Eigenmannia* sp. "A" and on the short arm of chromosome pair 14 in *Eigenmannia* sp. "B" (Figs. 5-6 box), which was also found to present size heteromorphism between the homologous chromosomes of the two species.

DAPI fluorescence was found in the centromeric regions of all chromosome pairs of both species, consistent with the C-banding results (Figs.7a, 8a-b). CMA_3 marked the short arm of chromosome pair 17 most intensely in *Eigenmannia* sp. "A" and the short arm of chromosome pair 14 most intensely in *Eigenmannia* sp. "B". Additionally, in *Eigenmannia* sp. "B" it was possible to observe intense marking of chromosome pairs 8 and 9, coincident with the CH blocks (Figs. 8c-d). FISH with 18S rDNA probes hybridized the region of the short arm of chromosome pair 17 in *Eigenmannia* sp. "A", and the region of the short arm of chromosome pair 14 in *Eigenmannia* sp. "B" (Figs. 8e-f).

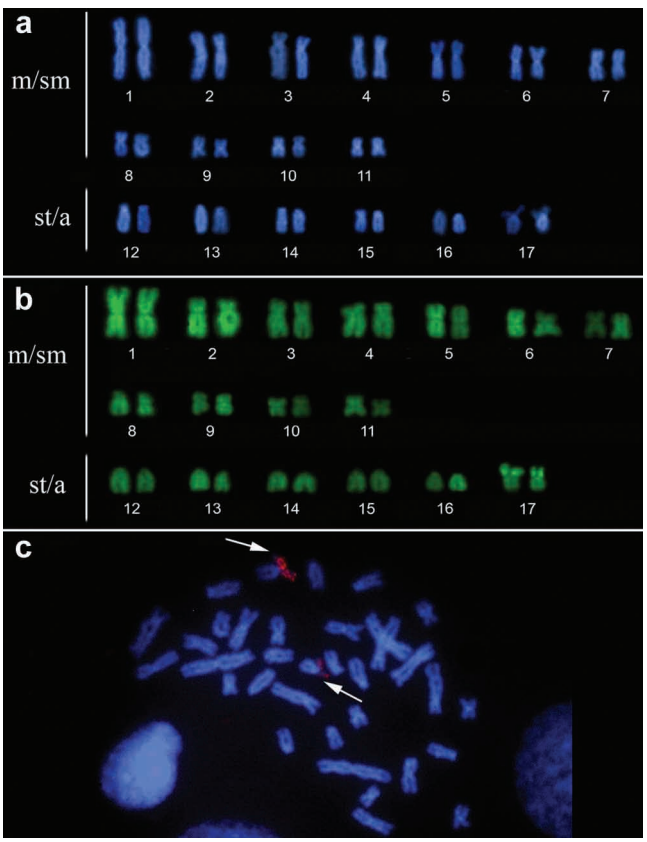

Fig. 7. (a) DAPI fluorescent banding coinciding with C banding. (b) CMA_3 fluorescent banding is coincident with the NOR region on short arm of pair 17 of *Eigenmannia* sp. "A". (c) FISH with 18S rDNA probes hybridizing on the short arm of chromosome 17 (arrows).

Discussion

Taxonomic considerations. The morphometric, meristic and osteological divergences among *Eigenmannia* sp. "A" and *Eigenmannia* sp. "B" indicate the occurrence of two distinct lineages, corroborated by the karyotypic differences. The karyotypic differences (2n = 34 *vs*. 2n = 38, XX/XY) are sufficient to act as a post-zygotic reproductive isolation mechanism (King, 1993). As such, we find that the two cryptic species currently occur in sympatry in the rio Quatipuru basin (specifically at igarapé do Açaiteuazinho), but that *Eigenmannia* sp. "A" also occurs allopatrically in the rio Marapanim basin.

The species *Eigenmannia* sp. "A" and *Eigenmannia* sp. "B" are distinct from *E. virescens*, *E. macrops*, *E. humboldtii*, *E. limbata* and *E. nigra* by the presence of three longitudinal stripes (*vs*. uniform colouration without stripes). Additionally, they are distinct from *E. humboldtii*, *E. limbata* and *E. nigra* due to the colouration pattern of the anal fin, which is hyaline (*vs*. darkened along the distal margin in *E. humboldtii* and *E. limbata* or uniformly darkened in *E. nigra*).

Eigenmannia sp. "A" shares the presence of stripes along the body with *E. trilineata*, *E. vicentespelaea* and *E. microstoma*. It differs from *E. trilineata* and *E. microstoma* in the size of the antero-dorsal process of the maxilla, being equivalent to half the size of the posterior naris (*vs*. equivalent to the size of the posterior naris) and in the suborbital height, 22.3-29.9% HL (*vs*. 32.5-46.6%; 29.9-40.8%, respectively). It is distinguished from *E. vicentespelaea* by the number of pectoral fin rays, 15-16 *vs*. 17-19 and by the number of scales above the lateral line, 12-14 *vs*. 7-8.

Similarly, *Eigenmannia* sp. "B" shares the presence of longitudinal stripes on the body with *E. trilineata*, *E. vicentespelaea* and *E. microstoma*. It differs from *E. trilineata* and *E. microstoma* in the suborbital height, 22.6-25.2% HL (*vs*. 32.5-46.6%; 29.9-40.8%, respectively), and can be distinguished from *E. microstoma* by the length of the superior maxilla, 22.3-24.0% HL *vs*. 17.5-19.2%; and by the head height at the supraoccipital, 72.1-72.5% HL *vs*. 76.1-85.1%. Finally, it differs from *E. vicentespelaea* in the number of scales above the lateral line, 12 *vs*. 7-8 and in the body height, 15.4-15.9% LEA *vs*. 10.5-14.5%.

Cytogenetic considerations. The analysis of Gymnotiformes has revealed significant cytogenetic variation between populations (Milhomem *et al*., 2008; Nagamachi *et al*., 2010). The karyotypic diversity encountered in the genus *Eigenmannia*, reflects to some extent the population structure where the formation of small aggregations with low dispersion favours the fixation of chromosome rearrangements (Moysés *et al*., 2005; Silva *et al*., 2009). The karyotype 2n = 34 (22 m/sm+12st/a) described by Moysés *et al*. (2010) for *Eigenmannia* sp. in the rio São Francisco basin is different from that of *Eigenmannia* sp. "A" (24 m/sm+10st/a) based on KF. This difference in KF could be a

result of a pericentric inversion event, which would also act as a post-zygotic isolation mechanism (King, 1993).

The CH encountered in the centromeric region of chromosomes of *Eigenmannia* sp. "A" and *Eigenmannia* sp. "B" is typical of Gymnotiformes (Silva *et al*., 2008; Milhomem *et al*., 2012a), as well as being observed in other vertebrate species (Sumner, 2003; Gomes *et al*., 2012). C bands are usually positively marked by DAPI as they are composed predominantly of A-T bases (Silva *et al*., 2009). However, in *Eigenmannia* sp. "B" the CH blocks in the interstitial regions of chromosomes 8, 9 and X were marked strongly with CMA_3 (indicating G-C rich regions), demonstrating that this region presents a distinct composition compared to other CH classes present in the autosomes.

In the genus *Eigenmannia*, the NOR is simple, frequently located on the short arm of a subtelocentric/acrocentric chromosome (Table 1), as was observed in both *Eigenmannia* sp. "A" and sp. "B" (17p and 14p, respectively). However, the species *Eigenmannia* sp. 1 and *Eigenmannia* sp. 2 (Almeida-Toledo *et al*., 1984, 1988, 2000), presented the NOR on the long arm of a metacentric chromosome, pair 10 (Table 1). As such, in order to confirm that the simple NOR is a shared character among the different species it is important to know whether the rDNA sequence sites are always located on the same chromosome in the different species, as demonstrated for *Gymnotus* gr. *carapo* (Milhomem *et al*., 2013).

In both species the NOR is positively stained by CMA_3, indicating that this region is interlaced with sequences rich in G-C bases (Nascimento *et al*., 2006; Silva *et al*., 2008; Milhomem *et al*., 2007, 2008, 2012a, 2012b). FISH with rDNA probes hybridized to different-sized regions between homologs, suggests that heteromorphism in the size of the NOR could either be associated with differences in transcription activity of the ribosomal genes or may be the result of differences in the copy numbers of the ribosomal genes (Oliveira *et al*., 2009; Milhomem *et al*., 2013).

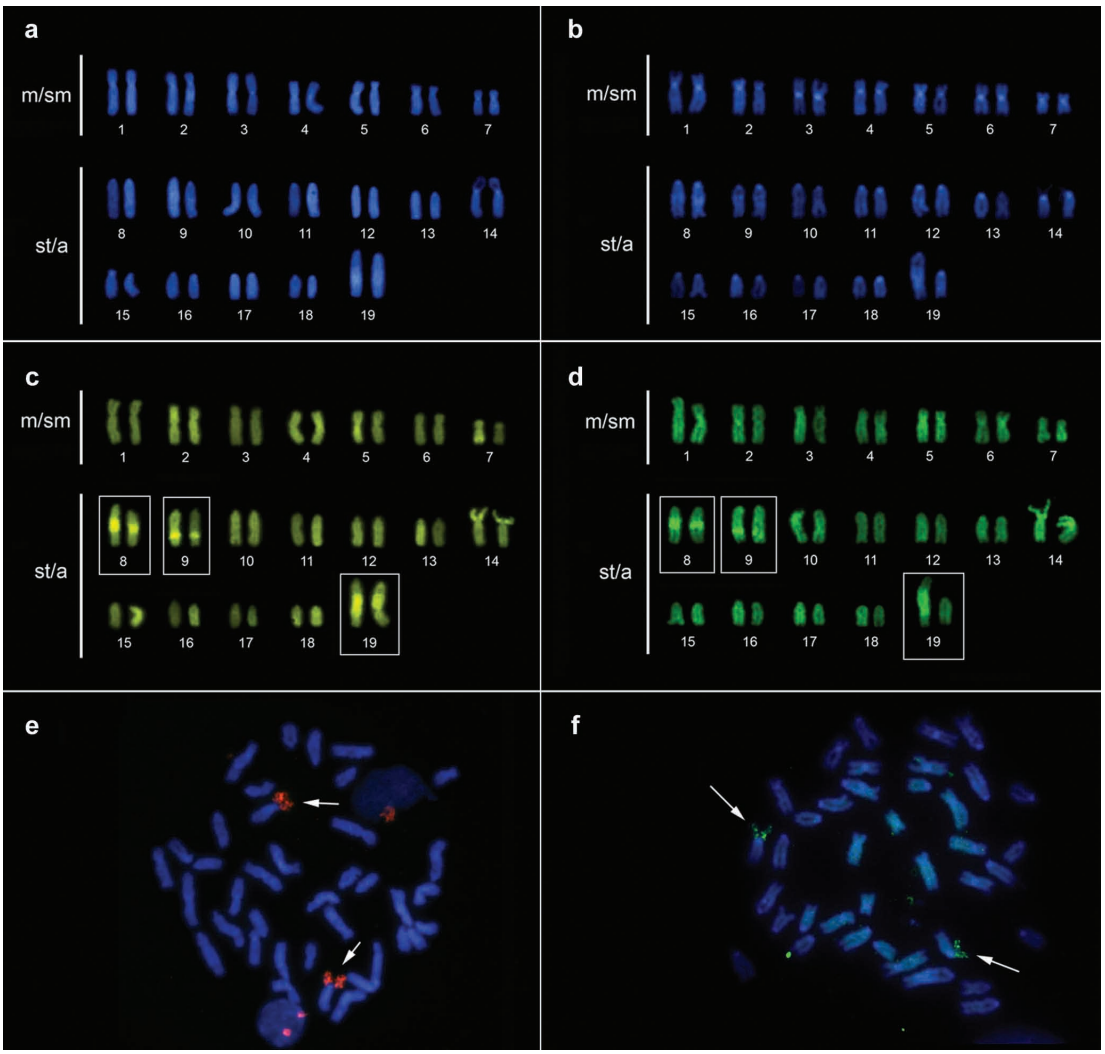

Fig. 8. DAPI fluorescent banding showing centromeric regions of both males (a) and females (b) of *Eigenmannia* sp. "B". CMA_3 fluorescent banding hybridizing areas, which coincide with the NOR (Fig. 6) on chromosome 14 of males (c) and females (d), and strong signal on pairs 8, 9 and the X – inset boxes. FISH with 18S rDNA probes of *Eigenmannia* sp. "B", hybridizing on the short arm of chromosome 14 (arrows) of males (e, f).

Sex chromosomes in *Eigenmannia*. In fishes, sex chromosomes are not present in basal taxa and their origin within genera or families is probably convergent. Additional information suggests that the origin of sex chromosomes in Neotropical fishes is recent, with diversification in some taxa approximately 7-10 Ma (Charlesworth *et al*., 2005; Cioffi *et al*., 2011; Henning *et al*., 2011).

Different sex chromosome systems have previously been described in *Eigenmannia*, including both simple XX/XY and ZZ/ZW systems and a multiple $X_1X_1X_2X_2/X_1X_2Y$ system (Table 1), suggesting that these different systems do not have a common origin. The species *Eigenmannia* sp. 2 (Almeida-Toledo *et al*., 1984, 1988, 2000) and *E. trilineata* (2n = 31/32) present the multiple $X_1X_1X_2X_2/X_1X_2Y$ system, where a centric fusion between two acrocentric chromosomes (pairs 6 and 11) in the male karyotype, resulted in a metacentric Y chromosome (neo-Y) in these species (Almeida-Toledo *et al*., 1984, 1988, 2000; Fernandes *et al*. 2010).

Karyotypes described for *E. virescens* (2n = 38) from the rio Mogi-Guaçu without sex chromosomes and karyotypes from the rio Tietê, with a simple XX/XY system (Table 1) (Almeida-Toledo *et al*., 2001), are similar to that described here for *Eigenmannia* sp. "B". Karyotypes of *Eigenmannia virescens* from the rio São Francisco, middle rio Amazonas, Island of Marajó, Abaetetuba, Belém and Benevides, present the ZZ/ZW system (Almeida-Toledo *et al*., 2002; Silva *et al*., 2009). Silva *et al*. (2009) suggest that the mechanisms involved in the differentiation of the W chromosome are the result of a pericentric inversion event in the proximal region of the short arm of an acrocentric chromosome, followed by a heterochromatization event. These events would have occurred in a distinct manner in independent populations, derived from an ancestral karyotype without differentiated sex chromosomes (Henning *et al*., 2011). It is highly possible that the sex chromosomes differentiation also contributed to species differentiation/divergence and worked as a reproductive isolation mechanism.

The events that occur during differentiation of sex chromosomes are still not well described. During the differentiation of sex chromosomes, the suppression or partial restriction of recombination between the sex determining pair of chromosomes should occur. This phenomenon is associated with the accumulation of heterochromatin on the sex chromosomes (Ohno, 1967). The absence of recombination favours the accumulation of repetitive sequences of DNA, permitting the morphological differentiation of the sex chromosomes (Almeida-Toledo *et al*., 2001; Cioffi *et al*., 2012). Such events are expected to have been involved in the morphological differentiation of the X and Y chromosomes of *Eigenmannia* sp. "B".

The investigation of the organization of repetitive sequences can provide evidence for the origin and evolution of sex chromosomes. Studies with chromosome painting, using sex chromosome specific probes and FISH with probes for repetitive DNA sequences, combined with phylogenetic analyses are fundamental to elucidate the mechanisms involved in the origin and differentiation of sex chromosomes. *Eigenmannia* represents a potential comparative model within the Gymnotiformes for such comparative analyses. The inclusion of diverse analytical methods using morphological, osteological and cytogenetic data can be extremely useful to clarify taxonomic problems in Neotropical fishes such as those encountered in *Eigenmannia*, and may well indicate further cryptic species.

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