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RESEARCH PAPER

Chromosomal and electric signal diversity in three sympatric electric knifefish species (*Gymnotus*, Gymnotidae) from the Central Amazon Floodplain

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Abstract We describe chromosomal and electric signal diversity in three sympatric species of *Gymnotus* (Gymnotidae) fish from the Central Amazon Floodplain. *Gymnotus arapaima* presents a karyotype of 2n = 44 (24 m-sm + 20st-a), *G. mamiraua* 2n = 54 (42 m-sm + 12st-a), and *G. jonasi* 2n = 52 (12 m-sm + 40st-a). No evidence for a chromosomal sexual system was observed in two species for which both males and females were analyzed (*G. mamiraua* and *G. arapaima*). In all three species the constitutive heterochromatin is located primarily in pericentromeric regions, but also at some other sites. *G. arapaima* and *G. mamiraua* exhibit simple nucleolar organizing regions (NORs) on short arms of chromosome pairs 19 and 24, respectively. *Gymnotus jonasi* exhibits a multiple interstitial NOR on the long arm of pairs 9 and 10, and on the short arm of pair 11. *G. arapaima* and *G. mamiraua* exhibit several additional similarities in their karyotypic formulas—reflecting the phylogenetic proximity of these species within a *G. carapo* group clade (based on molecular phylogenetic evidence). The chromosomal differences among these three sympatric species imply complete post-zygotic reproductive isolation. A prominent pattern of partitioning of the peak power frequency of the electric organ discharge of these three species indicates pre-zygotic reproductive isolation of mate attraction signals. We conclude by discussing the evolutionary events that may have promoted signal divergence and reproductive isolation in *Gymnotus* of the Central Amazon, and the role that chromosomal rearrangements may place in diversification.

Keywords Cytogenetics · Gymnotiformes · Reproductive isolation · Electric organ

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Introduction

Comparative cytogenetic studies of the populations of widely distributed species are beginning to be recognized as a tool for understanding the mechanisms underlying the generation of species diversity in fishes (Artoni and Bertollo 2001; Margarido et al. 2007; Milhomem et al. 2007, 2008; Silva et al. 2008, 2009; De Souza et al. 2009). Many studies point to the involvement of chromosomal rearrangements in post-zygotic reproductive isolation mechanisms (Navarro and Barton 2003; Cozzolino et al. 2004; Coghlan et al. 2005; Lukhtanov et al. 2005). In particular, several studies have used classical cytogenetic procedures to demonstrate that morphologically cryptic populations exhibit completely divergent karyotypes, implying post-zygotic reproductive isolation (Bertollo et al. 2000; Vicari et al. 2005; Milhomem et al. 2008, 2010; Silva et al. 2008). Moreover, Nagamachi et al. (2010) demonstrated with comparative genomic mapping (using chromosome painting) that the karyotype differences between two cryptic species of *Gymnotus carapo sensu stricto* are even greater than was revealed by classical cytogenetics alone (Milhomem et al. 2008)—suggesting that levels of cryptic diversity based on chromosomal divergence may be even greater than are currently recognized.

The nocturnally-active electric knife fish genus *Gymnotus* is an excellent model group for exploring the relative contributions of post-zygotic and pre-zygotic reproductive isolation to speciation and diversification. In addition to exhibiting extraordinary karyotypic diversity (Table 1) (including relative to some other knife fish groups, see e.g. Cardoso et al. 2011, for Rhamphichthyoidea), *Gymnotus*, like all gymnotiforms, employ an electric communication system that facilitates quantitative analysis of the extent to which populations or species are likely to be reproductive isolated at the pre-zygotic level. The electric communication signals of *Gymnotus* comprise pulsed, weak (<2 V) electric organ discharges (EODs) generated from a hypaxial electric organ. In combination with a cutaneous array of electroreceptors, these EODs permit both intra- and inter-specific electrocommunication, and also the location of nearby objects, including prey items (electrolocation) (Bullock et al. 2005). The electric communication modality of electric fishes is the dominant communication modality; olfactory and visual systems are substantially reduced, and *Gymnotus* is known to recognize individuals based on electric cues alone (McGregor and Westby 1992; Crampton and Albert 2006). Moreover, studies have documented that species, which co-occur in geographical sympatry, exhibit non-overlapping spectral and or temporal properties of their pulsed EODs, especially in mature individuals—indicating pre-zygotic reproductive isolation (Crampton 2006; Crampton et al. 2008, 2011).

Gymnotus is a monophyletic group, whose interspecific phylogenetic relationships are well known, and which is distributed in lowland freshwaters from southern Mexico to northern Argentina (Albert et al. 2005; Lovejoy et al. 2010). It is common to find multiple *Gymnotus* species co-occurring in local Neotropical fish communities, where invariably EOD parameters are partitioned among species (Crampton and Albert 2006). The highest known diversity of the genus has been recorded from the region of Tefé, Brazil, in the Central Amazon. Here 12 species were documented by W. Crampton during 7 years of multi-habitat and multi-season field work. Nine species are known from whitewater floodplain habitats in this area, primarily from the Mamirauá Reserve at the confluence of the Rio Solimões (Amazon) and Rio Japurá: *G. arapaima, G. carapo, G. jonasi, G. mamiraua, G. melanopleura, G. obscurus*, *G. onca, G. tigre,* and *G. varzea* (Crampton et al. 2011). This high diversity of *Gymnotus* from whitewater floodplains of the Tefé region is typical of the high levels of aquatic species richness and habitat-specialization found in Amazonian whitewater floodplains (Henderson et al. 1998; Queiroz 2005; Crampton 2011). Three additional species are restricted to terra firme stream systems of the Tefé region: *G. coatesi, G. coropinae,* and *G. curupira* (Crampton and Albert 2003, 2004; Crampton et al. 2005). Of these 12 species, four (*G. carapo*, *G. melanopleura*, *G. onca*, and *G. tigre*) are known only from a single immature specimen, or small numbers of immature specimens, and probably represent vagrants from upstream regions of the Amazon basin. The remaining eight species form resident, breeding populations in the area.

Despite the opportunities for simultaneously exploring pre- and post-zygotic reproductive isolating barriers in *Gymnotus*, studies that have combined observations of EOD variation and karyotypic variation are lacking. Here we present a karyotypic analysis of three species of *Gymnotus* (*G. arapaima*, *G. jonasi*,

Table 1 Summary of karyotypes from the electric knife fish genus *Gymnotus* published in the cytogenetic literature

Species	2n and FC	CB	NOR	Localities	References
Gymnotus carapo (female)	54 (54 m/sm)	−	2 p (m/sm)	Miracatu, São Paulo	1
G. carapo (male and female)	54 (54 m/sm)	−	2 p (m/sm)	Botucatu, São Paulo	1
G. carapo (male and female)	52 (50 m/sm + 2st/a)	−	2 p (m/sm)	Brotas, São Paulo	1
G. carapo	48 (34 m/sm + 14st/a)	−	–	Humaitá, Amazonas	1
G. carapo (male and female)	42 (32 m/sm + 10st/a)	−	18 p (st/a)	Belém, Pará	1
G. carapo (male)	81 (66 m + 12sm + 3st/a)	−	1 p (m)	Rio Mogi-Guaçu, São Paulo	2
G. carapo (male and female)	54 (52 m/sm + 2st/a)	−	1 p (m)	Coastal drainages of South Eastern Brazil	3–5
G. carapo	54 (52 m/sm + 2st)	+	1 p (m/sm)	Rio Grande, São Paulo	6
G. carapo (male and female)	42 (30 m/sm + 12st/a)	+	20 p (st/a)	Santa Cruz do Arari, Ponta de Pedras, São Miguel do Guamá, Capanema, Benfica, Pará	7, 8
G. carapo (male and female)	40 (28 m/sm + 12st/a)	+	19 p (st/a)	Almeirim, Pará	8
Gymnotus sylvius (male and female)	40 (28 m + 10sm + 2st/a)	−	18 p (sm)	Coastal drainages of South Eastern Brazil	3
G. sylvius (male and female)	40 (30 m/sm + 10st/a)	−	4 p (m/sm)	Miracatu e São Simão, São Paulo	9
G. sylvius	40 (38 m/sm + 2st)	+	2 (m/sm)	Rio Grande, São Paulo	6
G. sylvius (male and female)	40 (36 m/sm + 4st/a)	+	19 p (st)	Alfenas, Minas Gerais	10
G. sylvius (male and female)	40 (36 m/sm + 4st/a)	+	20 p (st/a)	Guaíra, Paraná	11
Gymnotus mamiraua (female)	54 (50 m/sm + 4st/a)	+	1 p (m/sm)	Santa Cruz do Arari, Pará	7
Gymnotus inaequilabiatus (male and female)	52 (40 m + 10sm + 2sta)	−	23 p (sm)	Coastal drainages of South Eastern Brazil	3
Gymnotus paraguensis (Macho e Fêmea)	54 (52 m/sm + 2st)	+	27 p (st/a)	Guaíra, Paraná	11
G. paraguensis (male and female)	54 (50 m/sm + 4st/a)	+	1 p (m/sm)	Alfenas, Minas Gerais	10
Gymnotus capanema (male and female)	34 (20 m/sm + 14st/a)	+	15 p (st-a)	Capanema, Pará	12
Gymnotus pantherinus (male and female)	52 (38 m + 8sm + 6st/a)	−	24 (st)	Paraná River, São Paulo	13
G. pantherinus (male and female)	52 (38 m + 8sm + 6st/a)	−	24 p (st/a)	Coastal drainages of South Eastern Brazil	3
Gymnotus pantanal (male and female)	40 (14 m/sm + 26st/a)	+	19 p + 1 do 1 q	Pantanal Matogrossense, Mato Grosso do Sul	14
G. pantanal (female)	40 14 m/sm + 26st/a)	+	16 p (a)	Guaíra, Paraná, Brazil	11
G. pantanal (male)	39 15 m/sm + 24st/a)				
Gymnotus sp. (female)	52 (50 m/sm + 2st/a)	−	–	Miracatu, São Paulo	1
Gymnotus sp. (male)	39 x_1x_2y (15 m/sm + 24a)	+	16 p (a)	Rio Paraná, Paraná	15, 16
Gymnotus sp. (female)	40 (14 m/sm + 26a)				
Gymnotus sp.	54 (48 m/sm + 6a)	+	1 p (m/sm)	Rio Paraíba do Sul, Rio de Janeiro	6

Table 1 continued

Species	2n and FC	CB	NOR	Localities	References
Gymnotus sp. (male)	50 (26 m/sm + 24st/a)	+	19 p (st)	Alfenas, Minas Gerais	10

Localities list region or municipality followed by state, or drainage region

Abbreviations: 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: (+) technique used in the karyotype analysis, (−) = technique not used in the karyotype analysis. All localities are in Brazil

References: 1—Foresti et al. (1984), 2—Fernandes-Matioli et al. (1998a), 3—Fernandes-Matioli et al. (1998b), 4—Fernandes-Matioli and Almeida-Toledo (2001), 5—Fernandes-Matioli et al. (1997), 6—Claro (2008), 7—Milhomem et al. (2007), 8—Milhomem et al. (2008), 9—Albert et al. (1999), 10—Lacerda and Maistro (2007), 11—Margarido et al. (2007), 12—Milhomem et al. (2011), 13—Marchetto et al. (1998), 14—Fernandes et al. (2005), 15—Sánchez et al. (2004), 16—Silva and Margarido (2005)

and *G. mamiraua*) from the central Amazon floodplain in the Mamirauá Reserve, and complement this with an analysis of the EODs of sexually mature males and females of each species (which serve as mate-attraction signals). We will demonstrate that these species of *Gymnotus* exhibit karyotypes that are reproductively incompatible, hence enforcing strict post-zygotic reproductive barriers. We will also demonstrate largely non-overlapping spectral and temporal EOD parameters among these three species, which imply that errors in mate recognition during reproduction are unlikely. Consequently, we hypothesize that the EODs, and associated mate preferences, of these species likely serve as pre-zygotic reproductive isolating barriers. Based upon our findings and the evolutionary history of *Gymnotus*, we will discuss scenarios for the evolution of pre and post-zygotic reproductive isolating barriers.

Materials and methods

Gymnotus specimens were collected from floating rafts of macrophytes along the margins of floodplain channels and lakes in the Mamirauá Sustainable Development Reserve (Mamirauá Reserve), Amazonas, Brazil, an area of floodplain forest at the confluence of the Solimões and Japurá rivers (Fig. 1). Cytogenetic analyses were conducted on 12 specimens of *G. arapaima* (10 males and 2 females), 23 specimens of *G. mamiraua* (10 males and 2 females), and 2 specimens (both females) of *G. jonasi*. These three species exhibit distinct morphologies (see original descriptions by Albert and Crampton 2001) and are phylogenetically distinct from each other, and from other resident sympatric species. *G. arapaima* and *G. mamiraua* are relatively phylogenetically proximate—both belonging to the "*G. carapo* group clade" *sensu* Lovejoy et al. (2010), while *G. jonasi* belongs to a distant "G1 clade". We present data for the species in the order *G. arapaima*, *mamiraua*, and *jonasi* in order to consider first those belonging to the *G. carapo* clade. We previously collected and performed cytogenetic analyses on specimens of *Gymnotus* cf. *mamiraua* from an additional site in the Eastern Amazon, near Santa Cruz do Arari (Milhomem et al. 2007; see Fig. 1).

Chromosomal preparations followed the methods described by Bertollo et al. (1978). In brief a live fish was administered an intraperitoneal injection of a 1 ml/100 g solution of active yeast to stimulate

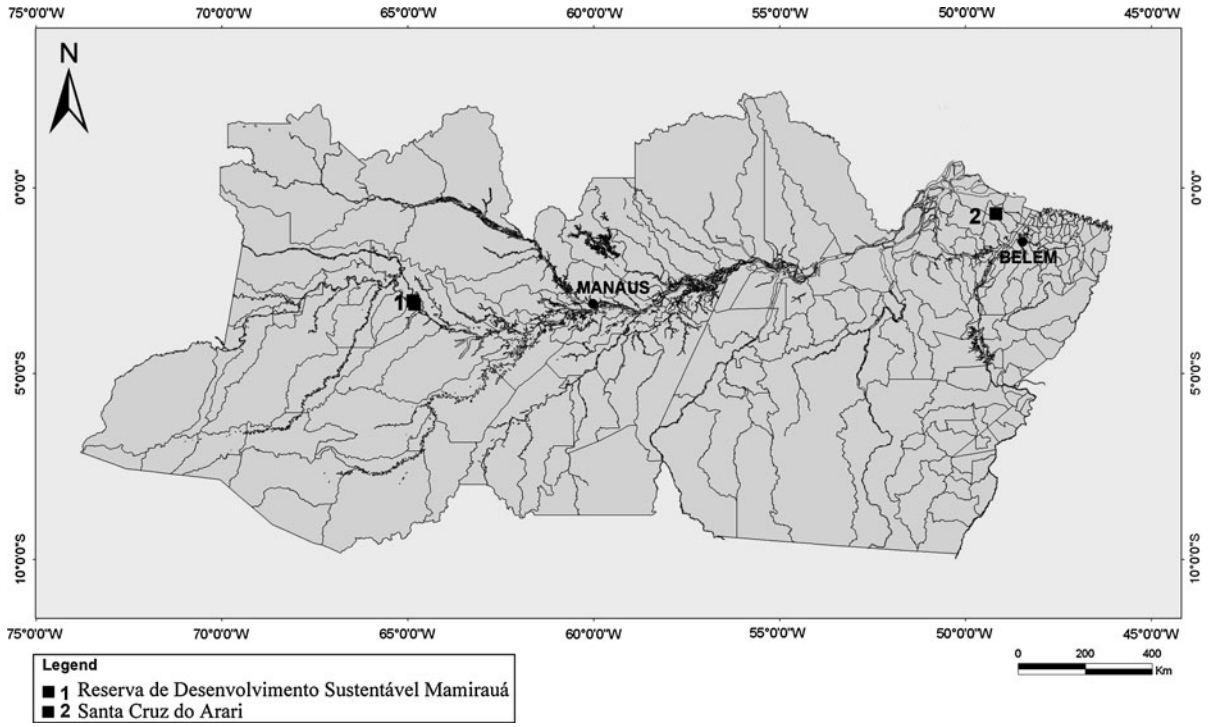

Fig. 1 Map illustrating location of the Mamirauá Reserve (Reserva de Desenvolvimento Sustentável Mamirauá) in Amazonas, Brazil and a site in the Eastern Amazon at Santa Cruz do Arari, Ilha do Marajó, Pará, Brazil, where *Gymnotus mamiraua* was previously sampled and subjected to cytogenetic analysis (see Milhomem et al. 2007)

Table 2 Summary of karyotypes and capture localities for three sympatric species of *Gymnotus* from the Central Amazon floodplain of Brazil

Species	IDSM lot	2n and KF	NOR	Coordinates
G. mamiraua	Ictio000773	54 (46 m-sm + 8st-a)	25 p (m/sm)	03°07′50.3″S 064°48′26.4″W
G. arapaima	Ictio000800	44 (26 m-sm + 18st-a)	20 p (m/sm)	03°02′11.8″S 064°51′16.6″W
	Ictio000805			
G. jonasi	Ictio000802	52 (12 m-sm + 40st-a)	9 q, 10 q, 11 p (st/a)	03°02′49.1″S 064°51′02.2″W

Abbreviations: 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

mitosis. Subsequently a 0.025% colchicine solution was injected in the proportion of 0.5 ml/100 g of body weight. 45 min later the fish was euthanized and the visceral cavity opened for the removal of the kidneys. The kidneys were macerated in a hypotonic solution of 0.075 M KCl and incubated at 37°C for 30 min. The cell solution was then suspended in a fixative (3:1 methanol:acetic acid) and centrifuged twice. The resulting pellet was then suspended in fresh fixative and dropped onto warmed slides. The slides were analyzed after conventional Giemsa staining, C-banding (Sumner 1972), Ag-NO_3 staining (Howell and Black 1980), CMA_3 staining (Schweizer 1980), DAPI staining (Pieczarka et al. 2006), and fluorescent in situ hybridization (FISH) with 18S rDNA probes from the Neotropical characiform fish *Prochilodus argenteus* (Hatanaka and Galetti Jr. 2004). The chromosomes were then classified following the scheme of Levan et al. (1964). All analyzed specimens were fixed with 10% formalin, preserved in 70% ethyl alcohol, and deposited in a voucher collection at the Instituto de Desenvolvimento Sustentável Mamirauá (IDSM) (Table 2). Sex determination was based on examination of the gonads with a stereomicroscope via a slit

opened with a scalpel along the ventral surface of the body cavity. Testes are a solid white or pink-white color, while ovaries have visible oocytes, which become more yellow with development. For the purposes of EOD analyses, mature specimens were classified as individuals with testes or ovaries at stages 2 or more, with a modified version of the Nikolsky scale (see Crampton et al. 2011, Suppl. Appendix 1). Immature adults were classified as individuals that exceeded the minimum size for reproduction for a given species, but which present resting or undeveloped gonads (stage 0 or 1 in the modified version of Nikolsky's scale). EOD data for juvenile specimens (individuals with stage 0 or 1 gonads that are below the minimum size for reproduction of a given species) are not presented here.

EOD recordings were taken from live specimens in the Mamirauá Reserve by W. Crampton during the period 1993–2002, and following the methodology of Crampton et al. (2008) and (2011). In brief, fishes were recorded within 48 h of capture in a nylon mesh envelope suspended in the center of an 88 × 37 cm insulated tank filled to a depth of 34 cm (114 l cooler). Water temperature was standardized to 27.0 ± 0.1°C, and conductivity to 55 ± 1 μScm^{-1}. Recordings were made from at least 1 h after sunset to 3 am, in near darkness, following acclimation in the tank for 5–15 min. Single head-to-tail EOD recordings were taken from tank-end Ag/Ag-Cl or NiCr electrodes, using an AC-coupled amplifier (CWE instruments model BMA-200, Ametek model SR-5113 [DC—30 kHz], or custom built—see Wells and Crampton 2006 [0.01 Hz–30 kHz]), and digitized with an analog–digital converter at 48–250 kHz (National Instruments model 6052E, Edirol model UA5, or Sony model TCD7/TCD8 digital audio tape recorder). Fishes with damaged or regenerated tails were excluded. Following recordings, specimens were euthanized humanely, fixed with 10% formalin, and measured for total length and weight before preservation in 70% ethanol. Peak power frequencies (PPFs) were calculated by Fast Fourier Transform using custom MATLAB software (The MathWorks, Natic, MA).

Results

Around 30 metaphase plates were examined for each specimen—revealing three distinct diploid numbers and karyotypes, corresponding to the three species: *G. arapaima* with 2n = 44 and karyotypic formula (KF) 24 m/sm + 20st/a (Fig. 2), *G. mamiraua* with 2n = 54 and KF = 42 m/sm + 12st/a (Fig. 3), and *G. jonasi* with 2n = 52 and KF = 12 m/sm + 40st/a (Fig. 4). Species in which males and females were analyzed (*G. arapaima* and *G. mamiraua*) did not exhibit sex-related chromosomal differences. We were unable to determine whether sex-related chromosomal differences occur in *G. jonasi* because we only encountered females (n = 2) of this species.

In all three species the constitutive heterochromatin (CH) occurs in the pericentromeric regions of the majority of the chromosome pairs (Figs. 2B, 3B, 4B). In *G. arapaima* (Fig. 2B) blocks were observed in the proximal region of the long arm of pair 1, on the entire

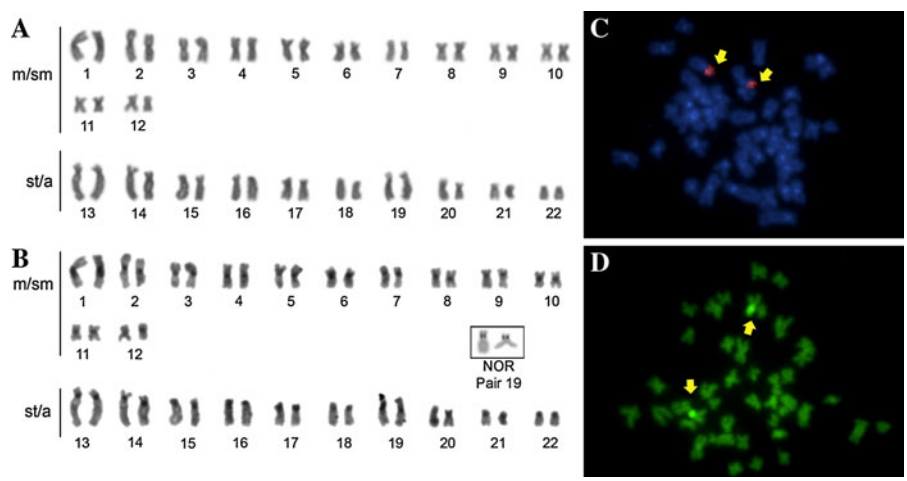

Fig. 2 Karyotype of *Gymnotus arapaima* from the Mamirauá Reserve, Amazonas, Brazil. **A** Conventional Giemsa-stained karyotype; **B** C-banding and NOR; **C** FISH with 18S rRNA probe and DAPI. **D** CMA_3. *Arrows* indicate the location of the NORs

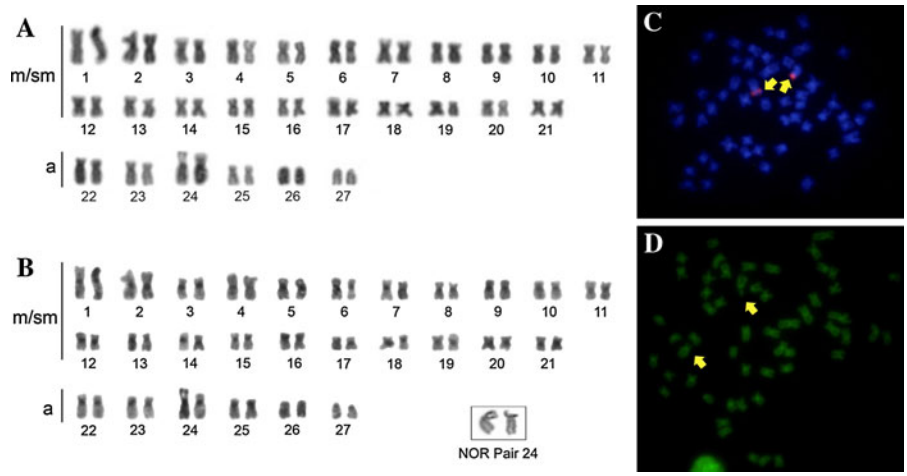

Fig. 3 Karyotype of *Gymnotus mamiraua* from the Mamirauá Reserve, Amazonas, Brazil. **A** Conventional Giemsa-stained karyotype; **B** C-banding and NOR; **C** FISH with 18S rRNA probe and DAPI. **D** CMA_3. *Arrows* indicate the location of the NORs

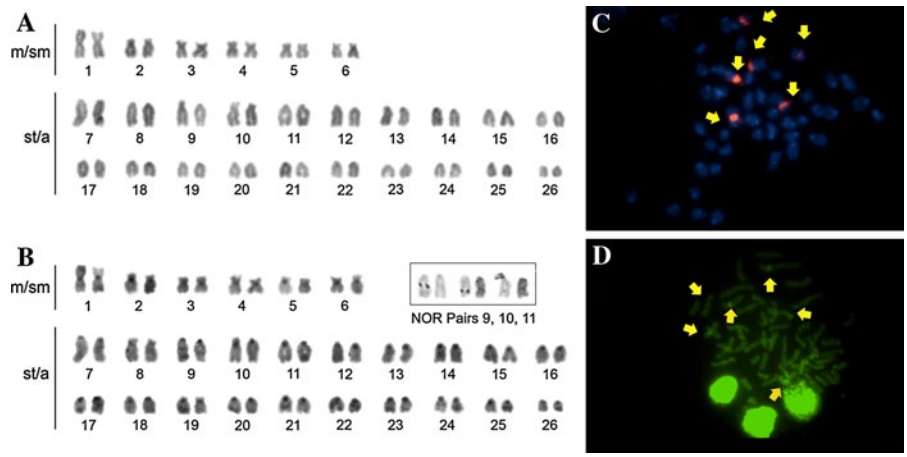

Fig. 4 Karyotype of *Gymnotus jonasi* from the Mamirauá Reserve, Amazonas, Brazil. **A** Conventional Giemsa-stained karyotype; **B** C-banding and NOR; **C** FISH with 18S rRNA probe and DAPI. **D** CMA_3. *Arrows* indicate the location of the NORs

short arm of pair 17, and in the distal region of the long arm of one of the chromosomes of pair 20. In *G. jonasi* (Fig. 4B), conspicuous blocks were observed on the long arms of pairs 7, 9 10, 16, 17, 21, 24 and 25. In all three species the CH regions are DAPI positive (Figs. 2C, 3C, 4C) indicating that they are rich in AT base pairs.

In *G. arapaima* the nucleolar organizing region (NOR) (revealed by Ag-NO_3 staining, FISH and CMA_3) occurred in the short arms of pair 19 (Fig. 2B–D). In *G. mamiraua* it was observed only by Ag-NO_3 staining and FISH in the short arms of pair 24 (Fig. 3B, C). In *G. jonasi*, Ag-NO_3 staining (Fig. 4B) revealed 3–5 NOR sites. However, FISH and CMA_3 (Fig. 4C, D) revealed six NOR sites, on the long arm of pairs 9 and 10, and on the short arm of pair 11.

The PPFs of the EODs of mature males, mature females and immature adults of each of the three species are presented in Fig. 5, along with the original EOD waveforms from each of these nine categories. All three species generate tetraphasic EODs in which the second (positive) and third (negative) phases are dominant. The PPFs of the three species overlap only slightly at each of the three developmental categories (mature male, mature female, immature adult), and exhibit no overlap at the interquartile range around the median PPF. The predominant pattern is therefore one of partitioning of PPF among the three species. For each development category *G. jonasi* exhibits the highest PPF (corresponding to the shortest EODs) and *G. mamiraua* the lowest PPFs (corresponding to the longest EODs).

Discussion

Gymnotus arapaima

Here we document the karyotype of *G. arapaima* (Fig. 2) for the first time, revealing a diploid number

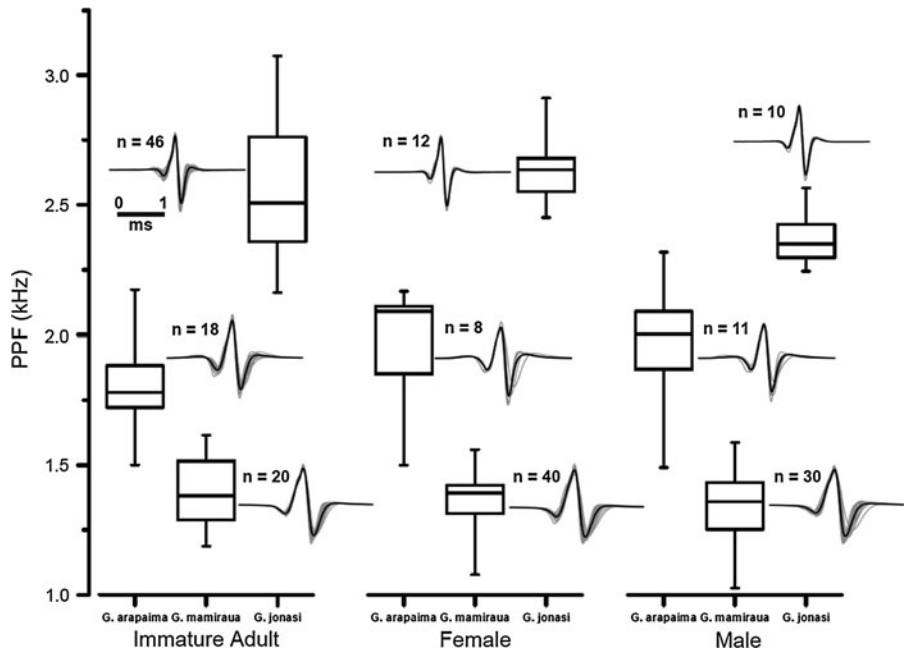

Fig. 5 Peak power frequency ranges for the pulsed electric organ discharges (EODs) of three species of sympatric *Gymnotus* from the Mamirauá Reserve: for immature adults, mature females and mature males. *Box plots* show range (*whiskers*), interquartile range (*box*) and median (*horizontal bar*) and are aligned vertically over the species labels on horizontal axis. The *inset* waveforms show individuals recordings (*grey*) and an averaged waveform for each group (*black*)

and karyotypic formula hitherto not documented for the genus (2n = 44) (24 m/sm + 20st/a). The molecular phylogeny of Lovejoy et al. (2010) places *G. arapaima* within a clade comprising the *G. carapo* species complex, *sensu-stricto* (see Albert and Crampton 2003 for definition—a group comprising *G. arapaima*, *G. ucamara*, and several cryptic species currently named *G. carapo*), from which it is distinguished primarily by possessing more rows of scales over the pterygiophores bones, and by attaining a larger body size (Albert and Crampton 2003). Cytogenetic studies of species from the *G. carapo* species-complex exhibit tremendous karyotypic diversity, with the diploid number varying from 34 to 54 (Table 1). This karyotypic diversity reinforces the notion that the *G. carapo* species-complex (including *G. arapaima*) is indeed a complex of reproductively isolated species, many of which are likely to be isolated at the post-zygotic level by chromosomal incompatibility. The diploid number of *G. arapaima* is within the range of other species in the *G. carapo* species-complex, but its karyotype is distinct in exhibiting a smaller number of bi-armed chromosomes.

Gymnotus mamiraua

The karyotype we document here for *G. mamiraua* in the Mamirauá Reserve (2n = 54, 42 m/sm + 12st/a) (Fig. 3) exhibits the same diploid number as specimens identified as *G.* cf. *mamiraua* from the eastern Amazon at Santa Cruz do Arari, Ilha do Marajó, Pará state, Brazil (Milhomem et al. 2007; Fig. 1 for location). Nonetheless, the form from the Eastern Amazon exhibits a distinct karyotypic formula (50 m/sm + 4st/a), suggesting chromosomal rearrangements, such as pericentric inversion and translocations. These two populations also differ in the composition of their NORs. *G.* cf. *mamiraua* from the eastern Amazon has interspaced segments of NOR rich in G–C base pairs with positive CMA_3. In contrast, *G. mamiraua* from the Mamirauá Reserve does not exhibit positive coloration for CMA_3 or DAPI, suggesting that the interspaced sequences between the ribosomal genes do not form A–T or G–C-rich clusters. Recent observations demonstrated cryptic species diversity based on chromosomal differences in the *G. carapo* species-complex (Milhomem et al. 2008; Nagamachi et al. 2010). It is therefore possible that the Central and Eastern Amazon forms of *G. mamiraua* may be reproductively isolated species, with the karyotypic difference representing a post-zygotic barrier to reproductive isolation, rather than intra-specific chromosomal polymorphism, since both are monomorphic for their respective karyotype. Alternatively, it is possible that these two populations represent the extremes of a geographical cline

polymorphism in chromosome structure (or other type of polymorphism); the westernmost and easternmost known localities for *G. mamiraua* are the Tefé region and Ilha do Marajó respectively (see Fig. 1). An investigation of the chromosomal composition of geographically intermediate populations, and an increase in sample sizes will be necessary to understand further this phenomenon.

Gymnotus jonasi

Here we also describe the karyotype of *G. jonasi* for the first time (Fig. 4). Its diploid number (2n = 52) is coincident with four other species that are phylogenetically disparate from *G. jonasi*—occurring well outside the "G1 clade" of small-bodied species to which *G. jonasi* belongs. These four species are: *G. carapo* from Brotas—São Paulo state, Brazil; *G.* sp. from Miracatu—São Paulo state; *G. inaequilabiatus* from southeastern coastal drainages of Brazil; and *G. pantherinus* from the Rio Paraná—Paraná state, Brazil. Nonetheless, while the diploid number of *G. jonasi* is coincident with these four species its KF (12 m/sm + 40st/a) is distinct. Three species: *G. carapo* from Brotas—São Paulo; *G.* sp. from Miracatu—São Paulo; and *G. inaequilabiatus* from southeastern coastal drainages of Brazil have a KF of 50 m/sm + 2st/a. In contrast, *G. pantherinus* from Rio Paraná, has a KF of 46 m/sm + 6st/a (see references in Table 1).

Gymnotus jonasi is unusual among all congeners in possessing the highest number of st/a chromosomes (40 in total), followed in second place by *G. pantanal* with 26 (Fernandes et al. 2005; Margarido et al. 2007). *G. jonasi* and *G. pantanal* (Fernandes et al. 2005) are unique among congeners in exhibiting multiple NORs. Although both *G. jonasi* and *G. pantanal* belong to the *G. pantherinus* group constructed by Albert et al. (2005) based on morphological data (a group which is being deconstructed by molecular analyses), the phylogenetic distance between these species is yet to be appraised by molecular analyses. Whether the unusual NOR condition of *G. jonasi* and *G. pantanal* is common to all members of the *G. pantherinus* group *sensu* Albert et al. (2005) or, alternatively, evolved independently, will require the cytogenetic profiling of additional species from this clade, and also resolution of phylogenetic uncertainties. Nonetheless, the unusual NOR of *G. jonasi* and *G. pantanal* clearly indicates that the NOR is not highly conserved in *Gymnotus*, as was previously postulated (Fernandes-Matioli et al. 1998b; Silva and Margarido 2005). In *G. jonasi* Ag-NO_3 staining shows 3–5 sites NOR but FISH demonstrated 6 NOR places.

Cytogenetic diversity and species diversification

The CH was similar in the three species examined here—occurring, as is the case in most other congeners, in the pericentromeric region of the chromosomes, and also exhibiting some unusual marking patterns (Milhomem et al. 2007, 2008). Also in all three species, the data obtained from CMA_3 and FISH allowed us to identify the active and inactive sites of the NOR and to demonstrate that the NOR is rich in G–C base pairs.

The data presented here are consistent with the growing notion (supported by the extensive classical cytogenetic literature, see Table 1) that *Gymnotus* is characterized by considerable chromosomal diversity (Foresti et al. 1984; Margarido et al. 2007; Milhomem et al. 2008, 2011). Using chromosome painting, Nagamachi et al. (2010) demonstrated the presence of multiple chromosomal rearrangements differentiating two cryptic species of *G. carapo*. Moreover, these authors demonstrated that the amount of genomic reorganization greatly exceeded estimates based on classical cytogenetic techniques for the same species (see Milhomem et al. 2008). Hence, classical cytogenetic techniques have apparently substantially underestimated the amount of chromosomal diversity in *Gymnotus*, and possibly in other fishes.

The three species studied here occur sympatrically and syntopically in the whitewater floodplain habitats of the Mamirauá Reserve. Two additional species form breeding communities in these habitats (*G. obscurus* and *G. varzea*) but are relatively rare. Here we note a general pattern of partitioning of the PPF of the EOD (i.e. the dominant frequency component) among not only males of the three species considered here, but also among females and immature adults (Fig. 5). Several studies have documented close matches between the PPF of electric fish EODs and the tuning of the tuberous electroreceptors involved in electrolocation and electrocommunication (e.g. Hopkins 1976; Watson and Bastian 1979). Divergences in PPF could therefore, in principle, be the evolutionary

consequence of reproductive character displacement in response to "reproductive interference"—in which peak power frequency (and also electroreceptor tuning and female mate preferences) diverge so as to either reduce the potential for costly interspecific reproductive encounters among species with confusingly similar signals (i.e. mismating) or to mitigate sensory masking (i.e. jamming) among either reproductive or non-reproductive individuals belonging to different species. Crampton et al. (2011) noted that when *G. obscurus* and *G. varzea* are included in signal analyses, there is considerable overlap in PPF among males and females of whitewater floodplain dwelling *Gymnotus*, from the Central Amazon (note also some overlap among the three species considered here, Fig. 5). However, a combination of PPF (spectral EOD properties) and waveform shape (temporal EOD properties) was in all cases completely non-overlapping among mature males and females, and also immature adults of these five syntopic species, but not smaller juvenile specimens (Crampton et al. 2011, Figure 5).

Crampton et al. (2011) documented an unusual form of reproductive character displacement in this community, driven specifically by the costs of mismating, where the signals of species which are close-by in signal space prior to maturation move further apart during maturation than do the signals of species which are already well-spaced prior to maturation. This phenomenon was generalized to waveform shape (which includes spectral properties of the EOD) but not to PPF itself (ruling out reproductive character displacement driven in this context by masking interference).

Crampton et al. (2011) also considered the evolutionary time frame over which this unusual form of reproductive character displacement might evolve. Because the *Gymnotus* species of the Tefé region form a polyphyletic assemblage of relatively old species (and not an in situ species radiation), they were likely dispersal-assembled, incrementally, over long periods of geological time, following speciation in allopatry. Therefore, the kinds of signal divergences observed to occur during maturation are likely a post-speciation phenomenon involving species that no longer exchange genes via hybridization events. Here the costs of interspecific mismating must include the loss of energy, resources or gametes during fruitless courtship and breeding episodes among heterospecifics (and not the costs of hybridization). In support of this supposition, hybrid phenotypes (both morphological and in terms of waveform) were not found in the region.

The three species considered in this paper are all phylogenetically distinct species. *G. arapaima* is relatively close to *G. mamiraua* (*G. mamiraua* is sister taxon to the *G. carapo*-species complex, which together form the sister taxon to a clade comprising *G. curupira*, *G. obscurus, G. tigre* and *G. varzea* inside the wider "*G. carapo* group clade"—see Lovejoy et al. 2010). *G. jonasi*, a representative of the G1 clade is distantly related to the *G. carapo* group clade members. Divergence time estimates indicate that *G. jonasi* and the *G. carapo group* clade diverged well before the Miocene, while *G. carapo* and *G. mamiraua* likely diverged subsequent to the late Miocene.

The non-overlapping properties of signals among *G. carapo, G. mamiraua* and *G. jonasi* involve not only mature specimens but also immature ones. These divergent patterns may have evolved in response to selection for reproductive character displacement although this cannot be tested with the ontogenetic test for reproductive character displacement described by Crampton et al. (2011) (and see also Crampton et al. 2011 for the difficulty of applying the classical geographical test for reproductive character displacement in cases of community-wide signal partitioning). Alternatively, the signals may have drifted with phylogenetic divergence, before contact in sympatry. Additionally, signals might be divergent due to sensory drive, where signal structure is correlated to physical aspects of the environment, and where co-existing species occur in different microhabitats (however, see Crampton et al. 2011 for arguments against sensory drive in electric fish).

The role that karyotypic differences play in speciation and in promoting reproductive isolation in *Gymnotus* is as yet unclear. The likely sequence of events involving community assembly, and reproductive character displacement of electric signals for *Gymnotus* species of the Tefé region suggests that these species probably accrued post-zygotic reproductive isolation independently of (and likely prior to) pre-zygotic reproductive barriers involving signals. The recent studies of cryptic diversity within the *G. carapo* species complex (Milhomem et al. 2008; Nagamachi et al. 2010) indicate that chromosomal rearrangements could lead to relatively rapid post-zygotic reproductive isolation among forms that have

not had time to diverge morphologically. In future research we will explore whether pairs of morphologically cryptic species in the *G. carapo* species-complex exhibit electric signal divergence in areas of sympatry relative to areas where they occur in allopatry, as is predicted to occur under a model of reproductive character displacement. Such studies will clearly need to be placed in the phylogenetic context, by incorporating all taxa into existing molecular phylogenetic hypotheses (Lovejoy et al. 2010), and by including population-level comparisons.

The high species, karyotypic, and electric signal diversity in *Gymnotus*—along with the now relatively large published data set on cytogenetics (reviewed in Table 1), signal diversity (reviewed in Crampton et al. 2011), alpha-taxonomy (reviewed in Crampton and Albert 2006), phylogenetic systematics (reviewed in Lovejoy et al. 2010), and electrophysiology (reviewed in Rodriguez-Cattaneo et al. 2008), make *Gymnotus* a particularly strong model group for these kinds of investigations.

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