Non-homologous sex chromosomes in two species of the genus *Eigenmannia* (Teleostei: Gymnotiformes)

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Abstract. The Neotropical genus *Eigenmannia* is a fish group with unknown species diversity where representatives possess a broad range of chromosomal sex determining systems namely XY/XX, X₁X₂Y/X₁X₁X₂X₂, ZZ/ZW as well as homomorphic sex chromosomes. To test the homology of two heteromorphic XY sex chromosome systems present in two sympatric populations, reciprocal cross-species FISH experiments were performed using probes de-

rived by microdissection of X and Y chromosomes present in analyzed specimens of *Eigenmannia virescens* and *Eigenmannia* sp.2, respectively. While X and Y paint probes hybridized to species-specific sex chromosomes, in reciprocal cross-FISH both probes hybridized exclusively to autosomes. The result suggests multiple independent origins of the XY systems in the analyzed populations.

Although conserved in groups such as mammals, birds and snakes, chromosomal sex determination systems have evolved independently in most major groups (Matsubara et al., 2006; Vallender and Lahn, 2006; Kawai et al., 2007). Broad-scale phylogenetic character mapping of sex determining mechanisms in Teleostei has revealed that only a minority of orders display a single sex determination system (Devlin and Nagahama, 2002; Mank et al., 2006). This led Mank et al. (2006) to conclude that 'most if not all of the broad categories of sex determination in teleosts had multiple evolutionary origins'. Notably, alternative chromo-

some systems can coexist in the same genus (Takehana et al., 2007a, b) and even in the same species of fish (Volf and Schartl, 2001).

The Neotropical weakly-electric eel *Eigenmannia* comprises a group of unresolved species presenting high diversity of karyotype constitutions and a broad range of chromosomal sex determining systems. In the two major river basins of the state of São Paulo (Brasil), several populations of *Eigenmannia virescens* bearing different karyotypes have been described, including two populations displaying heteromorphic sex chromosomes. These populations present overlapping distributions that have been referred to as *E. virescens* (2n = 38, XY/XX) and *E. sp.2* (2n = 31, X₁X₂Y/32, X₁X₁X₂X₂) (Almeida-Toledo and Foresti, 2001).

E. virescens (2n = 38, XY/XX) possesses an acrocentric X chromosome with a heterochromatinized distal region highly enriched with GC-rich sequences (Almeida-Toledo et al., 2001). Heterochromatinization has been suggested to play a central role in early instances of sex chromosome differentiation due to effects such as inhibition of recombination and position effect variegation (McKee and Handel, 1993; Hennig, 1999; Griffin et al., 2002). The Y chromosome

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Fig. 1. FISH of sex chromosome probes (a) EVX on a male specimen of *E. virescens* ($2n = 38, XY$) and (c) E2Y on a male specimen of *E. sp.2* ($2n = 31, X_1X_2Y$). Sex chromosomes are indicated on DAPI counterstained negative images (b and d). Note the homology of sex chromosomes X-Y in (a) and Y- X_1X_2 in (c). Note also the complex pattern of the X-specific heterochromatin in (b). The heterochromatic q arm of chromosome 8 (following Almeida-Toledo et al., 2001) reproducibly presented hybridization signals and is marked by an asterisk.

present in the karyotype of *E. sp.2* presumably originated from a Y-autosome fusion, as hypothesized by meiotic analysis and BrdU staining (Almeida-Toledo et al., 1988).

Chromosome painting techniques have been applied to fish in only a handful of studies. In all these cases, probes were obtained by chromosome microdissection (Reed et al., 1995; Campos-Ramos et al., 2001; Phillips et al., 2001; Harvey et al., 2002; Liu et al., 2002). Although probes obtained from flow-sorting are better suited for cross-species FISH (Ferguson-Smith et al., 1998), there are several impediments for its use such as the need for chromosomes which present differences in size and base content as well as high quality cell cultures.

In the present study, we assess the hypothesis of independent origins of two different sex chromosome systems (XY/XX and $X_1X_2Y/X_1X_1X_2X_2$) present in the karyotype of *Eigenmannia virescens* and *sp.2*, respectively, through the use of reciprocal chromosome painting applying sex chromosome-specific probes derived from microdissected chromosomes.

Materials and methods

Metaphase preparation

Male and female specimens of *E. virescens* and *E. sp.2* were collected in different small tributaries of the Tietê River, in the municipalities of São Miguel Arcanjo and Indaiatuba, respectively, of the state of São Paulo (Brasil) in 2006. Mitotic chromosome suspensions were prepared directly from kidney tissues as described previously (Almeida-Toledo et al., 2001).

Chromosome microdissection and DOP-PCR

One copy of the X and four copies of the Y chromosome were microdissected from the karyotype of *E. virescens* (female) and that of *E. sp.2* (male), respectively using the methodology previously described (Weimer et al., 1999) with minor modifications.

Briefly, chromosomes were dissected from Giemsa-stained coverslips with a micromanipulator (Eppendorf, Germany) attached to a inverted microscope (Zeiss Axiovert 100 or 135) using borosilicate needles prepared with a pipette puller model PB-7 (Narishige, Japan) and transferred to a micropipette containing a collection solution (1.5 $\mu\text{g}/\mu\text{l}$ proteinase K, 0.1% SDS, 0.1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl) following Weimer et al. (1999).

Pipette tips were broken in a 0.5 ml microtube containing 5 μl of a DOP-PCR (Telenius et al., 1992) mix consisting of dNTPs (200 μM), 5 μM 6-MW primer (5'-CCGACTCGAGNNNNNNATGTGG-3') and T7 reaction buffer (24 mM Tris-HCl, pH 7.5, 12 mM MgCl_2 , 30 mM NaCl).

The first eight cycles of DOP-PCR were conducted using T7 DNA polymerase (USB Biochemicals, USA) under the following profile: 1 min denaturation at 92°C, 2 min 20 s annealing at 25°C and 2 min extension at 34°C. An initial denaturation step (5 min) was included to inactivate proteinase. 0.3 U Sequenase was added at each cycle during annealing. Reaction volume was then increased to 50 μl containing 0.1 U Taq polymerase and 33 cycles were conducted with the following profile: 1 min denaturation at 92°C, 2 min annealing at 56°C and 2 min extension at 70°C followed by a final 5 min extension step. A detailed description of a DOP-PCR reaction profile can be found elsewhere (Guan et al., 1993; Weimer et al., 1999; Henning et al., in press). Hereafter, we refer to these two probes as EVX (for *E. virescens* X probe) and E2Y (for *E. sp.2* Y probe).

Fluorescence in situ hybridization

Primary amplification products were re-amplified in 22 additional DOP-PCR cycles catalyzed by Taq polymerase (as described above) in the presence of 0.1 mM biotin-16-dUTP. FISH was performed on chromosome preparations of *E. virescens* and *E. sp.2* specimens. The slides were prepared according to Henegariu et al. (2001), pre-treated for 5 min with pepsin (0.01% in 10 mM HCl) and aged (60°C for 1 h). Slides were denatured in 70% formamide/0.66 $\times$ SSC for 2 min at 72°C. For each slide, 12 μl of hybridization solution (containing 0.2 μg labeled probe, 50% formamide, 2 $\times$ SSC, 10% dextran sulfate and 5 μg salmon sperm DNA) was denatured for 10 min at 75°C and allowed to pre-hybridize for 1 h at 37°C. Hybridization took place for 72 h at 37°C. Slides were then washed at 42°C with 50% formamide/1 $\times$ SSC, 2 $\times$ SSC and 0.2 $\times$ SSC for 5–15 min each. Detection was carried out using avidin-FITC (Vector Labs, USA).

Fig. 2. Cross-species FISH of sex chromosome probes (a) E2Y on a male specimen of *E. virescens* ($2n = 38, XY$) and (c) EVX on a male specimen of *E. sp.2* ($2n = 31, X_1X_2Y$). Autosomes displaying hybridization signals are indicated with arrows on DAPI counterstained negative images (b and d). Note that recognizable sex chromosomes lack hybridization signals (large acrocentric in a and large metacentric in c). Additional signals can be seen in b in heterochromatic regions of chromosomes 3 and 6 (following Almeida-Toledo et al., 2001) and are marked by an asterisk.

Slides were counterstained and mounted using Vectashield® Mounting Medium with DAPI (Vector Labs) and visualized under an Olympus BX60 fluorescence microscope. FITC and DAPI images were captured separately as 16-bit black and white, colored and mounted using ImagePlus® (Media Cybernetics).

Results

The X chromosome-specific probe (EVX) obtained by chromosome microdissection hybridized to the X and Y chromosomes of the *E. virescens* karyotype, demonstrating the high level of homology of euchromatic regions of the X and Y chromosomes (Fig. 1a). DAPI counterstaining revealed a complex striped banding pattern on the X chromosome (Fig. 1b). This is in contrast to the original description of this karyotype, in which the heterochromatic block was found to be DAPI negative (GC-rich) (Almeida-Toledo et al., 2001). A strong hybridization bias towards repetitive sequences was observed, presumably because of the highly heterochromatic distal region of the X chromosome; this probably resulted in a probe highly enriched with repetitive sequences. Chromosome 8, which also contains a heterochromatic GC-rich region, was consistently painted by this probe.

The probe developed from four copies of *E. sp.2* Y chromosome (E2Y) hybridized to the Y chromosome as well as to two acrocentric chromosomes (X_1 and X_2), as shown in Fig. 1c.

Reciprocal cross-species FISH (EVX probe on *E. sp.2* karyotype and E2Y probe on to *E. virescens* karyotype) resulted in hybridization signals on autosomes (Fig. 2). E2Y hybridized completely to a metacentric pair and a small acrocentric pair. Heterochromatic blocks of chromosomes 3 and 8 were also reproducibly highlighted by E2Y (Fig. 2a). The EVX probe hybridized to a small acrocentric pair (Fig. 2c). None of the distinguishable sex chromosomes displayed consistent hybridization signals after cross-FISH.

Discussion

The lack of signals on recognizable sex chromosomes after reciprocal cross-species FISH using sex chromosome probes indicates a lack of homology between the $X_1X_2Y/X_1X_1X_2X_2$ and XY/XX systems found in *Eigenmannia*. Moreover, a previous phylogenetic map of sex chromosome systems based on mtDNA sequences supports the hypothesis of independent origins (Moysés, 2005). Non-homologous sex chromosomes have also been reported in species of *Oryzias* (Takehana et al., 2007b) and three salmonid genera (Phillips et al., 2001; Woram et al., 2003) and are presumably a widespread phenomenon in fish (Devlin and Nagahama, 2002; van Doorn and Kirkpatrick, 2007).

In the genus *Oryzias* (Beloniformes), the presence of different sex determining genes in congeneric species implies that multiple loci can assume the role of triggering sex determination (Matsuda, 2005; van Doorn and Kirkpatrick, 2007). On the other hand, the same sex determination re-

gion is apparently conserved in salmonid species (Salmoniformes) and has evolved through mechanisms of translocation or transposition (Woram et al., 2003). Nonetheless, evidence presented here shows that non-homologous chromosomes bear sex-determining regions in *Eigenmannia*. Homology of the sex-determining region per se can only be investigated using genetic or high resolution mapping.

Banding pattern obtained with DAPI counterstaining on the *E. virescens* karyotype contrasts to the original description of the X chromosome heterochromatic block (Almeida-Toledo et al., 2001). This demonstrates population-level variability in base composition of the X-specific heterochromatin. Hybridization patterns observed by using the EVX probe support the hypothesis that the X chromosome in *E. virescens* differentiated from the Y chromosome through the accumulation of repetitive sequences on the X and shares sequences with chromosome 8. Hybridization of the Y-derived probe (E2Y) to two acrocentric X chromo-

somes in *E. sp.2* adds further evidence to the origin by fusion hypothesis concerning the Y chromosome (Almeida-Toledo et al., 2000).

The remarkable variety of sex chromosomes, sometimes in the same nominal species such as in the unresolved genus *Eigenmannia*, warrants basal vertebrates as models for understanding early evolution of sex chromosome differentiation (Charlesworth, 2004). The general picture seems to point to a pattern of lack of phylogenetic constraints and multiple independent events (Eggert, 2004; Mank et al., 2006; Quinn et al., 2007).

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