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Differentiation and Evolution of the W Chromosome in the Fish Species of *Megaleporinus* (Characiformes, Anostomidae)

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Keywords

Chromosome painting · Cross-FISH · Microdissection ·
Sex chromosome evolution

Abstract

The W chromosome of *Megaleporinus trifasciatus* was isolated in order to analyze its behavior in the karyotype of this and other species of the family, including forms with differentiated and undifferentiated sex chromosomes. The chromosome was microdissected, and the WMt probe was prepared for the chromosome painting procedure. *M. trifasciatus* was also cross-hybridized (cross-FISH) using existing probes available for *M. macrocephalus* (WMm) and *M. elongatus* (WMe). Two *Leporinus* species and *Semaprochilodus taeniurus*, representing a clade close to the Anostomidae, were also cross-hybridized with the objective to better understand the evolution of the sex chromosomes. In the metaphase of female *M. trifasciatus*, the WMt probe highlighted the whole long arm of the W chromosome and a small, distal portion of the long arm of the Z chromosome. In males, the probe highlighted the distal portion of the long

arm of the Z chromosomes. The hybridization of female *M. trifasciatus* with the WMe and WMm probes revealed a pattern similar to that encountered using the WMt probe. The WMt, WMm, and WMe probes revealed broad similarities among the species of the genus *Megaleporinus*, which has a ZZ/ZW system of sex chromosomes, with only minor alterations becoming apparent when analyzed separately.

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The fish genus *Leporinus* was revised recently by Ramirez et al. [2017], who allocated the species with differentiated sex chromosomes to a new genus, denominated *Megaleporinus*. This genus includes *Megaleporinus obtusidens*, *M. reinhardti*, *M. macrocephalus*, *M. trifasciatus*, and *M. conirostris* with sex chromosomes of the ZZ/ZW type, and *M. elongatus*, which has a multiple system of sex chromosomes of the $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ type [Parise-Maltempi et al., 2007, 2013]. However, *Leporinus multimaculatus*, which has a ZZ/ZW system at an initial stage of differentiation [Venere et al., 2004], was maintained in the genus *Leporinus*.

The multiple sex chromosome system found in *M. elongatus* is closely related to the presence of C-positive heterochromatin, which is the diagnostic feature of the W chromosome [Parise-Maltempi et al., 2007, 2013].

The W chromosome of *M. elongatus* is a long subtelocentric chromosome, which is almost entirely heterochromatic. In the Z chromosome, only the distal third of the long arm is heterochromatic [Galetti and Foresti, 1986, 1987; Galetti et al., 1981, 1995; Parise-Maltempi et al., 2007].

The W chromosome of *M. elongatus*, *M. macrocephalus*, and *M. obtusidens* was mapped by Marreta et al. [2012] using a sequence of highly repetitive DNA (LeSpe I), which produced distinct signals in males and females, indicating that this sequence plays a role in the differentiation of these chromosomes. In a second study, Parise-Maltempi et al. [2013] used chromosome painting based on a W chromosome probe to compare the species with differentiated and undifferentiated sex chromosomes. They observed the complete painting of the W chromosomes in the females and almost all the Z chromosomes in the males, indicating a common origin of the sex chromosomes in at least 3 species (*M. elongatus*, *M. macrocephalus*, and *M. obtusidens*). This conclusion is based on the fact that *M. elongatus* does not share the same repetitive sequences with the species that have Z and W sex chromosomes. In the species with undifferentiated sex chromosomes (*L. friderici*, *L. striatus*, *L. lacustris*, *Schizodon borelii*, and *S. isognathus*), the microdissected W probe did not highlight any portion of the chromosomes [Parise-Maltempi et al., 2013].

There is consensus that the differentiation of the sex chromosomes in the anostomids has occurred primarily through the accumulation of repetitive elements [Nakayama et al., 1994]. These repetitive elements vary among some of the species [Silva et al., 2013] and were probably responsible for the relatively recent origin of the multiple $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ type system in *M. elongatus* [Parise-Maltempi et al., 2007]. It is still unclear, however, whether the increase in heterochromatic segments is the cause or a consequence of the origin of the heterochromatic segments through the accumulation of repetitive DNA, and the understanding of the evolution of this system of sex chromosomes in *Megaleporinus* and *Leporinus* is still incipient.

Up to now, a heteromorphic ZZ/ZW system of sex chromosomes has been found in only one of the anostomid species that occur in the Amazon basin, *M. trifasciatus* [Barros et al., 2017]. Given this, the present study used specific probes obtained from the chromosomal micro-

dissection of the W chromosome and crossed FISH (cross-FISH) to verify the similarity of the W sequence of *M. trifasciatus* with those of the species with undifferentiated sex chromosomes. Probes obtained from other species with the ZZ/ZW system were used to verify the similarities among the different W chromosomes in order to provide a better understanding of the evolution of the anostomid sex chromosomes.

Material and Methods

In the present study, W probes were obtained from *M. trifasciatus* (15 males and 5 females), collected in the region of the confluence of the Negro and Solimões rivers in the Brazilian state of Amazonas (3°08'49.7"S59°54'04.7"W). They were used together with the probes available for *M. macrocephalus*, obtained from specimens collected from the Paraguai River in the Brazilian state of Mato Grosso, and *M. elongatus*, collected from the Mogi-Guaçu River in Pirassununga, São Paulo state. These probes were mapped in their species of origin, each of the other 2 species, in other species that lack differentiated sex chromosomes, and in *S. taeniurus* (Prochilodontidae), which has a ZZ/ZW system, based on specimens also collected at the confluence of the Negro and Solimões rivers in the Amazonas state. In addition, 7 *L. fasciatus* (5 males and 2 females) and 7 *L. friderici* (4 males and 3 females) were obtained from the basin of the Tapajós River in the Brazilian state of Pará.

The specimens were euthanized using clove oil, and the mitotic chromosomes were obtained from kidney cells based on the protocol of Bertollo et al. [2015]. The specimens were identified, numbered, and fixed, and then deposited in the Ichthyology Collection of the Brazilian National Institute for Amazonian Research (INPA) under catalog numbers INPA-ICT 053210 to INPA-ICT 053218.

Chromosomal Microdissection and DNA Amplification

For the microdissection of the W chromosome, the protocol described in Parise-Maltempi et al. [2013] was used. Chromosome suspensions were dripped onto to slides wiped clean with 1% SDS. The slides were then washed in a solution of 1× PBS for 1 min and then incubated in a trypsin solution (35 mL of 1× PBS and 5 mL of trypsin) for 30 s. The slide were washed again in 1× PBS and then stained with 1% Giemsa in PBS. An Eppendorf TransferMan NK 2 (Eppendorf) coupled to a Zeiss Axiovert 40 CFL microscope was used for the microdissection with glass needles, which were made with a Nikon extruder and sterilized with UV radiation. Approximately 8–12 chromosomes were separated in 9 µL of ultrapure DNase-free water and amplified using the GenomePlex Single Cell Whole Genome Amplification kit (WGA4-Sigma), following the manufacturer's protocol.

FISH

The probes were marked with GenomePlex (WGA3 reamplification KIT; Sigma), following the manufacturer's protocol, except for the substitution of the kit's dNTP mix with a mixture of 1/2 T of the dNTPs, to which digoxigenin-11-dUTP (Roche) was added in the reaction. FISH was based on the protocol described by Rens

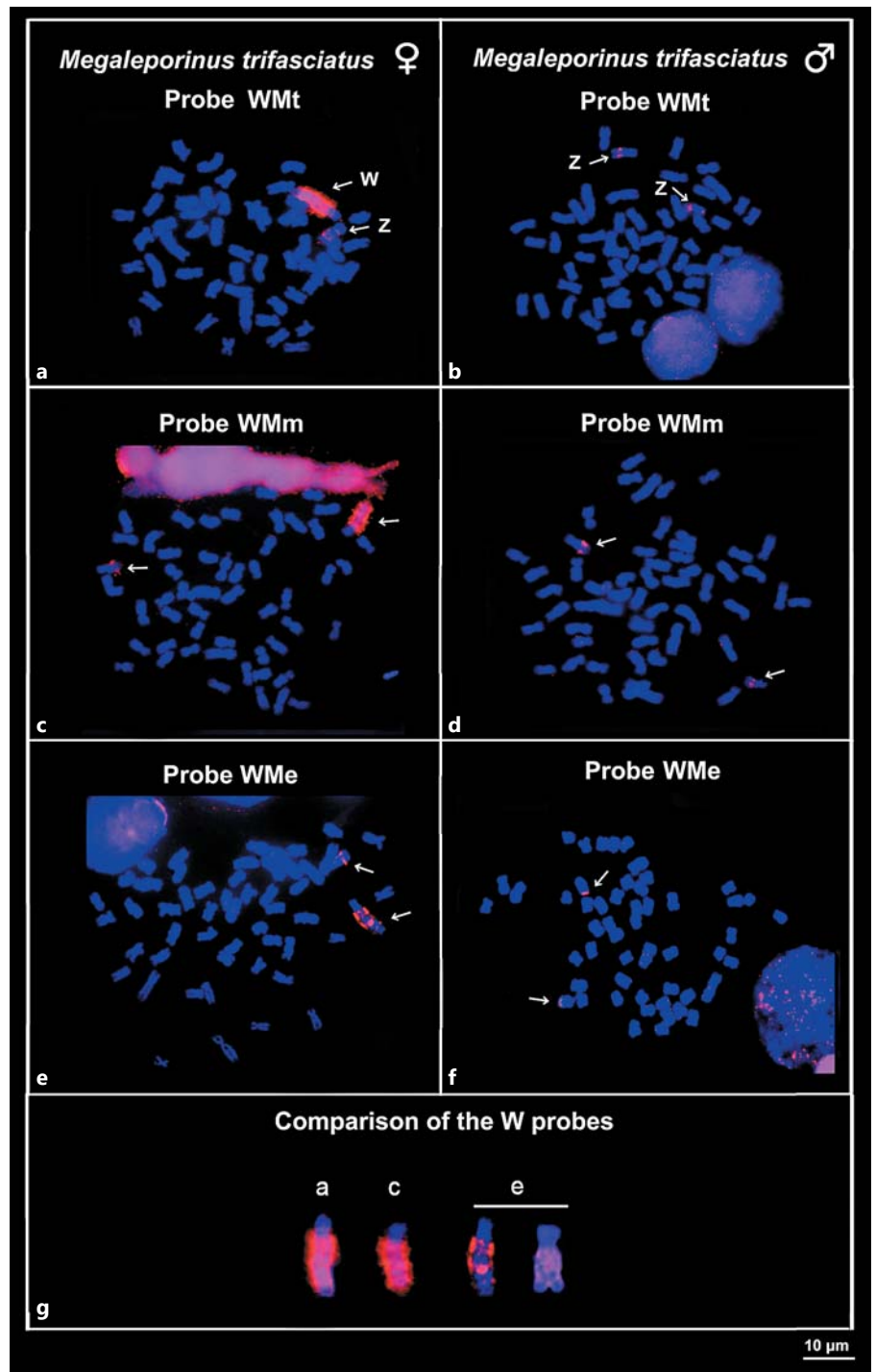


Fig. 1. Metaphases of *Megaleporinus trifasciatus* females (**a**, **c**, **e**) and males (**b**, **d**, **f**) analyzed by cross-FISH using the WMt, WMm, and WMe probes and counterstained with DAPI. **g** Comparison of the W probes.

et al. [1999, 2006], with a number of modifications. The slides with the chromosome preparations were treated with RNase (20 ng/µL) for 1 h at 37°C and dehydrated in an ethanol series (70, 90, and 100%). Afterwards, the chromosomes were denatured in 70% formamide in 2× SSC buffer at 70°C for 2 min and then dehydrated in a freezing ethanol series. A 3-µL aliquot of the marked *M. trifasciatus* probe was then added to 12 µL of the hybridization buffer

(7.5 µL formamide, 1.5 µL 20× SSC, and 3 µL 50% dextran sulfate). This mixture was denatured for 10 min at 65°C, with pre-hybridization at 37°C for 1 h, and then applied to each slide. The samples were hybridized at 37°C for 15 h with the species from which the probes were derived and for 2 nights with the cross species. Following hybridization, the samples were washed twice for 5 min in 50% formamide in 2× SSC, followed by two 5-min washes in 1×

SSC and 4-min wash in 4× SSC with 0.05% 100× Triton (4XT) at 42°C in the case of the species from which the probe was derived and at 45°C in the cross species. The probe was detected using the anti-digoxigenin-rhodamine antibody (Roche) for 1 h at 37°C. Once detected, the slides were washed 3 times in 4XT for 3 min at 42°C and mounted using the Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Vector). Images were obtained using an Olympus D71 digital camera attached to an Olympus BX51 microscope and processed in the DP Controller software.

Results

FISH Using the W Probe from M. trifasciatus (WMt)

In female *M. trifasciatus*, the WMt probe highlighted the whole long arm of the W chromosome but only a small distal area of the long arm of the Z chromosome (Fig. 1a). In male *M. trifasciatus*, the same distal pattern was found on the long arm of both Z chromosomes (Fig. 1b).

Hybridization in *M. trifasciatus* females with the W probes of *M. elongatus* (WMe) and *M. macrocephalus* (WMm) produced a pattern similar to that of the homologous probe (WMt), i.e., highlighting the whole long arm of the W chromosome but only a small portion of the long arm of the Z chromosome (Fig. 1c, e). In male *M. trifasciatus*, these probes (WMe and WMm) produced similar signals to those produced using the WMt probe (Fig. 1d, f).

When hybridized in male and female *L. friderici* and *L. fasciatus*, neither the WMt nor the WMm probe highlighted any of the chromosomes of the complement. A similar lack of signals was also found when the probes were hybridized in *S. taeniurus* (ZZ/ZW), a species of the family Prochilodontidae, a sister group of the Anostomidae (data not shown).

Discussion

The evolution of the sex chromosomes in fish has been discussed widely, and the different systems have been interpreted as the result of structural rearrangements, such as inversions, translocations, duplications, and even the addition of heterochromatin [Ayling and Griffin, 2002]. However, differentiated sex chromosomes have an unusual distribution in fish. In some cases, the same system is found in all the species of a group, such as the ZZ/ZW system found in all the species of *Triplotheus* [Artoni et al., 2001], and in the 10 species of *Megaleporinus*, which was created to distinguish the species with differentiated

sex chromosomes from those with undifferentiated sex chromosomes [Ramirez et al., 2017]. In most other cases, however, the presence of differentiated sex chromosomes is sporadic. Yet, sex chromosomes at an initial stage of differentiation are also found in some taxa [Venere et al., 2004; Parise-Maltempi et al., 2013].

The W chromosome is easily recognized in the *Leporinus* and *Megaleporinus* species because it is the largest chromosome of the complement and presents an extensive accumulation of heterochromatin [Galetti et al., 1981; Venere et al., 2004; Parise-Maltempi et al., 2007]. The considerable similarities in the morphology of the sex chromosomes found in the species of these 2 genera indicates that they have a common origin, with the accumulation of heterochromatin representing an initial step in the differentiation of the chromosomes [Galetti and Foresti, 1986].

In addition, when the W probes of *M. trifasciatus*, *M. elongatus*, *M. macrocephalus*, and *M. obtusidens* were crossed between species, the sequences were highly similar, which reinforces the conclusion that the systems of sex chromosomes found in *Megaleporinus* had a common origin [Marreta et al., 2012; Parise-Maltempi et al., 2013]. However, the W chromosome of *L. multimaculatus* (previously, *Leporinus* sp. 2) is characterized by additional distinct blocks of heterochromatin. It appears to represent a different type of chromatin that is not homologous with that found in the Z chromosome, and in fact, both the Z and W chromosomes in this species are more similar to autosomes, which suggests a different origin in comparison with the W of the *Megaleporinus* species [Venere et al., 2004].

One important finding of the present study is that the W probe of *M. trifasciatus* (WMt) highlighted only the long arm of the W chromosome and a small area of the Z chromosome. A similar pattern was observed in the case of the WMe and WMm probes in *M. trifasciatus*, although the hybridization pattern produced by the WMe probe was slightly different from that of the other probes, indicating a nonhomogeneous pattern, even though that of the Z chromosomes of the males was similar. The WMe probe highlighted the whole W chromosome in female *M. elongatus*, *M. macrocephalus*, and *M. obtusidens*, as well as a large portion of the Z chromosome in *M. macrocephalus*. Signals were also observed in the subtelomeric region of the Z chromosome of male *M. obtusidens* [Parise-Maltempi et al., 2013].

The small difference found on the overlap of WMt, Wme, and WMm (cross-FISH) probes can emphasize the importance of transposable elements (TEs). It is known

that TEs can accumulate in regions distant or close to genes [Kidwell, 2005]. Typically, this pattern of distribution is not random and seems to have some relation with specific characteristics of subregions of the host genomes [Kidwell and Lisch, 2000]. It may in fact influence the evolutionary mechanism involving the ZW sex chromosome system in the species analyzed here, with the deposition of heterochromatin over time being the most accepted hypothesis for its appearance in *Leporinus* sp. and *Megaleporinus* sp. Partially, this set of regulatory process would explain the different stages of chromosomal evolution of the ZW system observed in *Leporinus* sp2 (current *L. geminis*) [Ramirez et al., 2017], *L. elongatus* (current *M. elongatus*) [Parise-Maltempi et al., 2007, 2013], and in *M. trifasciatus*.

M. trifasciatus diversified early from the common ancestor in comparison with the other species of the genus. With the exception of *M. muyscorum*, all other species have a relatively recent common ancestor in comparison with *M. trifasciatus* [Ramirez et al., 2017]. In the present study, the differentiation of specific regions observed in the sex chromosomes of *M. trifasciatus* distinguished this species from the others analyzed using the same probes and technique. This indicates clearly that the system of sex chromosomes in this species is at a distinct stage of differentiation in comparison with the other *Megaleporinus* species, indicating different rates of evolution of the system in this new genus. Parise-Maltempi et al. [2007] described the presence of a multiple system of sex chromosomes in *M. elongatus* (previously, *L. elongatus*), which reinforces the idea that the sex chromosomes of *Megaleporinus* have evolved at distinct rates. The authors of this study concluded that the sex chromosomes might have originated from the same autosomes but through distinct processes.

The evidence from the WMt and WMm probes is inconsistent with the presence of sex chromosomes at an initial stage of differentiation in *L. friderici*, *L. fasciatus* (undifferentiated sex chromosomes), or *S. taeniurus* (ZZ/ZW), or even the sharing of some part of the sequence of the W chromosome of *M. trifasciatus* or *M. macrocephalus*. Parise-Maltempi et al. [2013] obtained similar results using the WLM or WLe (i.e., WMm and WMe in the present study) sequences in *L. friderici*, *L. striatus*, *L. lacustris*, *S. borelii*, and *S. isognathus*, i.e., they found no chromosomes that shared these sequences.

Ramirez et al. [2017] proposed *Megaleporinus* based on morphological, cytogenetic, and molecular traits, which includes the majority of the species allocated previously to *Leporinus*, that have differentiated ZZ/ZW and $Z_1Z_1Z_2Z_2/Z_1W_1Z_2W_2$ sex chromosomes [Galetti et al.,

1984; Parise-Maltempi et al., 2007]. The findings of the present study nevertheless indicate that these species are closely related in terms of the evolution of differentiated sex chromosomes, irrespective of the genus (*Leporinus* or *Megaleporinus*). As *Leporinus* is the most diverse anostomid genus [Britski and Birindelli, 2013] and less than half of its species have been karyotyped, it seems likely that additional cytogenetic analyses of a wider range of species of this and other anostomid genera may reveal a much greater diversity of sex chromosomes in this family. For these future studies, chromosome microdissection, combined with chromosome painting, would appear to offer a powerful tool for the definition of homologous chromosome regions between both closely related and more distant species [Nanda et al., 2011].

Given the diversity of the mechanisms of sex determination found in fish, the sex chromosome system of *Megaleporinus* appears to be relatively conservative and derived from a common ancestor. It seems likely that the sex chromosomes were already different, having been invaded by different repetitive sequences, given that the ZW chromosomes of *Leporinus* and *Megaleporinus* are found only in some closely related species.

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Statement of Ethics

The collection of all specimens was authorized by the Biodiversity Information System (SISBio) of the Brazilian government through license number 28.095-1.

Disclosure Statement

The authors have no conflicts of interest to declare.

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