

ORIGINAL ARTICLE

Highly conserved Z and molecularly diverged W chromosomes in the fish genus *Triportheus* (Characiformes, Triportheidae)

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The main objectives of this study were to test: (1) whether the W-chromosome differentiation matches to species' evolutionary divergence (phylogenetic concordance) and (2) whether sex chromosomes share a common ancestor within a congeneric group. The monophyletic genus *Triportheus* (Characiformes, Triportheidae) was the model group for this study. All species in this genus so far analyzed have ZW sex chromosome system, where the Z is always the largest chromosome of the karyotype, whereas the W chromosome is highly variable ranging from almost homomorphic to highly heteromorphic. We applied conventional and molecular cytogenetic approaches including C-banding, ribosomal DNA mapping, comparative genomic hybridization (CGH) and cross-species whole chromosome painting (WCP) to test our questions. We developed Z- and W-chromosome paints from *T. auritus* for cross-species WCP and performed CGH in a representative species (*T. signatus*) to decipher level of homologies and rates of differentiation of W chromosomes. Our study revealed that the ZW sex chromosome system had a common origin, showing highly conserved Z chromosomes and remarkably divergent W chromosomes. Notably, the W chromosomes have evolved to different shapes and sequence contents within ~15–25 Myr of divergence time. Such differentiation highlights a dynamic process of W-chromosome evolution within congeneric species of *Triportheus*.

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INTRODUCTION

Sex chromosomes are thought to have evolved from an autosomal pair when a sex-determining region or locus evolves on one of the homologs (Bull, 1983; Charlesworth, 1991). Interaction of sex-determining region or locus with sexually antagonistic polymorphisms maintained in linked genes is thought to have favored recombination suppression between the nascent sex chromosomes (Bachtrog, 2006) and the subsequent acquisition of neutral and deleterious mutations (genetic degeneration), amplification of repetitive DNA sequences and heterochromatinization of the sex-specific chromosome (Charlesworth *et al.*, 2005; Bachtrog, 2006). Therefore, differences in size and gene content can be found among the sex chromosomes, in which the Y (or W) may undergo variable degrees of degeneration (Graves, 2006).

Here we study sex chromosome evolution in a fish genus. In teleost fishes, although most species lack heteromorphic sex chromosomes, a variety of sex chromosome systems including simple and multiple ones can be found in some species (Devlin and Nagahama, 2002; Cioffi *et al.*, 2011). Among the ~10% of teleost fish species studied that have detectably heteromorphic sex chromosomes, most have female heterogamety (Devlin and Nagahama, 2002). We studied one such group, the genus *Triportheus* (Characiformes, Triportheidae) with ZW sex chromosomes in 12 species so far analyzed. The Z is a metacentric chromosome, the largest one in the karyotype, whereas

the W is always smaller than the Z, and varies in size and morphology among species (Bertollo and Cavallaro, 1992; Sánchez and Jorge, 1999; Artoni *et al.*, 2001; Artoni and Bertollo, 2002; Nirchio *et al.*, 2007; Diniz *et al.*, 2008; Yano *et al.*, 2014, 2016). Besides, the W chromosome is rich in heterochromatin and carries an 18S ribosomal DNA (rDNA) site on its long arms (Artoni and Bertollo, 2002; Nirchio *et al.*, 2007; Diniz *et al.*, 2009; Marquioni *et al.*, 2013; Yano *et al.*, 2014; Schmid *et al.*, 2016). Recently, a molecular phylogeny of the Triportheidae family was introduced based on the 16S rRNA and cytochrome *b* (*CytB*) mitochondrial genes, and on the recombination activating gene 1 (*Rag1*), recombination activating gene 2 (*Rag2*) and myosin heavy chain 6 cardiac muscle- α (*Myh6*) nuclear genes (Mariguela *et al.*, 2016). According to this study, *Triportheus* represent a monophyletic group originated at 26.2 ± 6.5 Myr, in which *T. auritus* is a direct representative of the first lineage that differentiated in the genus at 20.7 ± 6.5 Myr, and correspond to a sister group to all *Triportheus* species (Mariguela *et al.*, 2016), as demonstrated in the phylogenetic tree (Figure 1).

Molecular cytogenetics provides valuable tools and insights for comparative genomics research and has emerged as promising for understanding genome evolution and organization. In particular, whole chromosome painting (WCP) and comparative genomic hybridization (CGH) have been effective methods for the

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identification and characterization of sex chromosomes, tracking their origin and evolution among various taxa (Traut *et al.*, 1999; Phillips *et al.*, 2001; Ezaz *et al.*, 2005; Henning *et al.*, 2008; Ráb *et al.*, 2008; Cioffi *et al.*, 2013; Pazian *et al.*, 2013; Symonová *et al.*, 2015). However, the effectiveness of CGH technique can be limited to identify nascent sex chromosomes with very small sex-specific sequences, as demonstrated in the iguana *Oplurus fierinensis* (Altmanová *et al.*, 2016).

In a diversity of organisms, heterochromatinization, accompanied by amplification of tandem repeats, represents an important step in the morphological differentiation of simple sex chromosome systems, especially in the ZW ones (Nanda *et al.*, 2000; Kondo *et al.*, 2004; Marchal *et al.*, 2004; Peichel *et al.*, 2004; Charlesworth *et al.*, 2005; Ezaz *et al.*, 2009; Kejnovský *et al.*, 2009). Same type of studies provided evidence that rDNA cistrons can also occur on the sex chromosomes of distinct organisms (see, for example, Goodpasture and Bloom, 1975; Schmid *et al.*, 1983; Yonenaga-Yassuda *et al.*, 1983; Morielle and Varella-Garcia, 1988; Cioffi *et al.*, 2010). In this sense, the detection of the heterochromatin by C-banding procedures, as well as the mapping

of rDNA repeats, represent helpful approaches for sex chromosome identification and characterization.

Here we compared sex chromosomes of eight species from *Triporthus* genus using multiple molecular and conventional cytogenetic tools, such as C-banding to detect heterochromatin, rDNA mapping, CGH and WCP to characterize regions of homology between the Z and W chromosomes, which we assume represent the ancestral state, as well as the size of the W-specific region. Our study confirmed that the Z chromosomes are highly conserved and revealed remarkably divergent W-chromosome shapes and sequence content.

MATERIALS AND METHODS

Fish species and sample collection

Table 1 lists the individuals investigated, the collection locations, sexes and the numbers of cells analyzed in the cytogenetic experiments. Collections had the authorization of the Brazilian environmental agency ICMBIO/SISBIO (License number 48628-2). All species were identified and deposited in the fish museum of the Laboratory of Biology and Genetic of Fishes of the Universidade Estadual Paulista (UNESP; Botucatu, Brazil) (Table 1). The experiments followed ethical and anesthesia rules in accordance with the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (Process number CEUA 1853260315).

Chromosome preparations and C-banding

Mitotic chromosomes were obtained as described in Bertollo *et al.* (2015). Briefly, the animals were treated with an aqueous solution of colchicine for 50–60 min, anesthetized and killed, and the chromosomal preparations were obtained from cells of the anterior kidney. The C-positive heterochromatin was detected using barium hydroxide according to Sumner (1972).

Chromosome microdissection, probe preparation and fluorescence *in situ* hybridization (FISH)

Fifteen copies of the Z and 20 copies of the W chromosomes from *T. auritus* were microdissected, as it corresponds to a sister group to all *Triporthus* species (Mariguela *et al.*, 2016) and harbors the largest W chromosome. The chromosomes were manually microdissected and pooled before amplifying by degenerate oligonucleotide primed-PCR, following the protocol described in Telenius *et al.* (1992). Chromosome paints were prepared following Yang *et al.* (2009). The Z probes were labeled via PCR with SpectrumOrange-dUTP (Vysis, Downers Grove, IL, USA) and the W probes with SpectrumGreen-dUTP (Vysis) in a 30-cycle label PCR with degenerate oligonucleotide primer using 1 µl of the primary degenerate oligonucleotide primed-PCR products as template DNA (Yang *et al.*, 2009). 18S and 5S rDNA probes were obtained according to Cioffi *et al.* (2009) and Martins *et al.* (2006), respectively. The 18S rDNA probe was labeled with Cyanine 5-dUTP, whereas 5S rDNA was labeled with Spectrum Green-dUTP using nick-translation method (Roche, Mannheim, Germany).

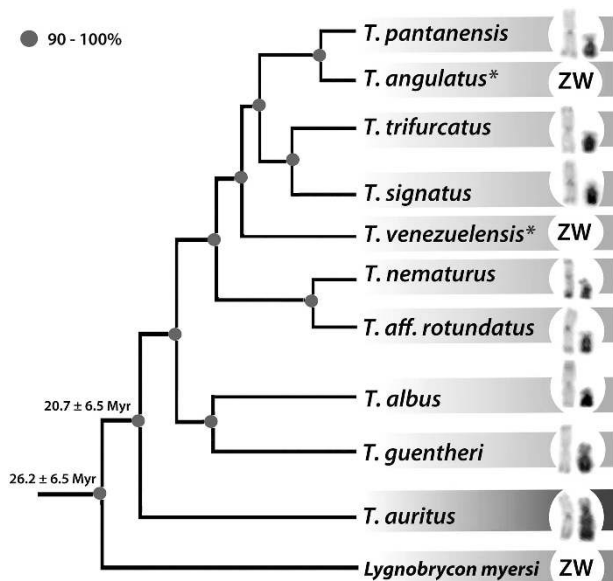


Figure 1 Adapted phylogenetic tree for the *Triporthus* genus, based on the phylogenetic data generated by Mariguela *et al.* (2016), with the respective C-banded Z and W chromosomes evidencing the divergence in the size of the latter. Besides the species analyzed in this study, *T. venezuelensis* and *T. angulatus* (indicated by asterisks) were also included. Note that *T. auritus*, which corresponds to a sister group to all *Triporthus* species, carries the larger W chromosome, whereas *T. albus* has the smaller one compared with its congeneric species.

Table 1 Brazilian collection sites of the *Triporthus* species, number of individuals and cells examined in this study

Species	Site	Basin	N	Number of cells	Deposit number
<i>Triporthus albus</i>	Araguaia river	Araguaia-Tocantins	(04 F; 04 M)	40	LBP18620
<i>Triporthus auritus</i>	Araguaia river	Araguaia-Tocantins	(05 F; 04 M)	25	LBP18622
<i>Triporthus guentheri</i>	Inhuma lake	São Francisco	(12 F; 06 M)	30	LBP18628
<i>Triporthus nematurus</i>	Paraguay river	Paraguay	(09 F; 07 M)	20	LBP18624
<i>Triporthus pantanensis</i>	Paraguay river	Paraguay	(01 F; 01 M)	30	LBP18623
<i>Triporthus aff. rotundatus</i>	Paraguay river	Paraguay	(19 F; 21 M)	25	LBP18625
<i>Triporthus signatus</i>	Piracicaba river	Tietê	(13 F; 24 M)	25	LBP18619
<i>Triporthus trifurcatus</i>	Araguaia river	Araguaia-Tocantins	(04 F; 11 M)	20	LBP18621

Abbreviations: F, female; M, male.

Three-color FISH for WCP

Cytogenetic preparations of males and females of the eight mentioned *Triportheus* species were used for a three-color FISH experiment, combining microdissected Z and W chromosomes, together with 18S rDNA probe, according to Yang *et al.* (2009). As commercial salmon sperm blocking DNA (Sigma-Aldrich, St Louis, MO, USA) was not sufficient to block the hybridization of high-copy repeat sequences, therefore, Cot1-DNA directly isolated from *T. auritus* female genome (prepared according to Zwick *et al.*, 1997) was used instead. Hybridization was performed for 16–18 h at 37 °C in a moist chamber. After hybridization, the slides were washed for 5 min with 1 × SSC at 65 °C, and in 4 × SSC/Tween using a shaker at room temperature and then rinsed quickly in 1 × phosphate-buffered saline. Subsequently, the slides were dehydrated again in an ethanol series (70, 85 and 100%) for 2 min each. After complete drying of the slides, the chromosomes were counterstained with DAPI/Antifading (1.2 mg ml⁻¹, Vector Laboratories, Burlingame, CA, USA).

Preparation of probes for CGH

The CGH experiments were performed in *T. signatus*, representing a species with one of the smallest W chromosomes found among *Triportheus* species. The female genomic DNA (gDNA) was labeled with digoxigenin-11-dUTP using DIG-nick-translation Mix (Roche), and the male gDNA was labeled with biotin-16-dUTP using BIO-nick-translation Mix (Roche), in which 1 µg of gDNA was used, each. Hybridization mixture for one slide (25–30 µl) was composed of 1 µg of labeled male gDNA, 1 µg of labeled female gDNA and 50 µg of sonicated salmon sperm blocking DNA (Sigma-Aldrich).

FISH for CGH

The CGH experiments followed the methodology described by Symonová *et al.* (2015). The hybridization signal was detected using a solution composed of anti-digoxigenin-fluorescein isothiocyanate (Roche) diluted in 0.5% bovine serum albumin in phosphate-buffered saline, and streptavidin-CY3 (Invitrogen Life Technologies, San Diego, CA, USA) diluted in phosphate-buffered saline containing 10% normal goat serum. The slides were then washed 4 times in 4 × SSC and 0.01% Tween, 7 min each at 42 °C. After complete drying, the chromosomes were counterstained and mounted in antifade containing 1.5 µg ml⁻¹ DAPI (Cambio, Cambridge, UK).

Two-color FISH with 18S and 5S rDNA probes

18S and 5S rDNA sequences were mapped on female chromosome preparations of *T. signatus* species, following the protocol described in Marquioni *et al.* (2013). For this experiment, the 18S and 5S rDNA probes were labeled with SpectrumOrange-dUTP and SpectrumGreen-dUTP, respectively, using nick-translation method (Roche).

Microscopic analyses

At least 20 metaphase spreads were analyzed per individual to confirm the diploid chromosome numbers, karyotype structure and FISH results. Images were captured by the CoolSNAP system software, Image Pro Plus, 4.1 (Media Cybernetics, Silver Spring, MD, USA), coupled to an Olympus BX50 microscope (Olympus Corporation, Ishikawa, Japan).

RESULTS

C-banding and rDNA mapping

C-positive heterochromatin was consistently localized in the centromeric regions of autosome pairs (data not shown). The Z chromosomes have additional heterochromatin in one or both telomeric regions, depending on the species, whereas the W chromosomes were almost entirely heterochromatic, except for the p arms in all species (Figure 1), in agreement with previous studies (Artori and Bertollo, 2002; Diniz *et al.*, 2009; Yano *et al.*, 2014).

The two-color FISH with 18S and 5S rDNA showed that both sites are colocalized on the p arms of the chromosome pair 3 in *T. signatus*, with an 18S rDNA additional site adjacent to the Wq telomere (Figure 2).

Comparative genomic hybridization

CGH using male and female gDNA probes developed from *T. signatus* identified Z- and W-specific sequences. In females, all chromosomes except the W, stained equally with these probes. The female gDNA probe painted the whole W chromosome, with a very bright signal on most of the Wq, as well as the telomeric region of both Z chromosome arms. In addition, the p arms of pair 3 also showed an extensive homology with the Wq region. With the male gDNA probe, the W chromosome showed signals on Wp and the proximal region of Wq. The merged images revealed that sequences from both sexes are shared on Wp and the proximal region of the Wq arms, whereas female-specific sequences are concentrated in the terminal region of the Wq (Figure 2).

Sex chromosome paint preparation and cross-species sex chromosome painting

The quality of both Z- and W-chromosome paints was validated by mapping them back onto *T. auritus* metaphase spreads with salmon sperm DNA and *T. auritus* female-specific Cot1-DNA as suppressor. The probe mixed with salmon sperm DNA produced nonspecific signals (data not shown), whereas 5 µg µl⁻¹ of *T. auritus* female-specific Cot1-DNA was sufficient to block nonspecific signals, giving clear hybridization signals on Z and W chromosomes, and identifying probes that are largely or completely Z or W specific (Figures 3 and 4).

Together with the Z and W probes, an additional 18S rDNA probe was used in a three-color FISH experiment to clearly identify the W chromosome in the metaphase plates, as the W chromosome carries a huge 18S rDNA cistron in the Wq arms of all *Triportheus* species. The Z probe from *T. auritus* completely painted the Z chromosome of all other species. The W chromosome of this species also displayed fluorescence signals in the p arms, but there were remarkable differences among species, with only *T. guentieri*, *T. nematurus* and *T. albus* showing strong hybridization signals across the whole Wp. A major part of the Wq arms was also painted with the Z probe in all species, with the exception of *T. aff. rotundatus* and *pantanensis* (Figures 3 and 4).

A high level of homology was found for the W chromosome among species, but with different hybridization patterns (Figures 3 and 4). Using the W probes, the W chromosome was homogeneously painted along its entire length in all species, except for the Wq telomeric region and the centromeric region of *T. aff. rotundatus* and *T. pantanensis* (Figures 3 and 4). In all species, the W probe also painted the Z chromosome in the centromeric and telomeric regions of the p arms, with the exception of *T. auritus*, in which only the telomeric region was painted (Figures 3 and 4). As the whole chromosome painting experiments were performed using a *T. auritus* female Cot1-DNA as a competitor, the W-specific region (terminal part of the q arms) was thus blocked and did not hybridize. Accordingly, on the euchromatic Z chromosome, only the centromere (a highly repetitive region) and the ancestral homologous region (terminal part of the p arms) were painted with the W probe, with the exception of the centromeric region of the Z chromosome of *T. auritus* that was also blocked or suppressed (Figure 4).

DISCUSSION

Our study demonstrated that the ZW sex chromosome system in *Triportheus* had a common origin, considering the homology found in all species using the Z- and W-chromosome probes from *T. auritus*; however, the W chromosomes have undergone rapid discordant differentiation, as differential hybridization patterns were verified for these chromosomes in WCP experiments. In fact,

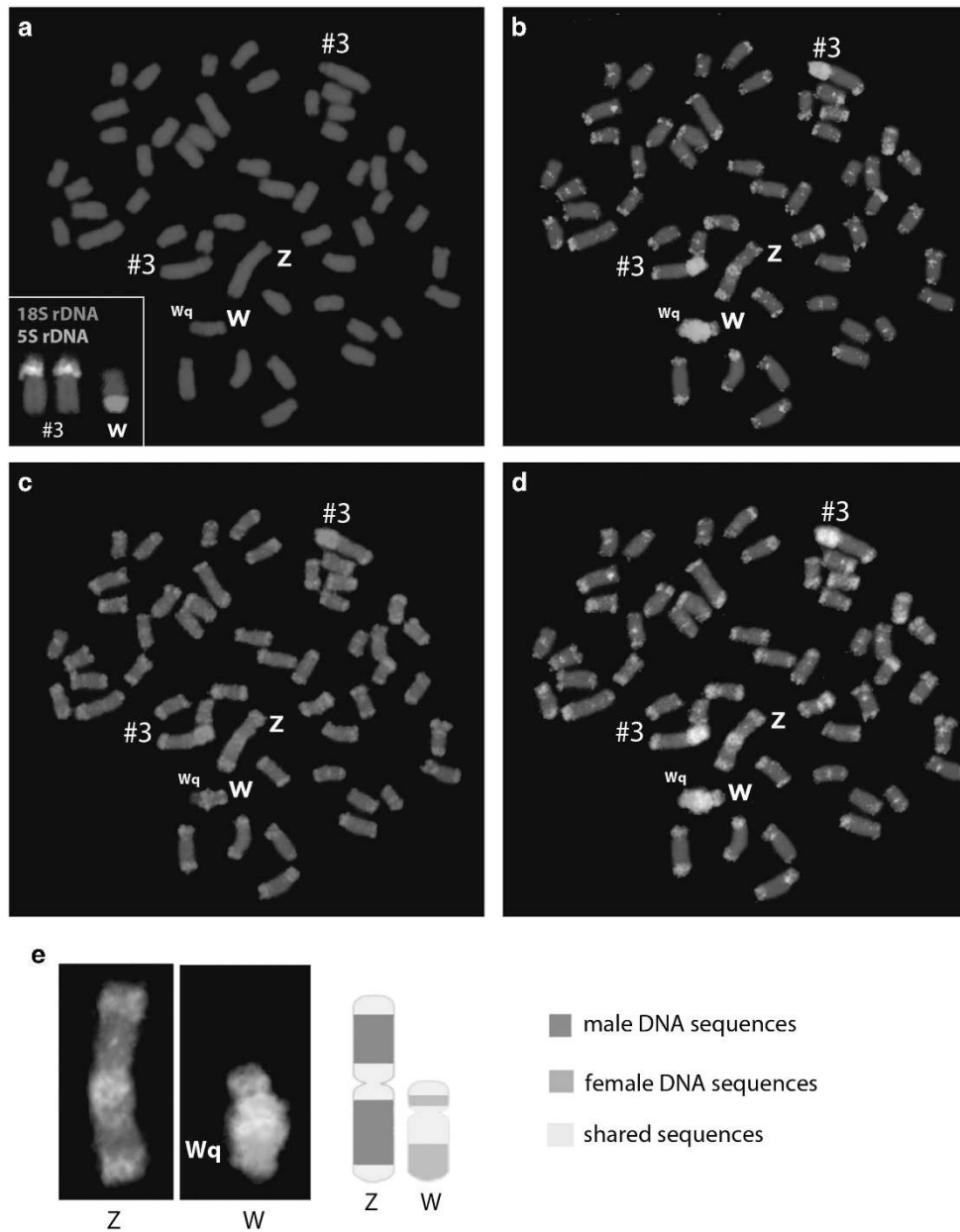


Figure 2 CGH on female metaphase of *T. signatus*, with emphasis on the Z and W chromosomes. The superposition of male and female gDNA probes highlights chromosomal regions sharing male and female sequences and the prevalence of female- or male-specific sequences. (a) DAPI staining. (b) Hybridization with gDNA female probe. (c) Hybridization with gDNA male probe. (d) Superposition of female and male gDNA probes. In (e), the sex chromosomes after the superposition of female and male gDNA probes are highlighted in enlarged forms, together with schematic diagrams summarizing the results, evidencing the accumulation of female-specific sequences in the terminal region of the Wq. The chromosome pair 3 and the W chromosome harboring 18S and 5S rDNA are boxed. Bar, 5 μ m.

the W chromosomes display a differential molecular composition, size and morphology among species despite their evolutionary relationships, highlighting the dynamic process that shapes the differentiation of the sex chromosomes. In addition, our study provided molecular cytogenetic evidence of the chromosomal rearrangement involving rDNA locus, highlighting its probable role in the evolution of the sex chromosomes by facilitating the reduced recombination and the subsequent accumulation of repetitive sequences on the W chromosome that ultimately led the evolution of highly differentiated sex chromosomes within this congeneric group.

Chromosomal rearrangements and sex chromosome differentiation

It is clear that size reduction and accumulation of heterochromatin are events that are associated with the differentiation of the W chromosome within *Triportheus* species (Figure 1). However, CGH and WCP experiments were able to clarify additional details on this evolutionary pathway. In fact, both techniques were critical to demonstrate the sequences that are still shared by both sex chromosomes or, otherwise, that those are more exclusive to one of them. Noteworthy, it was evidenced that the end of the Wq has a high concentration of female-specific sequences (Figures 2–4), where an 18S rDNA cluster is also located in all *Triportheus* species so far analyzed (Artoni and

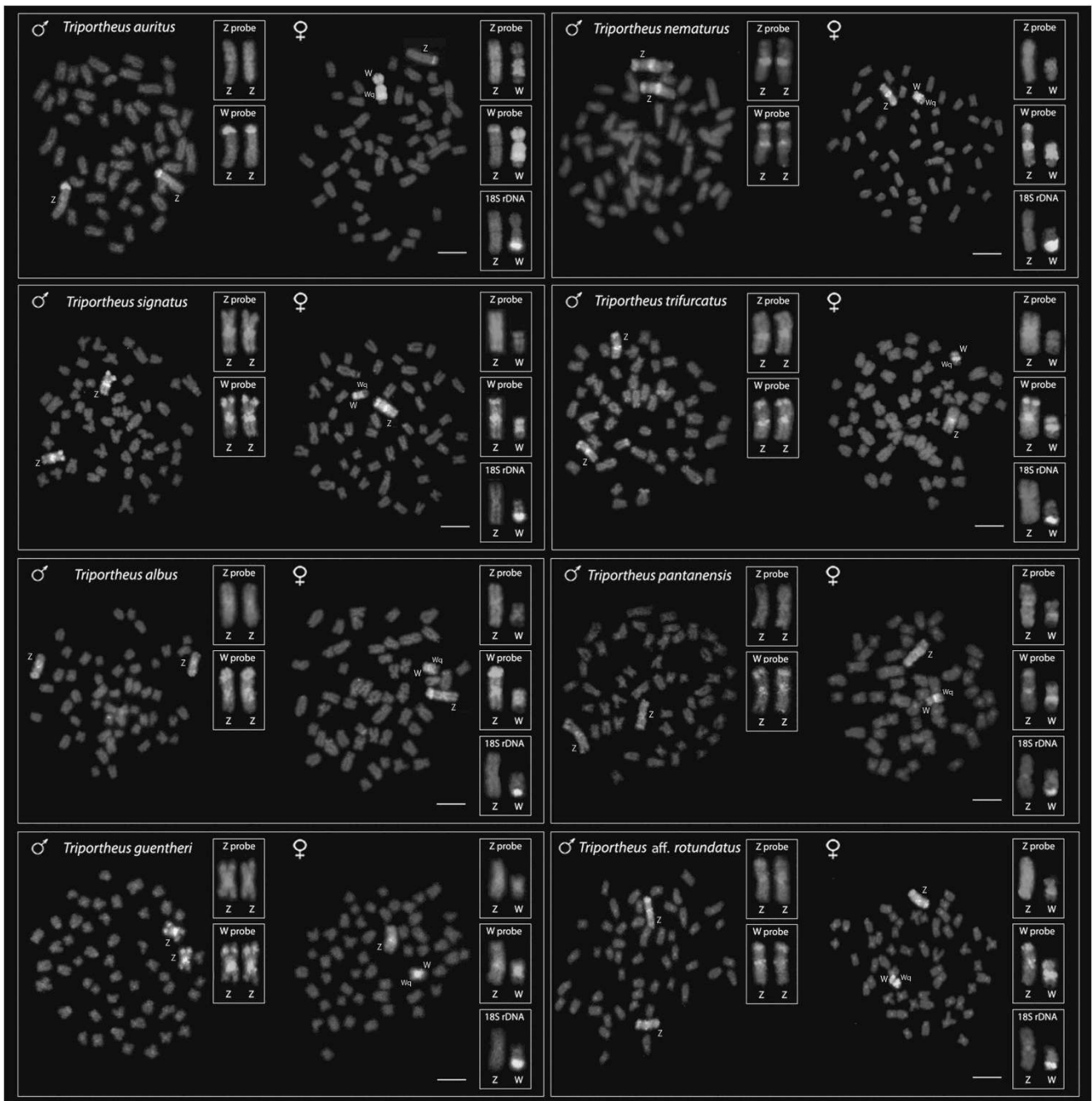


Figure 3 Cross-species chromosome painting using W-chromosome (green) and Z-chromosome (red) probes, both obtained from *T. auritus*, together with an 18S rDNA probe (yellow) in a three-color FISH experiment. The sex chromosomes are highlighted in boxes. Note the location of 18S rDNA sequences on the telomeric Wq. The additional 18S rDNA sites located on autosomes are not shown. Bar, 5 μ m. A full color version of this figure is available at the *Heredity* journal online.

Bertollo, 2002; Nirchio *et al.*, 2007; Diniz *et al.*, 2009; Marquioni *et al.*, 2013; Yano *et al.*, 2014). Sex chromosomes carrying 18S rDNA sequences have already been reported in several other vertebrates, such as *Characidium* fishes (Scacchetti *et al.*, 2015), cane toad *Bufo marinus* (Abramyan *et al.*, 2009), Chinese softshell turtle *Pelodiscus sinensis* (Kawai *et al.*, 2007) and tiger snake *Notechis scutatus* (O'Meally *et al.*, 2010). However, the *Triportheus* case deserves further considerations because of the unusual and particular location of these sequences that do not occur in both homologs of the sex pair (only on the W chromosome) and in some autosomes.

Unusually, the *Triportheus* sex chromosomes do not have 18S rDNA sequences on both homologs, but they are present only on the W chromosome. A huge 18S rDNA block is also located on the p arms of the third chromosome pair in all *Triportheus* species. Besides, in some species additional 18S rDNA sites are verified in other autosomes (Yano *et al.*, submitted). The 18S rDNA region of the third chromosome also showed high homology with the Wq, in our CGH experiments, suggesting that either part of the 18S rDNA block was transposed from the W chromosome to the 3p arms or vice versa. Evidence for an 18S rDNA cluster in the telomeric region of the

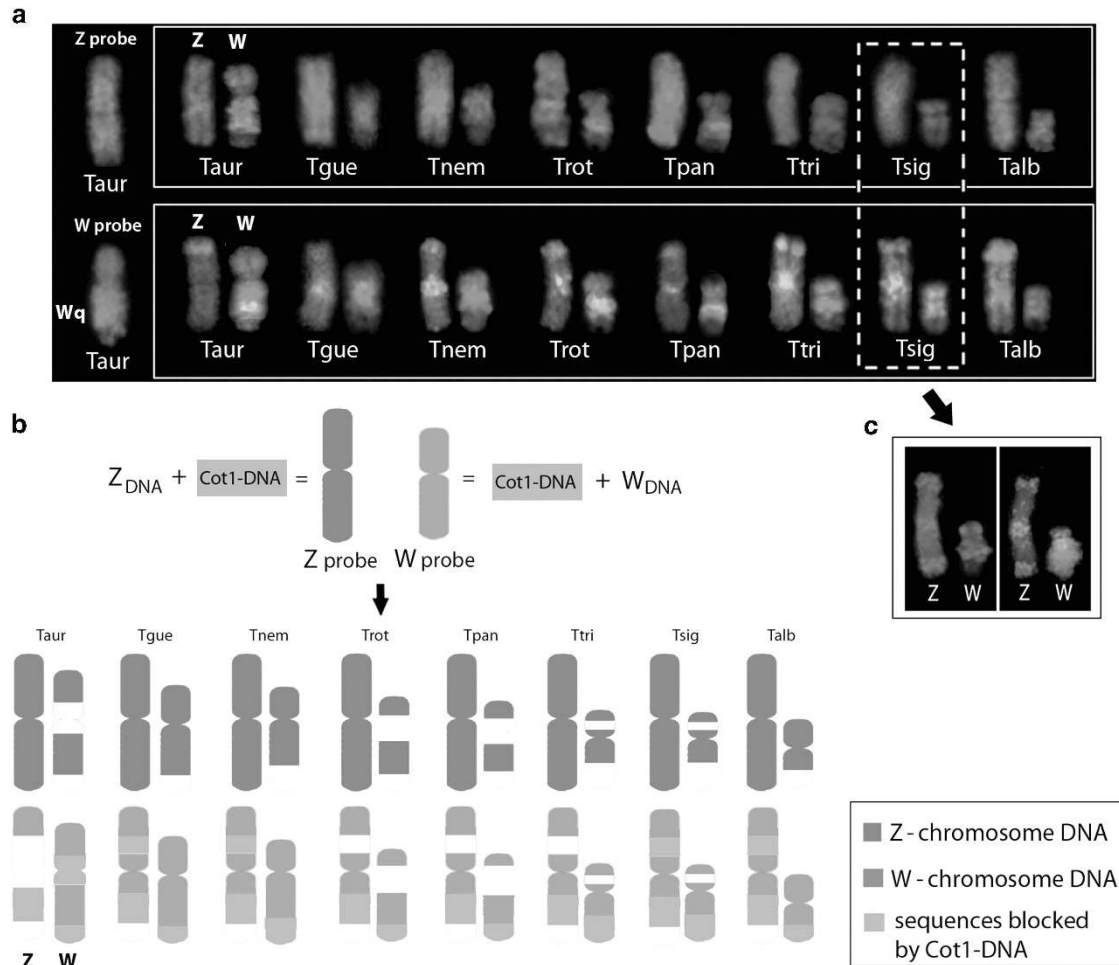


Figure 4 Hybridization patterns on the sex chromosomes of *Triportheus* species using Z- and W-chromosome probes. (a) Note that the sex chromosomes showed evident FISH signals with both Z and W probes, although with a variable pattern among species. In (b), the diagrams explaining the observed hybridization patterns, taking account of the use of Cot11-DNA from a female of *T. auritus* as a competitor. The W-specific regions (terminal part of the q arms) were thus blocked and did not hybridize. Accordingly, on the euchromatic Z chromosome, only the centromeric highly repetitive region and the ancestral homologous region (terminal part of the p arms) were painted with the W probe. In (c), ZW chromosomes after CGH experiments performed in *T. signatus* are highlighted.

Z chromosome was reported in *T. venezuelensis* and *T. angulatus* (Nirchio *et al.*, 2007; Marquioni *et al.*, 2013) that represent two of the most recently derived species, as their lineage originated at 5.2 ± 2.3 and 2.6 ± 1.4 Myr, respectively (Mariguela *et al.*, 2016). Considering the fact that most species, including those originated from older *Triportheus* lineages, do not show an 18S rDNA cluster on the Z chromosome, it is more plausible to assume that these sequences were firstly translocated onto W and latter transposed from the W to the Z chromosome in *T. venezuelensis* and *T. angulatus* in independent events. However, an alternative scenario in which these sequences were originally carried on both sex chromosomes, and that their present distribution reflects subsequent loss from the Z in some species, cannot be fully excluded.

It is tempting to speculate that maintenance and amplification of the rDNA sites on the W chromosome might have promoted reduced recombination between the ZW pair (see Figure 5). It has been proposed that polymorphisms in the rDNA locus in *Salvelinus* species may have acted in a similar way to limit crossing over near the sex locus (Reed and Phillips, 1997). Besides 18S rDNA, the W chromosome of *Triportheus* is rich in other repetitive DNA classes, as evidenced by the variable accumulation of microsatellites and U2 snDNA (Yano *et al.*, 2016; Yano *et al.*, submitted). Therefore, we

cannot rule out the possible involvement of distinct classes of repetitive DNA sequences in the differentiation process of W chromosome. Our WCP and CGH experiments clearly demonstrate that much of the Wq is a W-specific region (Figures 2–4), but that differentiation of the W chromosome morphology, associated with heterochromatinization, and probably with degeneration, evolved independently in different *Triportheus* lineages (Figure 5). These changes are specific to the W chromosome, whereas other chromosomes, including Z, remain similar in the *Triportheus* species so far analyzed, supporting the monophyletic status of this genus, but indicating that the female-specific sex chromosome is subject to particular evolutionary forces not shared by other chromosomes. Moreover, as the sex-specific chromosome can experience different evolutionary forces, parameters like effective population size and sexual selection may also affect how the recombination suppression evolves (Graves, 2006; Bachtrog *et al.*, 2011), even among closely related species as in the *Triportheus* case.

Common origin of the ZW sex system in *Triportheus* and differentiation of the W chromosome

The Triportheidae has four other genera, including *Lignobrycon*, in which *L. myersi* represents the only species currently described and

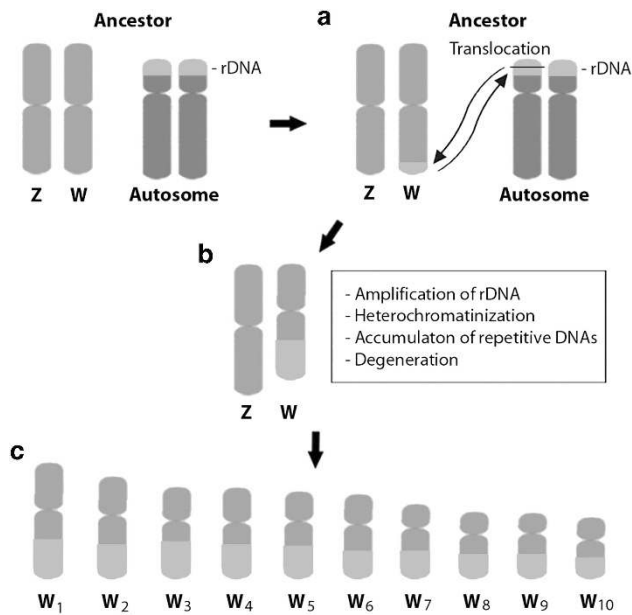


Figure 5 Schematic diagram summarizing the proposed chromosome rearrangements leading to the evolution of sex chromosomes in *Triportheus* species. (a) Fission and translocation event involving the p arms of an ancestral chromosome 3 occurred within an 18S rDNA cluster onto the W chromosome, or vice versa, in a common ancestor. (b) Subsequently, such 18S rDNA cistron was amplified in W chromosome. (c) Finally, whereas the Z chromosome was morphologically conserved, the W chromosome had extensive morphological and size variations because of rDNA expansion, heterochromatinization and repetitive DNA accumulation, in which the degree of degeneration and the differentiation later evolved independently in each *Triportheus* species. W1, *T. auritus*; W2, *T. guentheri*; W3, *T. signatus*; W4, *T. rotundatus*; W5, *T. nematurus*; W6, *T. pantanensis*; W7, *T. trifurcatus*; W8, *T. venezuelensis* (data from Nirchio *et al.*, 2007); W9, *T. angulatus* (data from Diniz *et al.*, 2009); W10, *T. albus*.

corresponds to the sister group of all other *Triportheidae* species (Mariguela *et al.*, 2016). Recent analyses indicate a similar ZW sex chromosome system in *L. myersi* (Rodrigues *et al.*, 2016), suggesting an early origin of the ZW system in the family, an unusual situation among fishes, where sex chromosomes have evolved independently among congeneric species, or even within the same species (Cioffi *et al.*, 2013).

The molecular divergence, demonstrated by cross-species WCP, and the variable morphological forms, underlines the process shaping the evolution of the sex-specific chromosome in *Triportheus*. Rapid degeneration of recently formed Y chromosomes has been demonstrated in *Drosophila* (Bachtrog *et al.*, 2008) and sticklebacks (Peichel *et al.*, 2001). However, there are few empirical data for W chromosomes. The data presented here suggest that the W chromosomes of *Triportheus* have evolved during a relative short (~15–25 Myr) divergence time (phylogenetic data from Mariguela *et al.*, 2016), displaying different genomic composition in terms of repetitive DNA sequences, size, and morphology among species, including accumulation of microsatellites, transposable elements and rDNAs on the W chromosomes (Yano *et al.*, 2014), like the situation in the W chromosomes of pyralid moths (Vítková *et al.*, 2007).

The current situation in *Triportheus* resembles the sex chromosome evolution in other taxa, such as birds and snakes. In primitive ratite birds, the W chromosome is almost the same size as the Z (Shetty *et al.*, 1999), and this may represent the ancestral condition from which other bird W chromosomes have evolved (Ferguson-Smith, 2007). Similarly, *T. auritus*, the earliest branching extant *Triportheus*

species (Mariguela *et al.*, 2016), has the largest W chromosome, with a size comparable to the Z (Figure 1). However, we detect no correlation between the size reduction of the W chromosome and the divergence time between *Triportheus* species (Figure 1). Similarly, various bird species also show variations in the morphology of the W chromosome, but no clear pattern of gradual reduction in size over time (Rutkowska *et al.*, 2012).

CONCLUSIONS

Overall, our study provides evidence for the common origin of a sex chromosome system within a congeneric group that corresponds to an uncommon event among fish species, even among closely related ones, where independent evolution is more common. It is also remarkable that rapid differentiation of the sex-specific chromosome occurred in size, shape and sequence content, likely favored by recombination reduction between the sex pair in view of the maintenance and amplification of specific genome sequences in the W chromosome. Our study provides a unique opportunity for fine-scale analysis of sex chromosome sequences in this group through sequencing and sequence analysis of microdissected sex chromosomes that will assist in discovering novel sex-determining genes and mechanisms of sex chromosome evolution in vertebrates in general.

DATA ARCHIVING

Description of the sampling sites and their GPS coordinates can be found at the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.8m201>.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- Abramyan J, Ezaz T, Graves JAM, Koopman P (2009). Z and W sex chromosomes in the cane toad (*Bufo marinus*). *Chromosome Res* **17**: 1015–1024.
- Altmanová M, Rovatsos M, Kratochvíl L, Pokorná MJ (2016). Minute Y chromosomes and karyotype evolution in Madagascan iguanas (Squamata: Iguania: Opluridae). *Biol J Linn Soc* **118**: 618–633.
- Artori RF, Bertollo LAC (2002). Evolutionary aspects of the ZZ/ZW sex chromosome system in the Characidae fish, genus *Triportheus*. A monophyletic state and NOR location on the W chromosome. *Heredity* **89**: 15–19.
- Artori RF, Falcão JN, Moreira-Filho O, Bertollo LAC (2001). An uncommon condition for a sex chromosome system in Characidae fish. Distribution and differentiation of the ZZ/ZW system in *Triportheus*. *Chromosome Res* **9**: 449–456.
- Bachtrog D (2006). A dynamic view of sex chromosome evolution. *Curr Opin Genet Dev* **16**: 578–585.
- Bachtrog D, Hom E, Wong K, Maside X, de Jong P (2008). Genomic degradation of a young Y chromosome in *Drosophila miranda*. *Genome Biol* **9**: R30.
- Bachtrog D, Mank JE, McDaniel SF, Pires JC, Rice W, Valenzuela N (2011). Are all sex chromosomes created equal? *Trends Genet* **27**: 350–357.
- Bertollo LAC, Cavallaro ZI (1992). A highly differentiated ZZ/ZW sex chromosome system in a Characidae fish, *Triportheus guentheri*. *Cytogenet Cell Genet* **60**: 60–63.
- Bertollo LAC, Cioffi MB, Moreira-Filho O (2015) Direct chromosome preparation from Freshwater Teleost Fishes. In: Ozouf-Costaz C, Pisano E, Foresti F, Almeida Toledo LF (eds), *Fish Cytogenetic Techniques (Chondrichthys and Teleosts)*. CRC Press: Enfield. pp 21–26.
- Bull JJ (1983). *Evolution of Sex Determining Mechanisms*. Benjamin/Cummings Publishing Company: Menlo Park.
- Charlesworth B (1991). The evolution of sex chromosomes. *Science* **251**: 1030–1033.

- Charlesworth D, Charlesworth B, Marais G (2005). Steps in the evolution of heteromorphic sex chromosomes. *Heredity* **95**: 118–128.
- Cioffi MB, Camacho JPM, Bertollo LAC (2011). Repetitive DNAs and differentiation of sex chromosomes in neotropical fishes. *Cytogenet Genome Res* **132**: 188–194.
- Cioffi MB, Liehr T, Trifonov V, Molina WF, Bertollo LAC (2013). Independent sex chromosome evolution in lower vertebrates: a molecular cytogenetic overview in the Erythrinidae fish family. *Cytogenet Genome Res* **141**: 186–194.
- Cioffi MB, Martins C, Vicari MR, Rebordinos L, Bertollo LAC (2010). Differentiation of the XY sex chromosomes in the fish *Hoplias malabaricus* (Characiformes, Erythrinidae): unusual accumulation of repetitive sequences on the X chromosome. *Sex Dev* **4**: 176–185.
- Cioffi MB, Martins C, Centofante L, Jacobina U, Bertollo LAC (2009). Chromosomal variability among allopatric populations of Erythrinidae fish *Hoplias malabaricus*: mapping of three classes of repetitive DNAs. *Cytogenet Genome Res* **125**: 132–141.
- Devlin RH, Nagahama Y (2002). Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* **208**: 191–364.
- Diniz D, Laudicina A, Bertollo LAC (2009). Chromosomal location of 18S and 5S rDNA sites in *Triportheus* fish species (Characiformes, Characidae). *Genetic Mol Biol* **32**: 37–41.
- Diniz D, Moreira-Filho O, Bertollo LAC (2008). Molecular cytogenetics and characterization of a ZZ/ZW sex chromosome system in *Triportheus nematurus* (Characiformes, Characidae). *Genetica* **133**: 85–91.
- Ezaz T, Moritz B, Waters PD, Graves JAM, Georges A, Sarre SD (2009). The ZW sex microchromosomes of an Australian dragon lizard share no homology with those of other reptiles or birds. *Chromosome Res* **17**: 965–973.
- Ezaz T, Quinn AE, Miura I, Sarre SD, Georges A, Graves JAM (2005). The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Res* **13**: 763–776.
- Ferguson-Smith M (2007). The evolution of sex chromosomes and sex determination in vertebrates and the key role of DMRT1. *Sex Dev* **1**: 2–11.
- Goodpasture C, Bloom SE (1975). Visualization of nucleolar organizer regions in mammalian chromosomes using silver staining. *Chromosoma* **53**: 3740.
- Graves JAM (2006). Sex chromosome specialization and degeneration in mammals. *Cell* **124**: 901–914.
- Henning F, Trifonov V, Ferguson-Smith MA, Almeida-Toledo LF (2008). Non-homologous sex chromosomes in two species of the genus *Eigenmannia* (Teleostei: Gymnotiformes). *Cytogenet Genome Res* **121**: 55–58.
- Kawai A, Nishida-Umehara C, Ishijima J, Tsuda Y, Ota H, Matsuda Y (2007). Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenet Genome Res* **117**: 92–102.
- Kejnovský E, Hobza R, Čermák T, Kubát Z, Vyskot B (2009). The role of repetitive DNA in structure and evolution of sex chromosomes in plants. *Heredity* **102**: 533–541.
- Kondo M, Nanda I, Hornung U, Schmid M, Scharf M (2004). Evolutionary origin of the medaka Y chromosome. *Curr Biol* **14**: 1664–1669.
- Marchal JA, Acosta MJ, Nietzel H, Sperling K, Bullejos M, Díaz de la Guardia R *et al.* (2004). X chromosome painting in *Microtus*: origin and evolution of the giant sex chromosomes. *Chromosome Res* **12**: 767–776.
- Marigueta TC, Roxo FF, Foresti F, Oliveira C (2016). Phylogeny and biogeography of *Triportheidae* (Teleostei: Characiformes) based on molecular data. *Mol Phylogenet Evol* **96**: 130–139.
- Marquioni V, Bertollo LAC, Diniz D, Cioffi MB (2013). Comparative chromosomal mapping in *Triportheus* fish species. Analysis of synteny between ribosomal genes. *Micron* **45**: 129–135.
- Martins C, Ferreira IA, Oliveira C, Foresti F, Galetti PM Jr (2006). A tandemly repetitive centromeric DNA sequence of the fish *Hoplias malabaricus* (Characiformes: Erythrinidae) is derived from 5S rDNA. *Genetica* **127**: 133–141.
- Morielle E, Varella-Garcia M (1988). Variability of nucleolus organizer regions in phyllostomid bats. *Rev Bras Genet* **11**: 853–871.
- Nanda I, Zend-Ajusich E, Shan Z, Grütznér F, Scharf M, Burt DW *et al.* (2000). Conserved synteny between the chicken Z sex chromosome and human chromosome 9 includes the male regulatory gene DMRT1: a comparative (re)view on avian sex determination. *Cytogenet Cell Genet* **89**: 67–78.
- Nirchio M, Oliveira C, Ferreira IA, Granado A, Ron E (2007). Extensive polymorphism and chromosomal characteristics of ribosomal DNA in the characid fish *Triportheus venezuelensis* (Characiformes, Characidae). *Genet Mol Biol* **30**: 25–30.
- O'Meally D, Patel HR, Stiglec R, Sarre SD, Georges A, Graves JAM *et al.* (2010). Non-homologous sex chromosomes of birds and snakes share repetitive sequences. *Chromosome Res* **18**: 787–800.
- Pazian MF, Shimabukuro-Dias CK, Pansonato-Alves JC, Oliveira C, Foresti F (2013). Chromosome painting of Z and W sex chromosomes in *Characidium* (Characiformes, Crenuchidae). *Genetica* **141**: 1–9.
- Peichel CL, Nereng KS, Ohgi KA, Cole BLE, Colosimo PF, Buerkle AC *et al.* (2001). The genetic architecture of divergence between threespine stickleback species. *Nature* **414**: 901–905.
- Peichel CL, Ross JA, Matson CK, Dickson M, Grimwood J, Schmutz J *et al.* (2004). The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome. *Curr Biol* **14**: 1416–1424.
- Phillips RB, Konkol NR, Reed KM, Stein JD (2001). Chromosome painting supports lack of homology among sex chromosomes in *Oncorhynchus*, *Salmo* and *Salvelinus*. *Genetica* **111**: 119–123.
- Ráb P, Rabova M, Pereira CS, Collares-Pereira MJ, Pelikanova S (2008). Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotoxic marker the largest subtelocentric chromosome pair as revealed by cross-species painting. *Chromosome Res* **16**: 863–873.
- Reed KM, Phillips RB (1997). Polymorphism of the nucleolus organizer region (NOR) on the putative sex chromosomes of Arctic char (*Salvelinus alpinus*) is not sex related. *Chromosome Res* **5**: 221–227.
- Rodrigues AS, Medrado AS, Diniz D, Oliveira C, Affonso PRAM (2016). ZZ/ZW sex chromosome system in the endangered fish *Lignobrycon myersi* (Teleostei: Characiformes: *Triportheidae*). *Comp Cytogenet* **10**: 245–254.
- Rutkowska J, Lagisz M, Nakagawa S (2012). The long and the short of avian W chromosomes: no evidence for gradual W shortening. *Biol Lett* **8**: 636–638.
- Sánchez S, Jorge LC (1999). A new report of the ZZ/ZW sex chromosome system in the genus *Triportheus* (Pisces, *Triportheinae*). *Cytologia* **64**: 395–40.
- Scacchetti PC, Utsunomia R, Pansonato-Alves JC, Vicari MR, Artoni RF, Oliveira C *et al.* (2015). Chromosomal mapping of repetitive DNAs in *Characidium* (Teleostei, Characiformes): genomic organization and diversification of ZW sex chromosomes. *Cytogenet Genome Res* **146**: 136–143.
- Schmid M, Haaf T, Geile B, Sims S (1983). Chromosome banding in Amphibia. VII. An unusual XY/XX-sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). *Chromosoma* **88**: 69–82.
- Schmid M, Steinlein C, Yano CF, Cioffi MB (2016). Hypermethylated chromosome regions in nine fish species with heteromorphic sex chromosomes. *Cytogenet Genome Res* **147**: 169–178.
- Shetty S, Griffin DK, Graves JAM (1999). Comparative painting reveals strong chromosome homology over 80 million years of bird evolution. *Chromosome Res* **7**: 289–295.
- Sumner AT (1972). A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* **75**: 304–306.
- Symonová R, Sember A, Majtánová Z, Ráb P (2015). Characterization of fish genomes by GISH and CGH. In: Ozouf-Costaz C, Pisano E, Foresti F, Almeida Toledo LF (eds), *Fish Cytogenetic Techniques. Ray-Fin Fishes and Chondrichthyan*. CCR Press: Boca Raton. pp 118–131.
- Telenius H, Carter NP, Bebb CE, Nordenskjöld M, Ponder BAJ, Tunnacliffe A (1992). Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* **13**: 718–725.
- Traut W, Sahara K, Otto TD, Marec F (1999). Molecular differentiation of sex chromosomes probed by comparative genomic hybridization. *Chromosoma* **108**: 173–180.
- Vítková M, Fuková I, Kubičková S, Marec F (2007). Molecular divergence of the W chromosomes in pyralid moths (Lepidoptera). *Chromosome Res* **15**: 917–930.
- Yang F, Trifonov V, Ng BL, Kosyakova N, Carter NP (2009). Generation of paint probes by flow-sorted and microdissected chromosomes. In: Liehr T (ed), *Fluorescence In Situ Hybridization (FISH) – Application Guide*. Springer: Berlin. pp 35–52.
- Yano CF, Bertollo LAC, Liehr T, Troy WP, Cioffi MB (2016). W chromosome dynamics in *Triportheus* species (Characiformes, *Triportheidae*): an ongoing process narrated by repetitive sequences. *J Hered* **107**: 342–348.
- Yano CF, Bertollo LAC, Rebordinos L, Merlo MA, Portela-Bens S, Liehr T, Cioffi MB, (submitted). Evolutionary dynamics of rDNAs and U2 snDNAs in *Triportheus* (Characiformes, *Triportheidae*): high variability and particular syntenic organization.
- Yano CF, Poltronieri J, Bertollo LAC, Artoni RF, Liehr T, Cioffi MB (2014). Chromosomal mapping of repetitive DNAs in *Triportheus trifurcatus* (Characidae, Characiformes): insights into the differentiation of the Z and W chromosomes. *PLoS One* **9**: e90946.
- Yonenaga-Yassuda Y, Assis MFL, Kasahara S, L'Abbate M, Souza MJ (1983). Nucleolar organizer regions in *Akodon arviculoides* (Cricetidae, Rodentia): evidence for the activity of rDNA genes in both X chromosomes of females. *Cytogenet Cell Genet* **35**: 143–147.
- Zwick MS, Hanson RE, McKnight TD, Islam-Faridi MH, Stelly DM, Wing RA *et al.* (1997). A rapid procedure for the isolation of COT-1 DNA from plants. *Genome* **40**: 138–142.