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Research Article

Sex chromosome system ZZ/ZW in *Apareiodon hasemani* Eigenmann, 1916 (Characiformes, Parodontidae) and a derived chromosomal region

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Abstract

Parodontidae fish show few morphological characteristics for the identification of their representatives and chromosomal analyses have provided reliable features for determining the interrelationships in this family. In this study, the chromosomes of *Apareiodon hasemani* from the São Francisco River basin, Brazil, were analyzed and showed a karyotype with 2n = 54 meta/submetacentric chromosomes, and a ZZ/ZW sex chromosome system. The study revealed active NORs located on pair 11 and additional 18S rDNA sites on pairs 7 and 22. The 5S rDNA locus was found in pair 14. It showed a pericentric inversion regarding the ancestral condition. The satellite DNA pPh2004 was absent in the chromosomes of *A. hasemani*, a shared condition with most members of *Apareiodon*. The W*Ap* probe was able to detect the amplification region of the W chromosome, corroborating the common origin of the system within Parodontidae. These chromosomal data corroborate an origin for the ZW system of Parodontidae and aid in the understanding of the differentiation of sex chromosome systems in Neotropical fishes.

Keywords: cytogenetics, FISH, 18S rDNA, 5S rDNA, Neotropical fishes.

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Introduction

Fishes are the largest group of vertebrates, with a wide variety of adaptive responses to diversified aquatic habitats and ecological limitations. Also with respect to sex determination, fishes exhibit much variety. Unlike the situation in most vertebrates, where the male and female sexes are represented by two different individuals (gonochorism), hundreds of fish species are known to be hermaphroditic (Devlin and Nagahama, 2002; Schartl, 2004).

Sex chromosomes are fundamental in many vertebrate species for the development of either a male or a female (Livernois *et al*., 2012). Few species of fish are carriers of heteromorphic chromosomal systems when compared to the enormous taxa diversity (Oliveira *et al*., 2007). However, fishes have a remarkable variety of sex determination chromosome systems (XY, X0, X_1X_2Y, XY_1Y_2, ZW, Z0, ZW_1W_2, $Z_1Z_1Z_2Z_2/Z_1Z_2$, W_1W_2, WXZ) (Moreira-Filho *et al*., 1993; Devlin and Nagahama, 2002; Oliveira *et al*., 2008), in which the ZZ/ZW simple system with female heterogamety is the most frequent one among Neotropical fishes (Artoni and Bertollo, 2002; Bellafronte *et al*., 2011; Machado *et al.* 2011).

The family Parodontidae occurs throughout South America and its species are classified in three genera: *Parodon*, *Apareiodon* and *Saccodon* (Pavanelli, 2003). Generally they are robust fish, with strong pectoral, ventral and caudal fins, a fusiform body, and a dorsal profile which is more arched than the ventral one (Travassos, 1957). Parodontidae taxonomy is controversial because the family members lack diagnostic morphological traits that are sufficiently reliable for accurate phylogenetic analysis (Pavanelli and Britski, 2003; Ingenito LFS, 2008, Ph.D Thesis, Universidade Federal do Rio de Janeiro, Brazil). Ingenito argued that existing morphological phylogenetic evidence for the genus *Apareiodon* is insufficient to support its maintenance, and that *Apareiodon* should be regarded as a junior synonym of *Parodon*.

Cytogenetic analyses of both *Parodon* and *Apareiodon* revealed a conserved diploid number of 54 chromosomes, but with remarkable heterogeneity in the distribution of heterochromatin, NOR activity , number and location of 18S rDNA, 5S rDNA and satellite DNA familiy sites, and morphologically differentiated sex chromosome systems (for a review see Bellafronte *et al*., 2011). From the Parodontidae species that have been cytogenetically ana-

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lyzed, six have sex chromosomes: ZZ/ZW$_1$W$_2$ for *Apareiodon affinis* Steindachner, 1879, and ZZ/ZW for *Parodon hilarii* Reinhardt, 1867, *Parodon moreirai* Ingenito & Buckup, 2005, *Apareiodon vladii* Pavanelli, 2006, *Apareiodon* sp. and *Apareiodon ibitiensis* Campos, 1944 (Moreira-Filho *et al.*, 1980, 1993; Centofante *et al.*, 2002; Vicente *et al.*, 2003; Rosa *et al.*, 2006; Vicari *et al.*, 2006; Bellafronte *et al*., 2009).

In Parodontidae, satellite DNA has great importance for the understanding of karyotype differentiation. So far, two satellite DNAs have been described for this fish group: pPh2004 (Vicente *et al*., 2003) and W*Ap* (Schemberger *et al*., 2011). The physical mapping of the pPh2004 sequence showed that this satellite DNA is present on chromosomes Z and W (also on autosomal chromosomes); however, it is not a part of the heterochromatic amplified region of the W chromosome heteromorphism (Bellafronte *et al*., 2011). The W*Ap* satellite DNA probe is capable of detecting the amplification region and the chromosome W heteromorphism, corroborating the common origin of this system in Parodontidae, apart from homologies to other chromosome sites (Schemberger *et al*., 2011).

The integrated analysis of chromosomal markers has also allowed to deduce the chromosomal differentiation of the family, which is organized in: (i) species without morphologically differentiated sex chromosomes (*Apareiodon piracicabae* Eigenmann, 1907 and *Apareiodon vittatus* Garavello, 1977), (ii) species with differentiated sex chromosomes and without satellite DNA pPh2004 (*A. ibitiensis*, *Apareiodon* sp., *A. vladii*), and (iii) species with proto sex chromosomes and/or heteromorphic sex chromosome systems and presence of satellite DNA pPh2004 (*A. affinis*, *P. hilarii*, *P. moreirai*, *Parodon nasus* Kner, 1859 and *Parodon pongoensis* Allen, 1942) (Schemberger *et al*., 2011).

Based on these data, the present study aimed at characterizing the species *Apareiodon hasemani* Eigenmann, 1916 (from the São Francisco River basin, Brazil) with respect to chromosome number, morphology, banding, sex system, presence or absence of certain molecular cytogenetic markers, as well as to establish karyoevolutionary relationships based in other cytogenetic studies performed in the family Parodontidae.

Materials and Methods

Chromosome studies were carried out in 18 specimens (7 males and 11 females) of *A. hasemani*, collected in the main channel of the São Francisco River, in Pirapora city, state of Minas Gerais, Brazil (17°21'17.47" S and 44°57'18.45" W). The analyzed samples were deposited in the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura (Nupelia), Universidade Estadual de Maringá, Brazil, with voucher number NUP11512. Samples were collected in compliance with the Ethics Committee on Animal Experimentation (Process CEUA 07/2011) of the Universidade Estadual de Ponta Grossa, Brazil) and with authorization to collect the biological material (Brazilian Federal license: Ministério do Meio Ambiente/Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis MMA/IBAMA/SISBIO number 10538-1) and the Instituto Estadual de Florestas de Minas Gerais.

Mitotic chromosomes were obtained from the anterior kidney, according to the methodology described by Bertollo *et al*. (1978). The heterochromatin was detected using the methodology of C-banding (Sumner, 1972). C-banded chromosomes were stained with propidium iodide (50 μg mL^{-1}) according to Lui *et al*. (2009). The nucleolar organizer regions were detected using the silver nitrate method (Ag-NOR) described by Howell and Black (1980).

Fluorescence *in situ* hybridization (FISH) experiments were performed in the Parodontidae specimens using probes for 18S rDNA (Hatanaka and Galetti Jr, 2004), 5S rDNA (Martins and Galetti Jr, 1999), pPh2004 satellite DNA (Vicente *et al*., 2003) and W*Ap* satellite DNA (Schemberger *et al*., 2011). The 18S rDNA, 5S rDNA and pPh2004 cloned probes were labeled with biotin or digoxigenin via PCR, using plasmid vector primers (T7 promoter and M13 reverse). The PCR amplification was done with: 20 ng of template DNA, 1X reaction buffer, 2 mM $MgCl_2$, 40 μM dATP, dGTP and dCTP, 28 μM dTTP, 12 μM biotin 16-dUTP, or digoxigenin 11 dUTP (Roche Applied Science), 0.3 μM of each primer and 1 U *Taq* DNA Polymerase (Invitrogen). The W*Ap* probe was labeled via degenerate oligonucleotide-primed polymerase chain reaction (DOP-PCR), using 11-dUTP- digoxigenin (Roche Applied Science). This PCR amplification was done with: 100 ng of template DNA, 1X reaction buffer, 2 mM $MgCl_2$, 40 μM dATP, dGTP and dCTP, 28 μM dTTP, 12 μM 11-dUTP-digoxigenin, 2 μM DOP primer, and 1 U *Taq* DNA polymerase (Invitrogen). The FISH procedure was performed under high stringency conditions (2.5 ng/ μL probe, 50% formamide, 2X SSC, 10% dextran sulfate) following the methodology described by Pinkel *et al*. (1986). The signal was detected using the anti-streptavidin antibody conjugated to Alexa Fluor 488 (Invitrogen) and an anti-digoxigenin antibody conjugated to rhodamine (Roche Applied Science). The chromosomes were counterstained with DAPI (0.2 μg/mL), covered with Vectashield mounting medium (Vector) and analyzed with the aid of an Olympus BX41 epifluorescence microscope equipped with a DP71 digital image capture system (Olympus).

Chromosomes were identified based on the system proposed by Levan *et al.* (1964) and classified as metacentric (m), submetacentric (sm), or subtelocentric (st).

Results

The species *A. hasemani* showed 2n = 54 meta-submetacentric chromosomes, with a fundamental number equal to 108 for both sexes (Figure 1). However, a hetero-

morphism in size and morphology was observed for chromosome pair 4, restricted to females, which had a medium-sized submetacentric chromosome (Z) and a large metacentric chromosome (W) (Figure 1). The C-banding technique revealed centromeric heterochromatin regions in some chromosomes, as well as interstitial and terminal bands (Figure 1b and 1d). The ZZ pair showed centromeric and terminal C-bands on both chromosome arms. In all metaphases of females, the W chromosome had similar centromeric and terminal heterochromatin bands on the long arm, when compared to the Z chromosome. The heterochromatin segments, however, occupied almost the entire short arm of the W chromosome, being twice as large as in the Z chromosome (Figure 1b, d). The nucleolar organizer regions stained with silver (Ag-NORs) showed terminal signals on the long arm of one chromosome pair, pair 11, which was subtelocentric (Figure 2, box), while FISH with 18S rDNA probes showed up to five terminal signals on the long arm of pairs 11 (larger and more evident marks), 7 and 22 (smaller in size), and all these signals generally coinciding with GC-rich regions (Figure 2a). FISH with 5S rDNA probes showed only one marked pair, located in the interstitial region of the long arm of chromosome pair 14 (Figure 2a). *Apareiodon hansemani* did not show pPh2004 satellite DNA sites (data not shown), but W*Ap* satellite DNA sites were found in the terminal regions of some chromosomes of the karyotype (Figure 2b). The Z chromosome showed the satellite DNA W*Ap* in the terminal region of the short and long arms and in the proximal region of the short arm (Figure 2bB). In turn, the W chromosome also showed a W*Ap* amplification from the proximal region of the short arm (Figure 2b).

Discussion

Karyotype analyses of *Apareiodon hasemani* (Figure 1) show a diploid number of 54 chromosomes, similarly to other species of Parodontidae, except for the population of *A. affinis* from the Upper Paraná River basin (Moreira-Filho *et al*., 1980), which has 2n = 54/55 chromo-

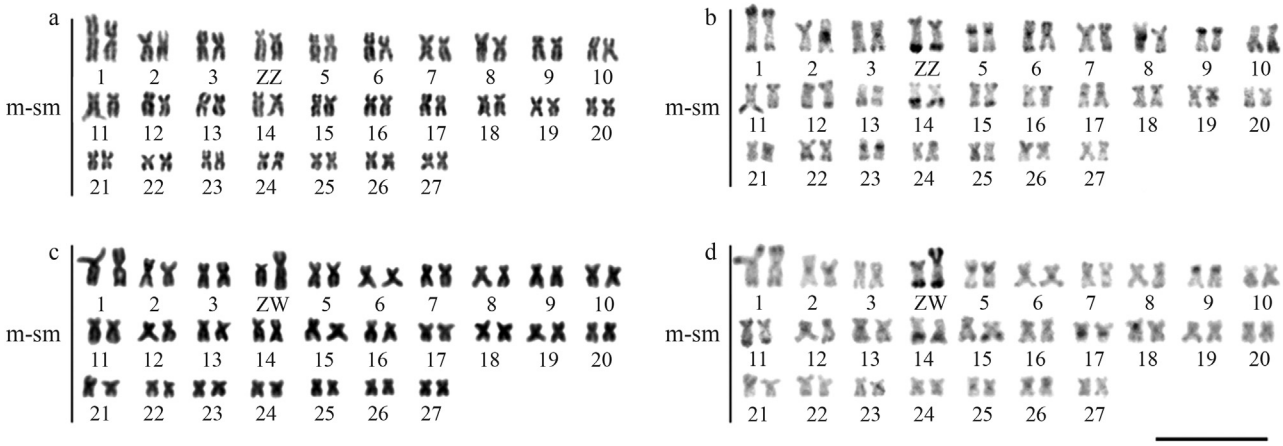

Figure 1 - Karyotypes of males and female *Apareiodon hasemani* arranged from Giemsa-staining (a, c) and C-banding (b, d). Bar = 10 μm.

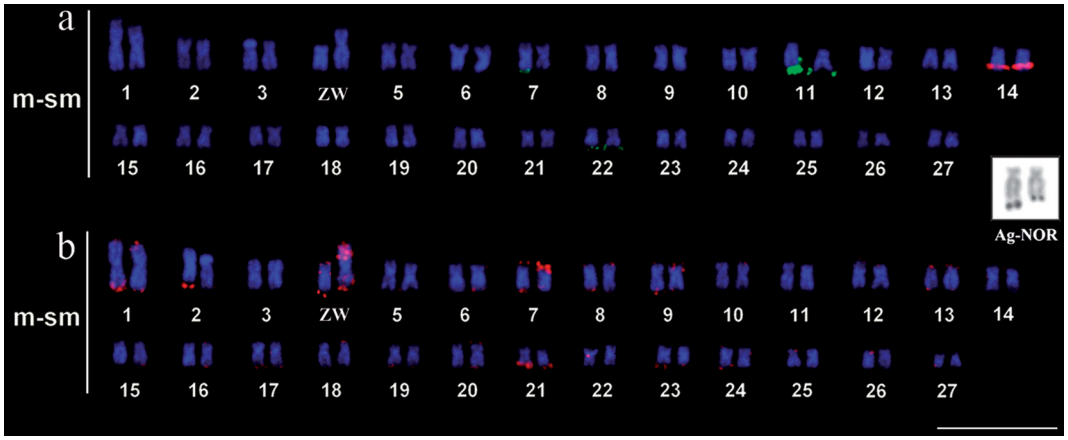

Figure 2 - Chromosomal markers in *Apareiodon hasemani* (a) representative double-FISH karyotype, showing five 18S rDNA sites and chromosome pair 14 bearing 5S rDNA sites. (b) representative the W*Ap* signals. The nucleolar organizer regions revealed by silver nitrate (Ag-NOR) staining are shown in the insert box. Bar = 10 μm.

somes. The chromosome morphology of *A. hasemani* consists mainly of meta-submetacentric chromosomes, a characteristic shared with *A. affinis* (Moreira-Filho *et al*., 1980), *P. hilarii* (Moreira-Filho *et al*., 1993) and *P. moreirai* (Centofante *et al*., 2002). In addition to meta-submetacentric chromosomes, other species also have subtelocentric chromosomes (Moreira-Filho *et al*., 1985; Jesus and Moreira-Filho 2000a, b; Bellafronte *et al*., 2005; Vicari *et al*., 2006; Rosa *et al*., 2006). Populations of *A. affinis* from the Lower Paraná and Paraguay River basins showed a structural polymorphism with the presence of acrocentric chromosomes, probably caused by pericentric inversions (Jesus *et al*., 1999; Jorge and Moreira-Filho, 2000).

The location of the major NORs sites of *A. hasemani* in the terminal region of the long arm of a subtelocentric chromosome pair, revealed by silver nitrate staining and the 18S rDNA probes (Figure 2a, box), is a character shared by other species of the genus *Apareiodon* and by *P. nasus* (Bellafronte *et al*., 2005; Vicari *et al*., 2006; Rosa *et al*., 2006; Bellafronte *et al*., 2009, 2011). In populations of *A. affinis* in the Lower Paraná and Paraguay basins, acrocentric chromosomes originated from pericentric inversions are NOR carriers (Jesus *et al*., 1999; Jorge and Moreira-Filho, 2004). Additional sites were also found in *A. hasemani*, in a characteristic shared with *A. ibitiensis* and *A. vittatus*. . Dispersal events may have originated the additional 18S rDNA sites in these species, since they are found in more than one chromosome pair (Jesus and Moreira-Filho, 2000a; Bellafronte *et al*., 2009, 2011, this study). Considering that NORs are generally associated with terminal heterochromatin, chromosome rearrangements and transpositions become more likely, which may result in dispersion in the genome (Moreira Filho *et al*., 1984).

FISH with 5S rDNA probes in Parodontidae revealed that these cistrons are often located on the short arm of one of the submetacentric chromosome pairs (Centofante *et al*., 2002; Vicari *et al*., 2006; Bellafronte *et al*., 2009, 2011) (Figure 2a). Additional sites may, however, occur in different locations in *A. vladii*, *P. hilarii*, *P. pongoensis* and *P. nasus*, which show a case of synteny with the 18S rDNA gene (Vicente *et al*., 2001; Bellafronte *et al*., 2005; Rosa *et al*., 2006). *Apareiodon hasemani* and most species of Parodontidae show only one submetacentric pair carrying 5S rDNA sites; however, the cistron is located in the subterminal region of the long arm. It is likely that this pattern originated from a pericentric inversion, since in other Parodontidae species and in the outgroup Anostomidae the 5S rDNA sites are located on the short arm (Bellafronte *et al*., 2011). A similar case was observed in the genus *Astyanax* (Kavalco *et al.* 2004), where *A. intermedius* shows all 5S rDNA sites located on acrocentric chromosomes, while *A. guiton* has a marked subtelocentric pair besides the aforementioned acrocentric chromosomes, probably originated by a pericentric inversion.

Apareiodon hasemani shares a ZZ/ZW heteromorphic sex chromosome system with *P. hilarii*, *P. moreirai*, *A. vladii*, *A. ibitiensis* and *Apareiodon* sp. (Bellafronte *et al*., 2011). The distribution of constitutive heterochromatin in *A. hasemani* (Figure 1b,d) is characterized by centromeric bands in some chromosome pairs, large interstitial and terminal blocks, and blocks adjacent to the NORs, which have also been found in several other species of Parodontidae (Moreira-Filho *et al*., 1984; Jesus and Moreira-Filho 2000a,b; Vicente *et al*., 2001, 2003; Centofante *et al*., 2002; Bellafronte *et al*., 2005; Rosa *et al*., 2006; Vicari *et al*., 2006). Moreover, the Z sex chromosome exhibited a correspondence of proximal and terminal bands on short arms with those of other parodontids bearing sex chromosomes (Bellafronte *et al*., 2011; Schemberger *et al*., 2011). Schemberger *et al*. (2011) inferred that the Z and W chromosomes of Parodontidae were initially a homologous pair, and that heterocromatinization through the accumulation of W*Ap* sequences was a decisive step in the differentiation of the W chromosome.

On the other hand, the terminal heterochromatic block in the long arm of the Z and W chromosomes of *A. hasemani* (Figure 2b) led to a size increase of the long arm of these chromosomes, in relation to other species in the group. Thus, with the increase in the long arm of the Z chromosome due to this terminal heterochromatin, the heterochromatic differentiation of the W chromosome in the short arm region did not lead to a sufficient size increase to exceed the total length of the long arm (Figure 3), a common state observed among the other parodontid carriers of heteromorphic sex chromosomes. In this proposed scenario, not only would the W chromosome change in shape and size, but the Z chromosome would also have increased in size in relation to a possible ancestral condition. This mechanism is in agreement with the model for the evolution of heteromorphic sex chromosomes (Muller, 1964; Charlesworth, 1978; Green, 1990).

During the evolution of the heteromorphic sex chromosomes XY or ZW, there is a suppression of meiotic recombination between a pair of homomorphic chromosomes (homologues), which progressively accumulate sex determining genes, followed by structural chromosomal rear-

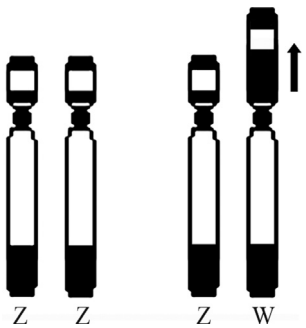

Figure 3 - Hypothetical derivation of the W chromosome from an ancestral chromosome similar to the Z chromosome in *Apareiodon hasemani*.

rangements such as deletions, insertions, accumulation of transposable elements, and expansion of repetitive sequences (Schartl, 2004; Kasahara, 2009; Livernois *et al*., 2012). In recent years, repetitive DNA sequences have been widely studied in fishes, providing important information on the differentiation and evolution of sex chromosomes. These studies have shown that the differentiation of sex chromosomes is frequently associated with the accumulation of such DNA sequences on chromosomes (Vicente *et al*., 2003; Parise-Maltempi *et al*., 2007; Machado *et al*., 2011). FISH with the sequence pPh2004 in *A. hasemani* showed that this satellite DNA is not present in the karyotype or in species with heteromorphic ZW sex chromosomes : *A. ibitiensis*, *Apareiodon* sp. and *A. vladii* (Bellafronte *et al*., 2011).

Furthermore, the W*Ap* satellite DNA probe (Schemberger *et al*., 2011) detected the W chromosome amplification region of *A. hasemani*, suggesting that this system is common to other parodontid carriers of sex chromosomes. In the family Parodontidae, all species studied so far have positive sites for this marker, suggesting a common origin of this satellite DNA and therefore of the sex chromosome systems (Schemberger *et al*., 2011). The simple ZZ/ZW sex chromosome systems may have originated from a paracentric inversion of a W*Ap* terminal site to the proximal region of the short arm of a metacentric chromosome pair, with the subsequent amplification of this sequence leading to differentiation of the W sex chromosome in most species. Through the use of chromosome markers, Schemberger *et al*. (2011) demonstrated the existence of closely related species groups: (i) those without heteromorphic sex chromosomes (*A. piracicabae* and *A. vittatus*); (ii) those with proto sex chromosomes and the presence of satellite DNA pPh2004 (*P. nasus* and *P. pongoensis*); (iii) those with a heteromorphic sex chromosome system (*A. ibitiensis* and *A. vladii*); (iv) those with a heteromorphic sex chromosome system and the presence of satellite DNA pPh2004; and (v) a species with a multiple sex chromosome system and satellite DNA pPh2004 (*A. affinis*). *Apareiodon hasemani* shows chromosomal markers similar to those found in *A. ibitiensis*, *Apareiodon* sp. and *A. vladii* (Bellafronte *et al*., 2011; Schemberger *et al*., 2011); however, the location of the 5S rDNA can be considered derived in *A. hasemani*.

The main Brazilian rivers are separated by barriers that prevent the dispersal of species and populations, favoring the occurrence of events that cause the isolation of groups (Weitzman *et al*., 1988). By such conditions, unique karyotypes and distinct sex chromosomes systems in fish may have become fixed independently in different species of a genus, promoting diversification and contributing to the speciation process (Centofante *et al*., 2001). Although allopatry may have contributed to the origin of sex chromosome systems, Moreira-Filho *et al*. (1985) reported the occurrence of four species of Parodontidae in sympatry and syntopy, so the different sex chromosomes systems in some species would play an important role in their isolation. This study found that the repetitive DNA in Parodontidae seemed to play an important role in the chromosome diversification of the studied species, as in *A. hasemani.* Furthermore, *A. hasemani* presented derived chromosomal regions with respect to 5S rDNA, contributing to the genetic isolation of the population and differentiation of strains.

Acknowledgments

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