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Karyotype description and evidence of multiple sex chromosome system $X_1X_1X_2X_2/X_1X_2Y$ in *Potamotrygon* aff. *motoro* and *P. falkneri* (Chondrichthyes: Potamotrygonidae) in the upper Paraná River basin, Brazil

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Cytogenetic analysis of *Potamotrygon* aff. *motoro* and *P. falkneri* indicated the occurrence of an $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system in both species, with 2n = 66 chromosomes for females and 2n = 65 chromosomes for males. The nucleolus organizer regions (NORs) identified using Ag-NOR technique showed that both species have multiple Ag-NORs (5 to 7 chromosomes stained). C-banding technique indicated the presence of heterochromatic blocks in the centromeric regions of almost all chromosomes in both species. Through this study there was evidence of heterogeneity in the karyotypes, which suggests that chromosomal rearrangements such as inversions and/or translocations occurred during the chromosomal evolution in two species of this genus.

Análises citogenéticas de *Potamotrygon* aff. *motoro* e *P. falkneri* identificaram a ocorrência de um sistema múltiplo de cromossomos sexuais do tipo $X_1X_1X_2X_2/X_1X_2Y$, em ambas as espécies, com 2n = 66 cromossomos em fêmeas e 2n = 65 cromossomos nos machos. As regiões organizadoras de nucléolos (RONs) identificadas pela reação Ag-RON, evidenciaram marcações múltiplas em ambas as espécies (com variações de 5 a 7 RONs). A técnica de bandamento C, revelou a presença de blocos heterocromáticos localizados nas regiões centromérica em quase todos os cromossomos nas duas espécies em estudo. Através do presente estudo foi evidenciada uma heterogeneidade nos cariótipos, permitindo sugerir que rearranjos cromossômicos, como inversões e/ou translocações, ocorreram durante a evolução cromossômica nas duas espécies desse gênero.

Key words: Cytogenetic, NOR, C-band, Fish, Stingrays.

Introduction

Although cytogenetic studies in fish have considerably increased lately, the Chondrichthyes remain among the least studied groups (Rocco *et al*., 2004), with only about 6% of approximately 1,100 currently living species karyotyped (Stingo & Rocco, 2001). Cytogenetic data on marine rays suggest a variation in the diploid number from 2n = 28 for *Narcine brasiliensis* (Donahue, 1974) to 2n = 104 for *Okamejei meerdervoortii* (Makino, 1937). The cytogenetic data available in literature for freshwater rays of the family Potamotrygonidae suggest that the species *Potamotrygon motoro* and *P. orbignyi* have the same diploid number of 2n = 66 , whereas *Paratrygon aiereba* presents 2n = 90 (Valentim *et al*., 2006).

Fish represents an extremely heterogeneous group regarding to sex determination presenting eight systems of sex determination controlled by sex chromosomes (Devlin & Nagahama, 2002). In the Neotropical region species with morphologically distinct chromosomes show a great variety of systems (Oliveira *et al*., 2009). However, cytogenetic studies with freshwater rays have not shown the occurrence of heteromorphic sex chromosome systems in this group (Valentim *et al*., 2006).

The present study aimed to investigate the karyotypic structure of *P.* aff. *motoro* and *P. falkneri*, as well as to identify chromosome differences involved in the diversification process of these species, using chromosome banding methods.

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Material and Methods

Cytogenetic studies were carried out with *Potamotrygon* aff. *motoro* and *P. falkneri* specimens from the upper Paraná River basin, collected in Porto Rico, Paraná State (22º47'42.4"S 53º20'29.7"W) and Ilha Solteira, São Paulo State (20º47'42.4"S 51º38'29.7"W), Brazil. We collected 30 *P.* aff. *motoro* specimens, of which 10 females and 13 males were from Porto Rico and 3 females and 4 males from Ilha Solteira. As regards *P. falkneri*, we collected 34 specimens, 12 females and 14 males from Porto Rico and 3 females and 5 males from Ilha Solteira. Voucher specimens were deposited at collection of fish the Laboratório de Biologia e Genética de Peixes (LBP 5202 , LBP 5203, LBP 6716, LBP 6717).

Chromosomes were obtained from gill and kidney tissues using the technique described by Foresti *et al.* (1993). Meiotic metaphases were obtained using the technique described and adapted for fish by Bertollo *et al.* (1978). Silver staining of the nucleolus organizer regions (NOR) followed the technique of Howell & Black (1980), and C-banding was performed according to Sumner (1972). Chromosome morphology was determined based on arm ratio, as proposed by Levan *et al.* (1964), and chromosomes were classified into metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a). Fundamental numbers (FN) were calculated considering metacentric, submetacentric, subtelocentric chromosomes as biarmed and acrocentric as uniarmed.

Results

The species *Potamotrygon* aff. *motoro* and *P. falkneri* had 2n = 66 chromosomes in females and 2n = 65 chromosomes in males. This heteromorphic chromosome found, is due to the occurrence of a sex chromosomes system of the type $X_1X_1X_2X_2/X_1X_2Y$ and as also evidenced by the data of meiosis. The two analyzed species showed different fundamental numbers, as shown in Table 1.

Analysis of meiotic preparation of *P. falkneri* males revealed the presence of a trivalent, besides the 31 bivalents (Fig. 1), reinforcing the hypotheses of occurrence of multiple sex chromosomes.

The Ag-NORs technique indicated the presence of multiple Nucleolus Organizer Regions in both species. The samples of *P.* aff. *motoro* from Porto Rico have stains in the terminal regions of five pairs of chromosomes; they were identified in the short arm of pair two, in the short arm of pair four, in the long arm of pair six, in the long arm of pair sixteen, and in the pair twenty-six (Fig. 2). The samples from Ilha Solteira have stains in the terminal regions of six chromosomes: in the short arm of pair two, in the long arm of pair six, in the long arm of pair twenty, and in of pair twenty-six (Fig. 3). The samples of *P. falkneri* from Porto Rico have stains in the terminal regions of six metacentric chromosomes

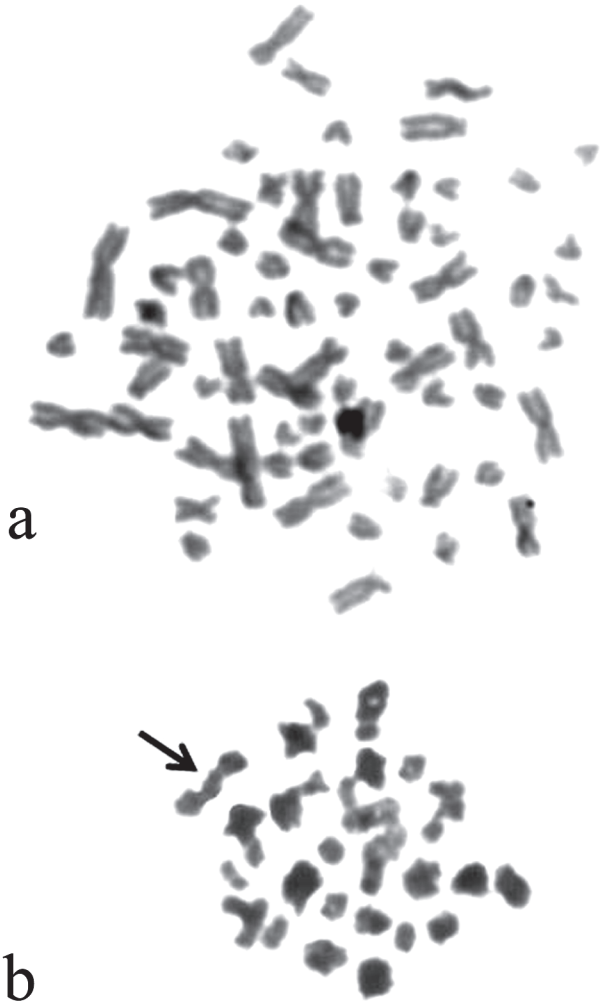

Fig. 1. Meiotic chromosomes of *Potamotrygon falkneri* sample from Ilha Solteira. Spermatogonial metaphase (2n = 65 chromosomes) after Giemsa staining (**a**) and metaphase I, with 31 bivalents and a trivalent (arrow) (**b**).

Table 1. Data and cytogenetic karyotypic formulas analyzed in populations of specimens of species of rays *Potamotrygon* aff. *motoro* and *Potamotrygon falkneri* (DN = diploid number, FN= fundamental number).

Species	Location	DN	FN	Karyotypic formulae	Ag-NOR	C-Banding	Figures
P. aff. *motoro*	Porto Rico - PR	? 66	116	22m+8sm+20st+16a	7	Centromeric	Fig. 2
		? 65	114	21m+9sm+19st+16a	7	Centromeric	
P. aff. *motoro*	Ilha Solteira - SP	? 66	122	20m+10sm+26st+10a	6	Centromeric	Fig. 3
		? 65	120	19m+11sm+25st+10a	6	Centromeric	
P. falkneri	Porto Rico - PR	? 66	110	20m+10sm+14st+22a	6	Centromeric	Fig. 4
		? 65	108	19m+10sm+14st+22a	6	Centromeric	
P. falkneri	Ilha Solteira - SP	? 66	114	20m+10sm+18st+18a	7	Centromeric	Fig. 5
		? 65	112	19m+10sm+18st+18a	7	Centromeric	

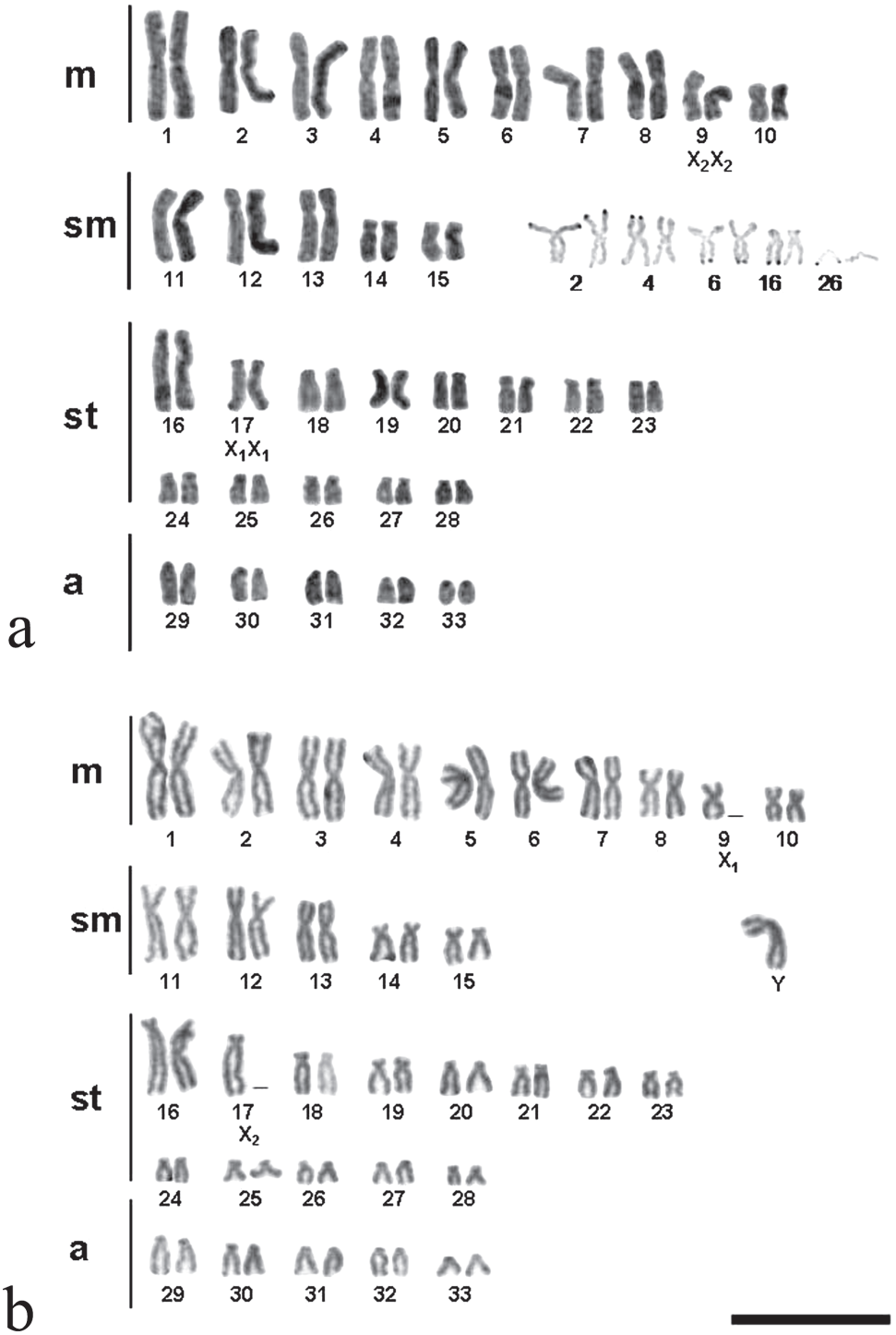

Fig. 2. Karyotypes of female (**a**) and male (**b**) of *Potamotrygon* aff. *motoro* sample from Porto Rico, highlighting the sex chromosomes after conventional and the chromosomes marked by NOR. Scale bar = 10 μm.

and one acrocentric chromosome (Fig. 4). In the sample from Ilha Solteira, stains were also found in the terminal regions of five metacentric and two acrocentric chromosomes (Fig. 5). C-banding pattern indicated that *P*. aff. *motoro* and *P. falkneri* (Fig. 6) have large C-band positive segments located in the centromeric region of most chromosomes.

Discussion

Although many vertebrate species have sex chromosomes morphologically different between males and females, most fish species show no chromosomal difference between sexes; thus, there is great difficulty in identifying

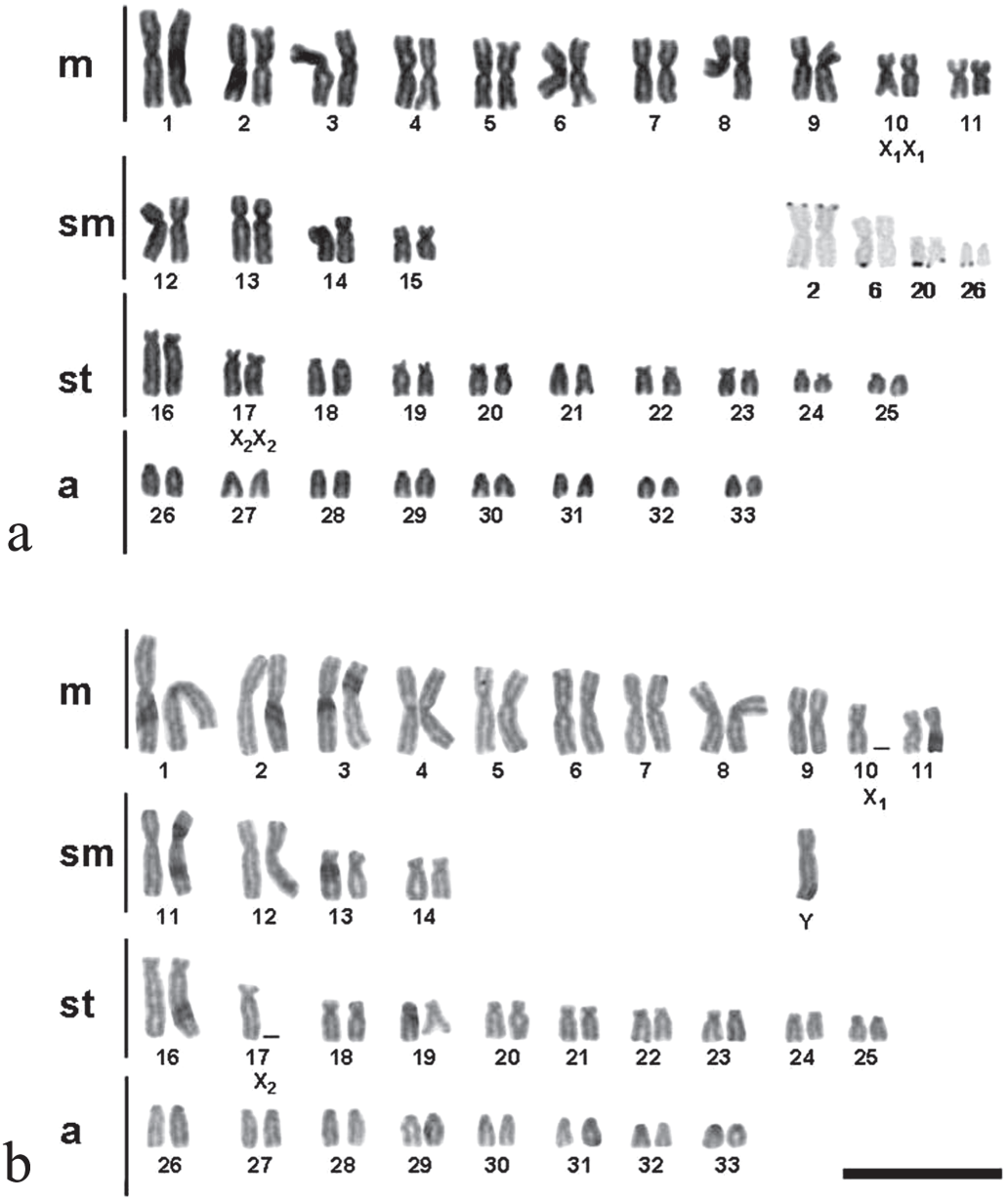

Fig. 3. Karyotypes of female (**a**) and male (**b**) of *Potamotrygon* aff. *motoro* sample from Ilha Solteira, highlighting the sex chromosomes after conventional and the chromosomes marked by NOR. Scale bar = 10 μm.

sex using karyotypic analysis.

In this study, all samples of *Potamotrygon* aff. *motoro* and *P. falkneri* have 2n = 66 chromosomes for females and 2n = 65 chromosomes for males. Such difference in the chromosome number is due to a multiple system of sex determination of the type $X_1X_1X_2X_2/X_1X_2Y$.

Both species have similar distribution of C-band positive segments in the centromeric regions of almost all chromosomes, as already evidenced by Valentim *et al*. (2006) for *Paratrygon aiereba*, *Potamotrygon motoro*, and *P. orbigny*.

In some fish species such as *Pseudotocinclus tietensis* (Andreata *et al*., 1992), *Microlepidogaster leucofrenatus* (Andreata *et al*., 1993), and *Characidium* cf. *fasciatum* (Maistro *et al*., 1998) sex chromosomes are easily identified using techniques like C-banding. However, in this study, the chromosomes involved in sex determination could not be identified through the constitutive heterochromatin patterns obtained for *P*. aff. *motoro* and *P. falkneri* since in their sex chromosomes positive C-band segments are restricted to the centromeric regions.

The analyzed *P*. aff. *motoro* samples have several karyotypic formulae and fundamental numbers, but the sample from Ilha Solteira had a karyotypic formula different from that of the remaining samples due to the presence of different numbers of acrocentric and subtelocentric chromosomes. These variations in the fundamental number

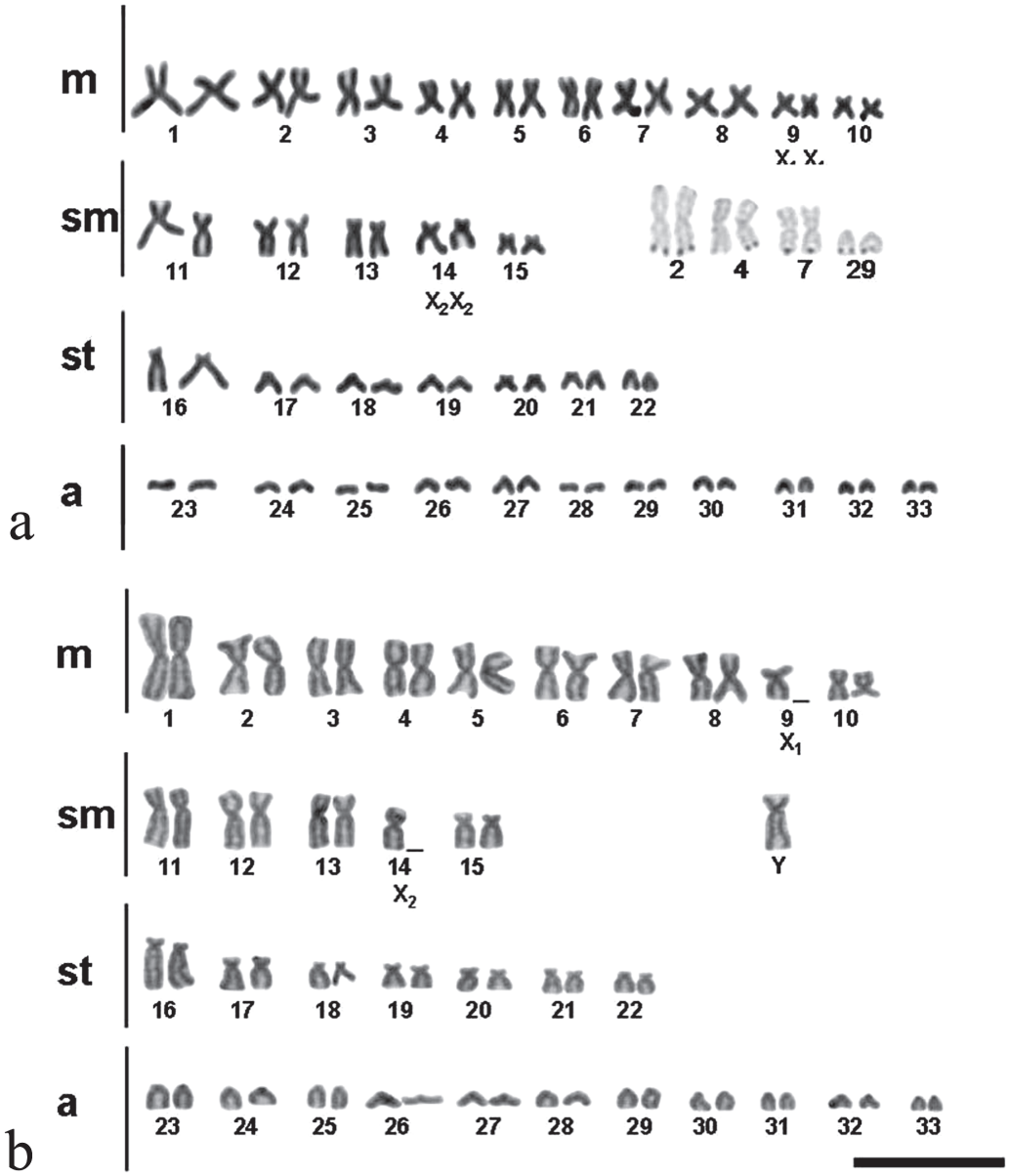

Fig. 4. Karyotypes of female (**a**) and male (**b**) of *Potamotrygon falkneri* sample from Porto Rico, highlighting the sex chromosomes after conventional and the chromosomes marked by NOR. Scale bar = 10 μm.

may be the result of chromosomal rearrangements such as pericentric inversions since the diploid number is conserved. This kind of rearrangement has been pointed as very important in fish chromosome differentiation (Oliveira *et al*., 2009).

Potamotrygon aff. *motoro* and *P. falkneri* had eight to ten chromosomes stained with silver nitrate (Ag-NORs) always in terminal regions of similar size chromosomes. Multiple NORs were also observed in other rays (Valentim *et al*., 2006) and are very common in fishes (Oliveira *et al*., 2007). The differences in NOR sites found in these samples could be explained by Robertsonian rearrangements, resulting in dispersion and/or loss of ribosomal genes.

The occurrence of ribosomal DNA in sex chromosomes has been observed in several groups of organisms. Cistrons of 5.8 S, 18S and 28S ribosomal genes, which form the NORs in animals and are also stained with silver nitrate, were found in sex chromosomes of insects, such as *Drosophila* (Parise-Maltempi & Avancini, 2001), beetles (Juan *et al*., 1993), mammals (Yonenaga-Yassuda *et al*., 1983; Oshida *et al*., 1999), and plants (Nakayama *et al*., 2001). In fish, reports of NORs in sex chromosomes are restricted to *Fundulus diaphanus* (Howell & Black, 1979), *Salvelinus alpinus* (Reed & Phillips, 1995), *Triportheus guentheri* (Artoni & Bertollo 1999), *Hoplias malabaricus* (Born & Bertollo, 2000) and *Hisonotus* sp. *A* (Andreata, 2002).

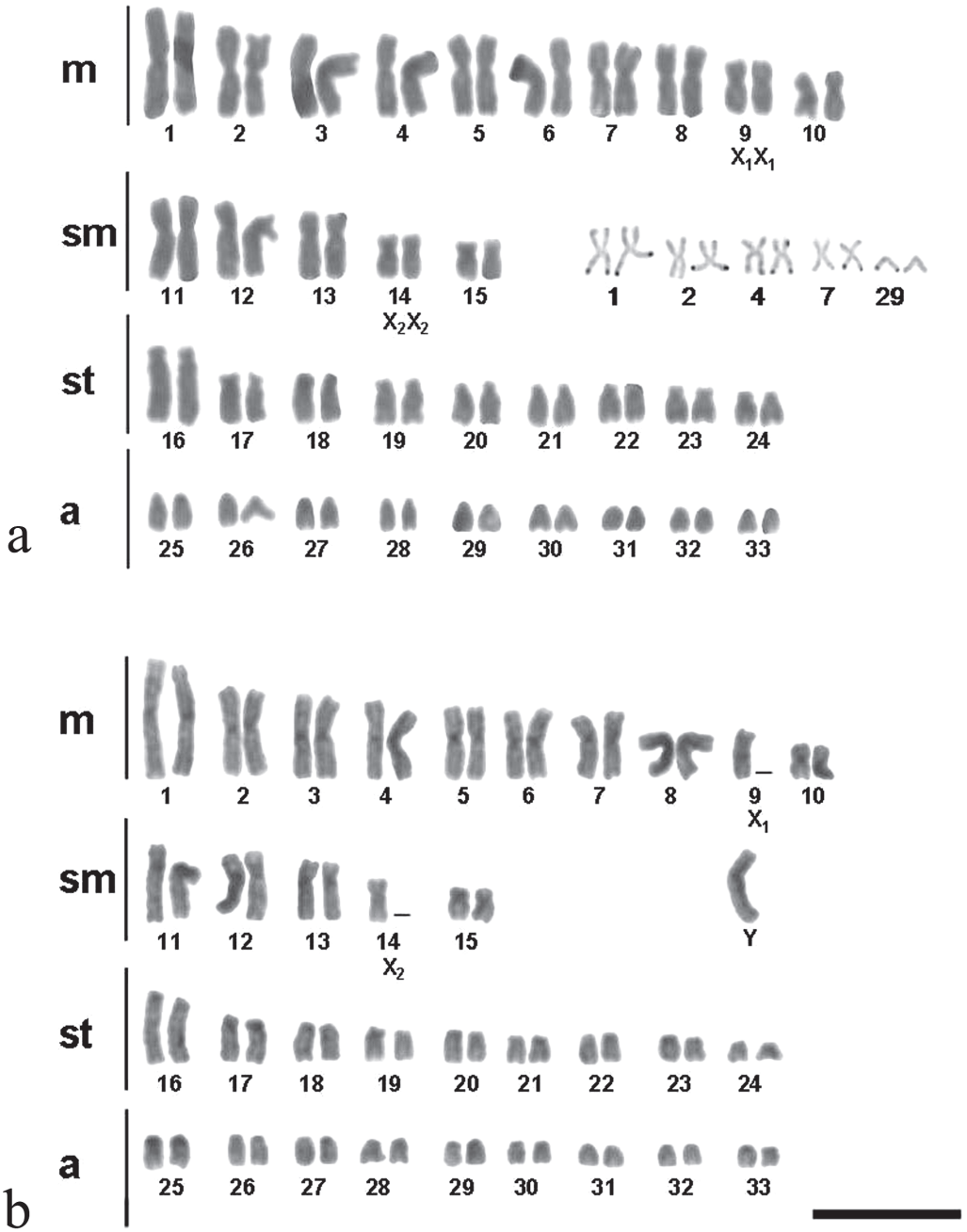

Fig. 5. Karyotypes of female (**a**) and male (**b**) of *Potamotrygon falkneri* sample from Ilha Solteira, highlighting the sex chromosomes after conventional and the chromosomes marked by NOR. Scale bar = 10 μm.

The formation of a multiple sex chromosome system in fish is usually due to a preexisting simple system in a particular group, such as ZZ/Z_0, XX/X_0, ZZ/ZW, or XX/XY. In the studied ray species, the found $X_1X_1X_2X_2/X_1X_2Y$ system could have arisen from reciprocal translocations or from an ancestral system with a simple such as XY where the Y chromosomes fused with an autossomal chromosome giving origin to new Y and X_2 chromosomes, and the old X chromosome is identified as X_1 chromosome. In some fish species, the origin of Y is clear since it has the same characteristics of chromosomes (X_1) and (X_2), such as in *Eigenmannia* species (Almeida-Toledo *et al*., 1984, 2000).

Valentim (2001) reported a possible sex chromosome system of the type XX/X_0 in *Potamotrygon* sp. from the middle rio Negro, Amazonas State, where the female had 2n = 68 chromosomes and the male 2n = 67 chromosomes due to a putative absence of a homologous pair 2 (metacentric) in the male. Different mechanisms of sex determination in

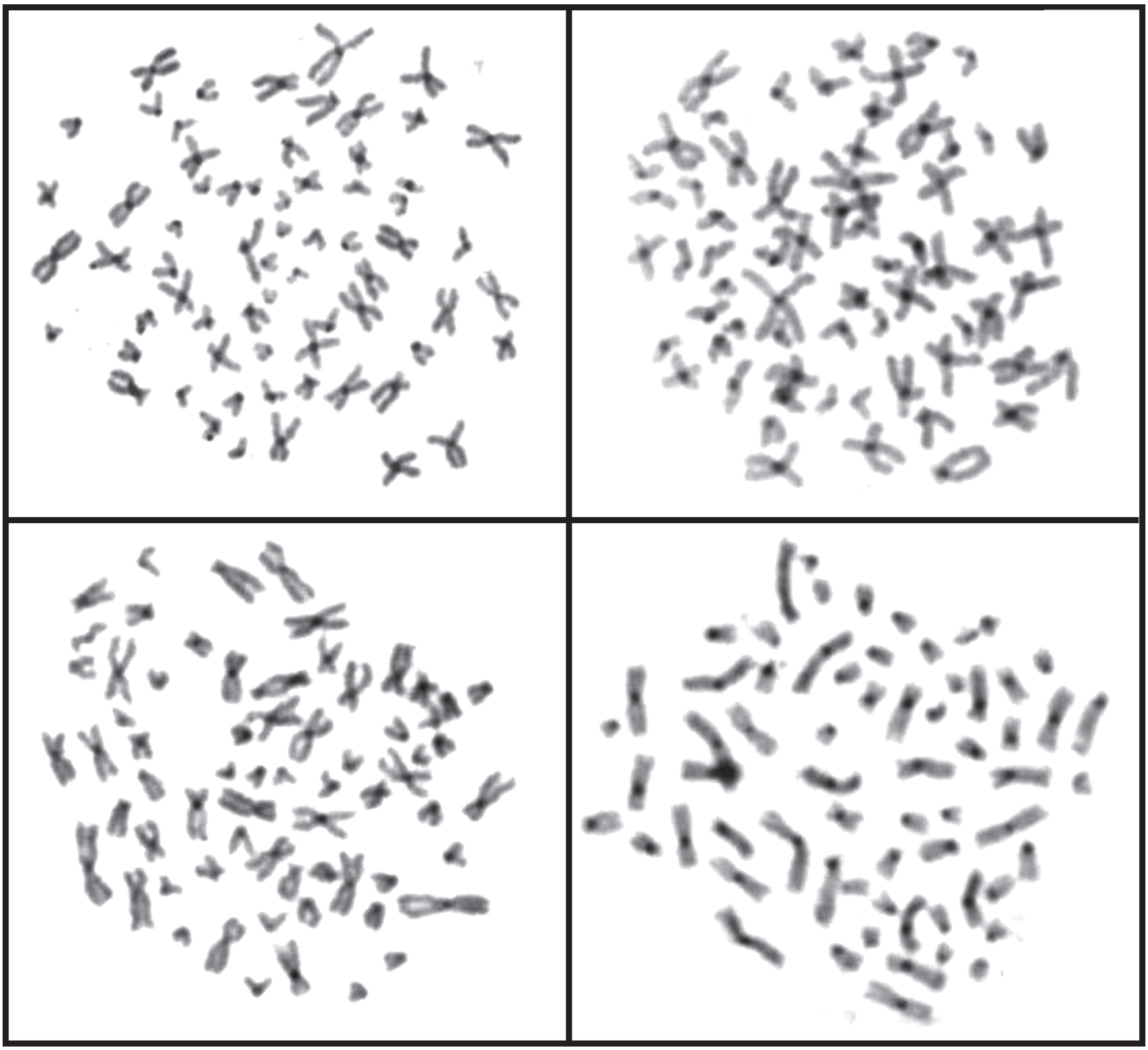

Fig. 6. Somatic metaphases of *Potamotrygon* aff. *motoro*, the population of Porto Rico (**a**), population of Ilha Solteira (**b**), identification of constitutive heterochromatin. Metaphases of *Potamotrygon falkneri* sample from Porto Rico (**c**) and Ilha Solteira (**d**), analysis of constitutive heterochromatin after C-banding technique.

phylogenetically related groups are common in Neotropical fish, as evidenced in several groups such as Gymnotiformes (Almeida-Toledo *et al*., 1984, 2000, 2001) and Loricariidae (Andreata *et al*., 1992, 1993), in which different sex chromosome systems were identified among its representatives.

This study indicated that there is great uniformity concerning the diploid number in the studied species. However, variations in the number of chromosome arms suggest that extensive chromosomal rearrangements such as pericentric inversions occurred during the chromosomal evolution in the two analyzed species. If, on the one hand, the uniformity in diploid numbers and mechanisms of chromosome heteromorphisms related to sex showed a closer relationship between what is shown for *P*. aff. *motoro* and *P. falkneri*, it can be assumed that the occurrence of such chromosomal polymorphism may reflect the existence of not only two freshwater ray species in this environment, but also a complex group of species that need to be further studied. A consistent understanding of the evolutionary relationships between variable karyotypes and the mechanisms of *P*. aff *motoro* and *P. falkneri* sex chromosomes will depend on new cytogenetic and molecular information from other species and samples of freshwater rays.

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