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Research Article

Multiple interstitial ribosomal sites (NORs) in the Brazilian squirrel *Sciurus aestuans ingrami* (Rodentia, Sciuridae) with 2n = 40. An overview of *Sciurus* cytogenetics

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Abstract

This is the first time the karyotype of the Brazilian squirrel *Sciurus aestuans ingrami*, with 2n = 40, is described. The karyotype of this species comprises 18 pairs of biarmed and one minute pair of acrocentric autosomes, a medium-sized submetacentric X and a medium-sized acrocentric Y. Four pairs have an interstitial secondary constriction, co-located with nucleolar organizer regions (NORs), identified by silver-staining technique and fluorescent in situ hybridization (FISH) with ribosomal 18S/28S probes. The occurrence of multiple interstitial NORs is rare in rodents, and this is one of the few examples, identified by a molecular cytogenetics approach.

Key words: Squirrels, chromosomes, karyotype, NORs, Rodentia.

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Introduction

Squirrels of the genus *Sciurus* Linnaeus (1758), especially those from Central America, present a wide geographical variation in pelage coloration. Systematics of this group is rather puzzling. Currently, 50 genera and 273 species of squirrels are recognized in the world. Only 4 genera and 19 species from Neotropical forests were known. Hoffmann *et al.* (1993) recognized four species in Brazil: *S. ignitus* (westernmost region), *S. igniventris* (Amazon region), *S. spadiceus* (southern Amazon), and *S. aestuans*. There are some taxa included in *S. aestuans* which have been considered as full species by some authors*: S. a. aestuans*, found mainly in the northern Amazon; *S. a. gilvigularis*, from the southern Amazon and Venezuela; *S. a. alphonsei*, found along the coast, from southern Pará to Pernambuco; and *S. a. ingrami*, found all along eastern Brazil, from Bahia to Rio Grande do Sul (Emmons, 1997).

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Chromosome data on Brazilian squirrels are scarce in the literature. Lima and Langguth (2002) described the karyotypes of the Brazilian species *S. alphonsei* and *S. spadiceus*, with 2n = 40 and FN = 76. North-American squirrels display different degrees of chromosomal variation. Terrestrial squirrels show a striking diversity, mainly due to Robertsonian rearrangements and pericentric inversions (2n = 30 to 50). In contrast, tree squirrels of the genus *Sciurus* exhibit a remarkable chromosomal homogeneity, with a 2n = 40 karyotype. Species from the Brazilian Atlantic forest (*S. alphonsei*) and Amazon forest (*S. spadiceus*) were observed to have 2n = 40 and FN = 76 (Lima and Langguth, 2002). Only one South-American squirrel (*Sciurus granatensis*) was described with a 2n = 42 karyotype (Nadler and Hoffmann, 1970).

It is difficult to assess the basic or ancestral karyotype of Sciuridae, because centric fusions cannot be distinguished from fissions, but it is generally accepted that South-American sciurids evolved from ancestral North-American stock (Nadler and Hoffmann, 1970). In this paper, we present the karyotype of two Brazilian *S. a. ingrami* (2n = 40) females and one male, which presented a

pattern of multiple interstitial NORs that is unusual in rodents.

Material and Methods

Our sample is composed of three Brazilian specimens: one female from the southernmost region, city of Venâncio Aires, state of Rio Grande do Sul (29°36'W, 52°19'S); and two specimens from the southeastern region, state of São Paulo: one female from the city of Piedade (23°42'W, 45°25'S), and one male from the city of Juquitiba (23°55'W, 47°04'S). Metaphase preparations were obtained from bone marrow, after *in vivo* injection of colchicine, and from fibroblasts of tail biopsy (one female), cultured in Dulbecco's Modified Eagle medium supplemented with 20% fetal bovine serum, according to conventional procedures. Metaphase cells were spread onto clean slides, air-dried, and stored at -20 °C until use. Slides were stored for several months. C-banding and silver-nitrate staining were performed according to routine techniques. We were unable, however, to obtain G-banding patterns with a good resolution.

We also mapped ribosomal genes, using *in situ* hybridization with a probe containing 18/28S rDNA of *Xenopus laevis* (HM456). Ag-staining was performed according to Howell and Black (1980), with some modifications. For FISH, slides were treated with RNAse and pepsin, denatured (70% formamide/2xSSC at 70 °C for 5 min), and 200 ng of biotinylated probe (50 µL) in a hybridization mixture (50%formamide/2xSSC, 10% dextran sulfate) were applied onto the slides. Hybridization was performed overnight in a moist chamber at 37 °C. FITC-avidin and biotinylated anti-avidin antibody (Vector) were used to detect the probe hybridization signals. After detection, slides were mounted in antifade (Vectashield) staining solution containing propidium iodide (0.5 µL/mL) and DAPI (0.8 µL/mL). Metaphases were analyzed in a Zeiss Axiophot microscope, using dual-band pass filter, and photographed with 400 ISO film (Fuji).

For the localization of telomeric sequences, FISH of the digoxigenin-labeled $(TTAGGG)_n$ oligomer (Oncor Inc.) was performed following the recommended protocol. Chromosomes were photographed using ASA 400 Kodak Kodachrome film.

Results

Eighteen biarmed chromosome pairs of gradually varying sizes and one minute acrocentric pair (n. 19) of autosomes, one medium-sized submetacentric X, similar in size to pair n. 5, and the single medium-sized acrocentric Y compose the karyotype of *S. a. ingrami* (2n = 40, FN = 74). Eight secondary constrictions were observed interstitially on the long arms of four chromosome pairs: the six large submetacentrics of pairs n. 1, 2, and 3, and the two small submetacentrics of pair n. 17 (Figure 1).

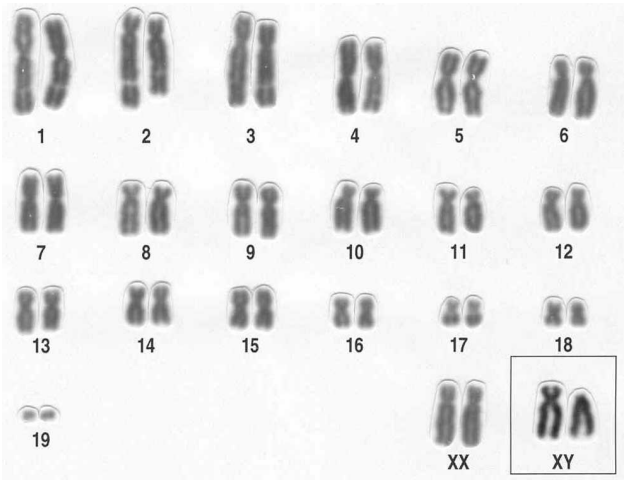

Figure 1 - Giemsa-stained karyotype of a *S. a. ingrami* female (2n = 40, NF = 74) showing secondary constrictions on long arms of pairs 1, 2, 3, and 17. In the square : the XY chromosomes of a male.

C-banding revealed slightly stained pericentromeric heterochromatin on some pairs of autosomes (1, 2, 3, 4, 6, 8, 9, 10, and 14) and on the X chromosome. Heterochromatin is also observed at the secondary constriction of the small submetacentric chromosome 17 (Figure 2). The Y chromosome is not heterochromatic (data not shown). Figure 3a shows unbanded chromosomes after DAPI staining, which could explain the absence of G-banding after trypsin treatment.

Silver nitrate staining revealed Ag-NORs co-located to secondary constrictions in pairs 1, 2, 3, and 17. In 20 metaphases analyzed from one female, there was a variation from 5 to 7 Ag-NORs per cell, their frequency being of 100% on pairs 1 and 3, 97.5% on pair 17, and 22.5% on pair 2. The FISH technique showed 8 signals, which were coincident with secondary constrictions in all cells analyzed

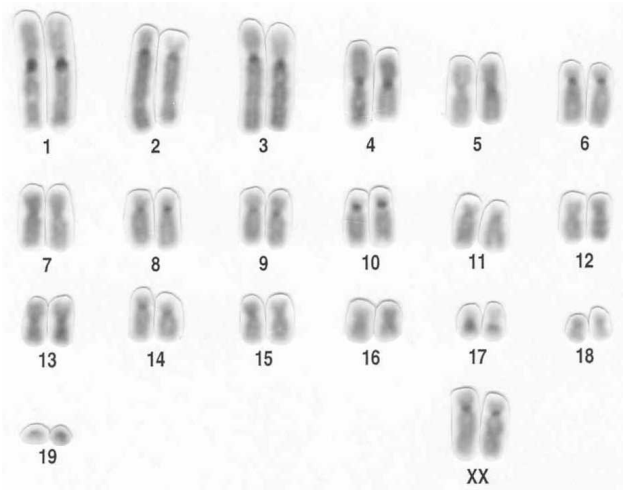

Figure 2 - C-banded karyotype of a *S. a. ingrami* female (2n = 40, FN = 74) showing small pericentromeric heterochromatin in pairs 1, 2, 3, 4, 6, 7, 9, 10, and 11, and at the secondary constriction of the submetacentric 17.

(Figure 3b). Localization of telomeric sequences revealed signals restricted to termini of all chromosomes only (Figure 3c).

Discussion

Chromosome data on squirrels from the New World are scarce. Most studies have focused on Eurasian, Japanese and South African squirrels. This is the second paper presenting chromosomal data of a Brazilian squirrel.

Arboreal squirrels were thought to present a striking karyotype homogeneity (Nadler and Hoffmann, 1970), but a review of squirrel chromosome data, presented in Table 1, showed that there is no such homogeneity. The diploid number 40 is a general feature in animals of the genus *Sciurus*, but their karyotypes are clearly different, as shown by conventional staining, C-banding and Ag-NOR patterns.

The karyotype of *S. a. ingrami* (2n = 40, NF = 74) differs from those of the Brazilian species *S. alphonsei* and *S. spadiceus* (2n = 40, FN = 76) in that its chromosome pair 19 is acrocentric instead of metacentric. This variation could be due to a pericentric inversion occurred in this pair. Chromosome pairs 1, 2, and 3 of *S. a. ingrami* presented interstitial secondary constrictions on the long arms, whereas, in the two Brazilian species, they were observed only in pair 1. The highly condensed morphology of the chromosomes of *S. spadiceus* and *S. alphonsei* may have been an impediment for the visualization of secondary constrictions. The X and Y chromosomes seem to be similar in these species. *S. a. ingrami* differs from the Venezuelan species *S. granatensis* (2n = 42, FN = 78) basically by the presence of 19 meta/submetacentric autosomes and one small acrocentric in the latter.

As compared to European/Asian species, *S. a. ingrami* differs from the Iranian *S. anomalus* (2n = 40, NF = 76) basically by the presence of one minute acrocentric and four pairs of chromosomes with secondary constrictions, in contrast with one small subtelocentric in the latter. *S. a. ingrami* differs from the Korean *S. vulgaris coreae* (2n = 40, NF = 72) by the presence of 17 meta/submetacentrics and two acrocentric pairs of autosomes in the latter. NORs were not described in the species *S. anomalus, S. granatensis and S. vulgaris coreae*. Regarding heterochromatin, *S. vulgaris coreae* presented blocks at the centromeres and telomeres of all autosomes, whereas only some weak signals were observed in 10 pairs of chromosomes of the *S. a. ingrami* presented here.

Pericentric inversion seems to be the most frequent mechanism to explain the differences among squirrel karyotypes.

The telomeric DNA distribution in the genome of the antelope ground squirrel *Ammospermophilus harrisii* (2n = 32), which presented large C-banded regions, showed that all C-banded segments, except a few intercalary segments, hybridized to this DNA (Pathak *et al*., 1998). *S. a. ingrami*

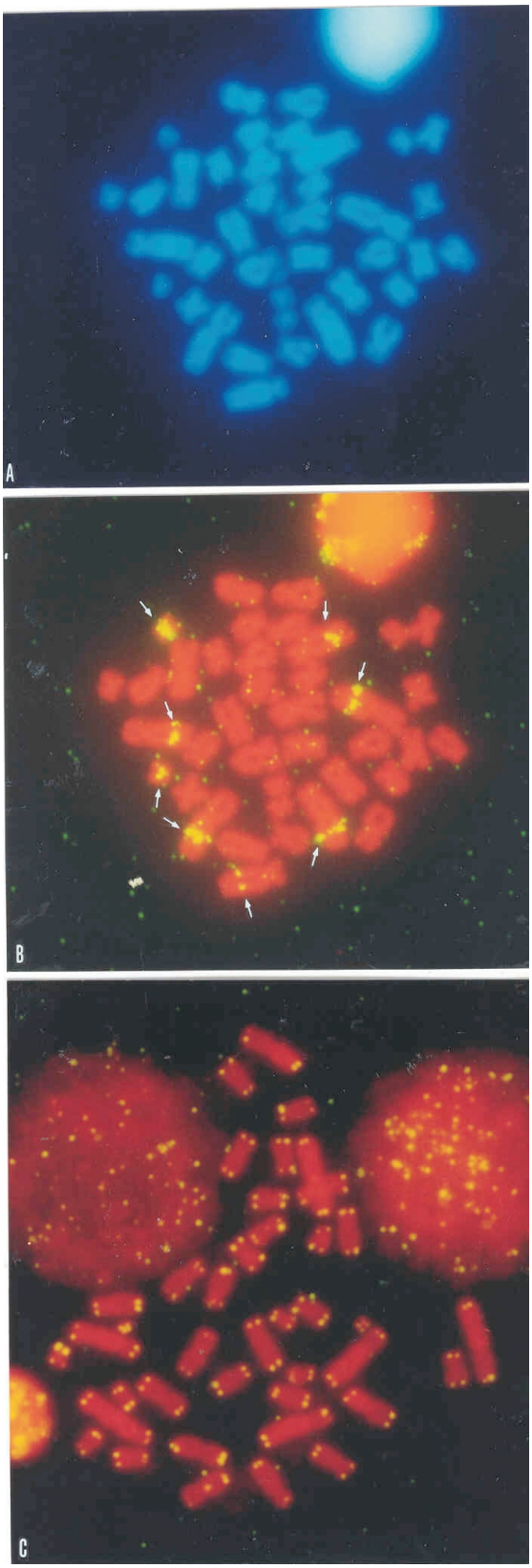

Figure 3 - (*In situ* hybridization) FISH in *S. a. ingrami* (2n = 40): a) DAPI staining; b) same metaphase using ribosomal gene probes, showing eight signals at secondary constrictions on pairs 1, 2, 3, and 17; c) another metaphase using telomere probes, showing terminal signals on all chromosomes.

Table 1 - Diploid numbers (2n), fundamental numbers (FN) and location of Ag-NORs of terrestrial and arboreal representatives of the subfamily Sciurinae.

Habit	Species	2n	FN	location of the Ag-NORs	References
Terrestrial	*Ammospermophilus harrisii*	32		Not examined	Pathak *et al.* 1998
	Ammospermophilus sp	32	60	Not examined	Nadler and Sutton, 1962
	Tamias (Eutamias) sp	38	58-60	Not examined	Nadler *et al.* 1969
	Tamias sibiricus asiaticus	38	60	Not examined	Kim and Lee, 1990
	Tamias striatus	38	58	Not examined	Nadler, 1964
	Spermophilus perotensis	32	58	Not examined	Uribe-Alcocer and Ahumada, 1990
	Spermophilus (spermophilus) sp	30-46	56-68	Not examined	Nadler, 1966
	Spermophilus spilosoma	32	58	Not examined	Uribe-Alcocer and Ahumada, 1990
	Spermophilus (Urocitellus) sp	32-34	60-64	Not examined	Nadler, 1969
	Spermophilus (Ictidomys) sp	32-34	60-64	Not examined	Nadler, 1966a
	Spermophilus (Poliocitellus) franklinii	42	66	Not examined	Nadler, 1966b
	S. (Otospermophilus) sp	38	72	Not examined	Nadler, 1966b
	S. (Callospermophilus) sp	42	78	Not examined	Nadler, 1966b
	S. (xerospermophilus) sp	36	68	Not examined	Nadler, 1966b
	Spermophilus suslicus	36 34	72 68	Terminal region of 3 autosome pairs	Koralev *et al.* 1991; Koralev, 1994
	Cynomys (cynomys) ludovicianus	50	96	Not examined	Nadler and Hoffmann, 1970
	Cynomys (Leucocrossuromys) sp	40-50	72-96	Not examined	Nadler and Hoffmann, 1970
	Marmota sp	36-42	62-66	Not examined	Nadler, 1968
	Spermophilopsis leptodactylus	38	70	Not examined	Nadler *et al.* 1969
Arboreal	*Callosciurus flavimanus*	40	74	Not examined	Nadler and Hoffmann, 1970
	Menetes berdmorei	62	76	Not examined	Nadler and Hoffmann, 1970
	Dremomys rufigenis	38	62	Not examined	Nadler and Hoffmann, 1970
	Petaurista petaurista grandis	38	72	Not examined	Oshida *et al.* 1992
	Petaurista petaurista melanotus	38	72	Not examined	Oshida *et al.* 1992
	Petaurista alborufus castaneus	38	72	Interstitial at the secondary constriction and at distal end on the long arm of pairs 8, 12, and 13	Oshida *et al.* 2000
	Petaurista alborufus lena	38	72	Satellite region on the short arm of pair 16 and distal end on the short arm of pair 17	Oshida *et al.* 1992, Oshida *et al.* 2000
	Petaurista leucogenys	38	72	Interstitial at the secondary constriction of pair 3 and at distal end of pair 6	Oshida and Obara, 1993; Koralev *et al.* 1991
	Pteromys volans orii	38	68	At distal end of pair 17	Oshida and Yoshida, 1996
	Sciurus vulgaris coreas	40	72	Not examined	Kim and Lee, 1990
	Sciurus sp	40	74-76	Not examined	Nadler and Sutton, 1967
	Sciurus anomalus	40	76	Not examined	Nadler and Hoffmann, 1970
	Sciurus granatensis	42	78	Not examined	Nadler and Hoffmann, 1970
	Sciurus aestuans ingrami	40	74	Interstitial at the secondary constriction of pairs 1, 2, 3, and 17	Present paper
	Sciurus alphonsei	40	76	Not examined	Lima and Langguth, 2002
	Sciurus spadiceus	40	76	Not examined	Lima and Langguth, 2002
	Tamiasciurus sp	46	80-88	Not examined	Nadler *et al.* 1969

presented some pericentromeric heterochromatin and absence of signals on non-telomeric sites.

The presence of multiple interstitial (nucleolus organizer regions) NORs coincident with secondary constrictions, as observed in *S. a. ingrami*, is rare in rodents. Examples are the giant flying squirrels *Petaurista alborufus castaneus* and *P. a. lena*, with secondary constrictions on two chromosome pairs (8 and 13) and Ag-NORs, detected interstitially and at the distal end of pairs 8, 12, and 13 (Oshida *et al*., 2000).

Ag-NORs were analyzed in some squirrel species (Table 1). Some data on arboreal squirrels were found in the literature, revealing interstitial and terminal Ag-NORs. The spotted ground squirrel *Spermophilus suslicus* (2n = 36 and 2n = 34) had one interstitial (pair 3) and two telomeric (pairs 6 and 9) Ag-NORs (Koralev, 1994). The Siberian flying squirrel *Pteromys volans orii* (2n = 38, NF = 68) presented one pair of terminal Ag-NORs on the short arm of chromosome 17. A size variation of Ag-NORs was observed, indicating a variable NOR activity in this species (Oshida and Yoshida, 1996).

The use of FISH allowed us to detect eight chromosomes with NORs in *Sciurus a. ingrami*, while silver staining revealed only seven, suggesting that these techniques should be used together, to the best understanding of the variability and differential activity of ribosomal genes.

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