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Heterogeneity and meiotic behaviour of B and sex chromosomes, banding patterns and localization of $(TTAGGG)_n$ sequences by fluorescence *in situ* hybridization in the neotropical water rat *Nectomys* (Rodentia, Cricetidae)

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A cytogenetic study using fluorescence *in situ* hybridization (FISH) of telomere probes, CBG, GTG and RBG banding patterns and synaptonemal complex data was carried out in 41 specimens of *Nectomys* from three Brazilian states: Pernambuco, Mato Grosso and São Paulo. The specimens presented $2n = 52$, 53, 56 and 57, and the differences in chromosome number were due to the presence of three different supernumeraries and also to the occurrence of tandem fusions. The tandem fusions involved chromosome pairs $3 + 11$ and $5 + 24$ from karyotype with $2n = 56$ that originated pairs 1 and 4 in specimens with $2n = 52$. Sex chromosome polymorphism was also detected, and the X presented three different morphologies, which could be explained by heterochromatin addition/deletion. FISH results revealed interstitial telomeric bands (ITBs) in a submetacentric B, but no ITB was detected in the chromosomes originated by tandem fusion. The supernumeraries presented a remarkable heterogeneity of size and morphology, constitutive heterochromatin pattern and localization of telomeric sequences. Synaptonemal complex by light and electron microscopy showed the supernumerary as an autopaired univalent. The sex chromosome pairing in meiotic cells involved the heterochromatic short arm of the X chromosome and the short arm of the Y chromosome.

Key words: fluorescence *in situ* hybridization, *Nectomys*, synaptonemal complex, supernumerary, tandem fusion

Introduction

The water rats from genus *Nectomys* present a wide distribution in the neotropical region. Cytogenetic studies have been performed in samples of *Nectomys* from different geographical localities in South America and an ample variability of chromosome number was detected, including specimens with $2n = 16$ and 17 in samples from north-western Venezuela and Trinidad; $2n = 38$, 42 and 52 in samples from Peru; and $2n = 52$ to $2n = 59$ in different localities from Suriname, western Venezuela, Argentina and Brazil (Gardner & Patton 1976, Baker *et al.* 1983, Maia *et al.* 1984, Yonenaga-Yassuda, *et al.* 1988, Barros *et al.* 1992).

In Brazilian populations, two basic karyotypes were detected: $2n = 52$ and 56 in samples identified as *Nectomys squamipes*. Specimens collected from the states of Amazonas, Pará, Maranhão, Piauí (Bonvicino *et al.* 1996) and Brasília (Yonenaga-Yassuda *et al.* 1988) presented $2n = 52$; $2n = 56$ was found in the states of São Paulo (Yonenaga-Yassuda *et al.* 1988), Minas Gerais, Rio de Janeiro and Bahia (Bonvicino *et al.* 1996); both $2n = 52$ and $2n = 56$ were detected in the states of Pernambuco and Mato Grosso do Sul (Maia *et al.* 1984, Yonenaga-Yassuda *et al.* 1988, Bonvicino *et al.* 1996). The main difference between the two basic karyotypes of $2n = 52$ and $2n = 56$ occurred on account of tandem fusions involving four pairs of chromosomes (Yonenaga-Yassuda *et al.* 1988). In addition, a remarkable variability of $2n = 53$, 54, 55, 57, 58 and 59 was observed because of the presence of 1, 2 or 3 supernumeraries in addition to the basic chromosome complement. Robertsonian rearrangement of two standard autosomes resulting in a $2n = 55$ karyotype was also detected plus a case of mosaicism ($2n = 56/57$) involving a B chromosome; morphological variability as well as monosomy of sex chromosomes ($2n = 55$, X0) were also observed (Maia *et al.* 1984).

Although karyological comparisons between $2n = 52$ and $2n = 56$ have shown a remarkable similarity, Bonvicino *et al.* (1996) demonstrated that individuals with the two different basic karyotypes should be considered different species because their hybrid showed a severe meiotic arrest and back-crosses between females and F1 confirmed that hybrids were infertile.

In the present paper, we report the cytogenetic analysis of 41 individuals of *Nectomys* with $2n = 52$, 53, 56 and 57 collected in the states of São Paulo, Mato Grosso

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and Pernambuco in Brazil. This study includes fluorescence *in situ* hybridization (FISH) of telomeric probes and CBG, GTG and RBG banding in mitotic metaphases and synaptonemal complex analysis of meiotic cells using light and electron microscopy.

Materials and methods

Cytogenetical analysis was carried out in 24 males and 17 females of *Nectomys* collected from three different states of Brazil: Sáo Paulo (SP), Mato Grosso (MT) and Pernambuco (PE) (Table 1).

Mitotic metaphases

Mitotic metaphases were obtained from bone marrow and spleen after an *in vivo* colchicine treatment. Fibroblast cultures from ear and tail biopsies were established using Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum. CBG, GTG and RBG banding were performed using routine cytogenetic techniques.

Fluorescence *in situ* hybridization (FISH)

For localization of telomeric sequences, oligonucleotides $(TTAGGG)_7$ in both the sense and the antisense orientation (Gibco BRL) were used as probes for FISH experiments, after 3′ end-labelling with biotin-11-dUTP (Boehringer). Hybridization was performed overnight at 37°C, and the signals were detected by incubation with fluorescein isothiocyanate (FITC)-labelled anti-biotin. FISH experiments using the commercially available all human telomeres (P5097-DG5, Oncor), digoxigenin labelled, were also performed. The slides were counterstained with propidium iodide in fluorescence antifade solution. Chromosome signals were visualized in a total of 40 metaphases from four specimens using a Zeiss Axiophot microscope equipped with a FITC filter and photographed using Ektachrome 400 (Kodak) colour slide film.

Meiotic cells and synaptonemal complex (SC)

Testis chromosome spreads from 24 males were obtained, and around 7–10 cells per individual were photographed. Synaptonemal complexes from 10 specimens (five of each $2n = 56$ and $2n = 57$) were obtained (Loidl & Klein 1991) and stained with silver nitrate, and different meiotic phases were identified according to Greenbaum *et al.* (1986).

Results

Twenty-four males and seventeen females were cytogenetically analysed, including 39 specimens from Sáo Paulo with $2n = 56$ and 57 chromosomes and two from Pernambuco and Mato Grosso, which had, respectively, $2n = 52$ and $2n = 53$ (Table 1).

The autosomes showed banding patterns similar to those described by Yonenaga-Yassuda *et al.* (1988). Supernumerary chromosomes were found in the two basic karyotypes ($2n = 52$ and $2n = 56$) and presented different morphologies and size as follows: a large subtelocentric (larger than the X chromosome) in the specimen with $2n = 53$ and a medium-size submetacentric or acrocentric in specimens with $2n = 57$ (Figure 1). The acrocentric form could just be identified after CBG, GTG and RBG banding patterns because it had similar size and morphology to the medium-sized autosomes, which presented gradual decreasing of size.

FISH

FISH with telomeric probes performed in four individuals revealed signals at the telomeres of all chromosomes, including the supernumeraries and the pairs 1 and 4 in specimens with $2n = 52$ and 53 that

Table 1. Locality, diploid number ($2n$), fundamental number (FN) and morphology of the sex and supernumerary chromosomes

Locality	2n	FN	Sex pair	Supernumerary
Fazenda Intervales (SP) 24°12′S, 48°30′W	56	56	(1) XaYb; (3) XbYa; (4) XbYb; (3) XcYc; (3) X*Y*; (2) XbXb; (2) XbXc; (3) XcXc	
	57	58	(2) XbYa; (1) XbYc; (1) XcYb; (1) XbYb; (2) XaXc; (1) XbXb; (4) XbXc	Medium submetacentric
Iporanga (SP); 24°07′S, 47°38′W	56	56	(1) XbYc	
	57	58	(1) XcYa	Medium submetacentric
Ariri (SP); 25°12′S, 48°02′W	56	56	(1) XbYa	
Caraguatatuba (SP); 23°37′S, 45°24′W	56	56	(1) XaYa; (1) XbYa	
Araçariguama (SP); 23°26′S, 47°03′W	57	57	(1) XaXb	Medium acrocentric
Juruena (MT); 10°19′S, 58°29′W	53	54	(1) XcXc	Large subtelocentric
Sáo Lourenço (PE); 7°56′S, 35°01′W	52	52	(1) XcXc 24 males; 17 females	

Xa, large submetacentric; Xb, large subtelocentric, arm ratio, 3.7; Xc, large subtelocentric, arm ratio, 4.7; Ya, medium submetacentric; Yb, medium subtelocentric; and Yc, small subtelocentric. Morphologies of sex chromosomes from three specimens with $2n$, 56 were not obtained (X*Y*).

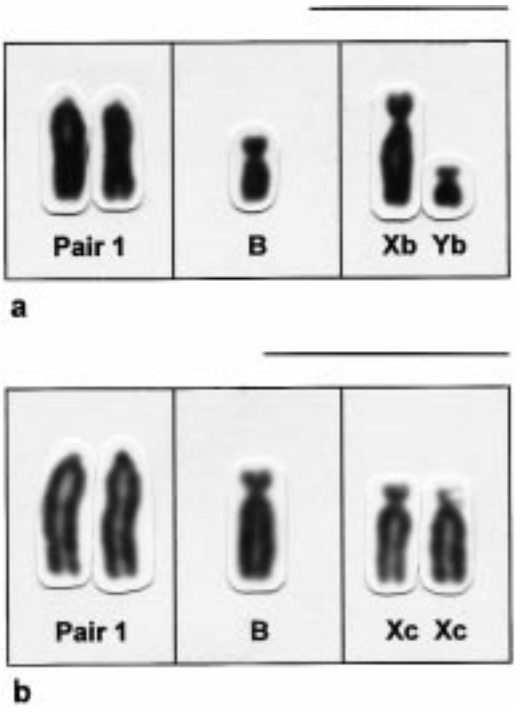

Figure 1. Giemsa staining demonstrating chromosomal size of pair 1, supernumerary (B) and sex chromosomes. **a** $2n = 57$; **b** $2n = 53$. Bar = 10 μm.

were involved in tandem fusion events. In addition, the submetacentric B presented a strong interstitial telomeric band (ITB) at the proximal region of the long arm in two specimens analysed. ITBs were not observed in the acrocentric or subtelocentric Bs (Figure 2).

Banding patterns in the supernumeraries

The banding patterns of the supernumerary chromosomes are shown in Figure 3a–c. After CBG banding, the submetacentric B had the long arm entirely heterochromatic; the acrocentric presented a heterochromatic block at the end of the long arm; the large subtelocentric was almost totally heterochromatic, except for the less heavily C-banded short arm. In addition, the proximal region of the long arm appeared to be more heavily stained than the other ones.

After GTG banding, the submetacentric B showed an evident negative G-band at the proximal region of the long arm. The acrocentric B presented a large G-positive block taking half of the distal region of the long arm. The subtelocentric, in spite being morphologically quite similar to the X chromosomes, presented a distinctive G-banding pattern with a proximal darker positive band, which was equivalent to the strongly stained C-band.

RBG banding pattern showed that the submetacentric and subtelocentric were late replicating after 5-BrdU incorporation in all their extension. The acrocentric, on the other hand, was early replicating, except in relation to a small negative R-band at proximal region of the long arm.

Sex chromosome variability

Sex chromosomes were classified according to the arm ratio (Levan *et al.* 1964) and presented, each one, three different morphologies: Xa = submetacentric, arm ratio = 2.7; Xb and Xc = subtelocentrics with arm ratio = 3.7 and 4.7 respectively; Ya = medium submetacentric, arm ratio = 2.2, Yb = medium subtelocentric, arm ratio = 3.7; Yc = small subtelocentric, arm ratio = 5.0 (Figure 4a).

CBG banding showed heteromorphic pericentromeric blocks of constitutive heterochromatin in the X; the short arm showed an intermediate stained C-band, less heavily stained than the pericentromeric regions. The Y was C-banded at the distal region of long arm and at the pericentromeric region (Figure 4b). The X chromosomes had two positive G-bands in the long arm, and the Y had a dark distal band in the long arm. The short arm of X and the entire Y chromosome were late replicating after 5-BrdU incorporation (not shown).

Meiotic cells and synaptonemal complex (SC)

Diakinesis after conventional staining and CBG banding, from specimens with $2n = 56$, revealed 27 autosomal bivalents plus the sex pair, which had the typical end-to-end arrangement involving the short arms (Figure 5). Individuals with $2n = 57$ presented 28 bivalents plus a univalent corresponding to the supernumerary. Meiotic data from females $2n = 52$ and 53 were not obtained.

Synaptonemal complexes were analysed from specimens with $2n = 56$ and 57 by both light and electron microscopy. It was possible to visualize that autosomal synapsis initiated from extremities of the homologous axes during mid-zygonema and progressed bidirectionally from both ends towards the central region; the axes of sex chromosomes were more darkly stained than the autosomal axes and remained unpaired, nevertheless appearing very close at one of the ends, presumably the telomeres of the short arms of X and Y (Figure 6a).

Most of synapsis occurred during the transition from late zygonema to early pachynema and autosomal axes in various degrees of synapsis were observed. Ag-NORs associated to some autosomal SCs could be observed. The axes of the sex chromosomes were clearly distinguishable (Figure 6b), and the images we analysed varied from synaptic initiation to partial synapsis. The synapsis progressed in a unidirectional fashion initiating from the telomeric region in the direction to the non-homologous axes of X and Y.

At mid-pachynema the axes were totally paired

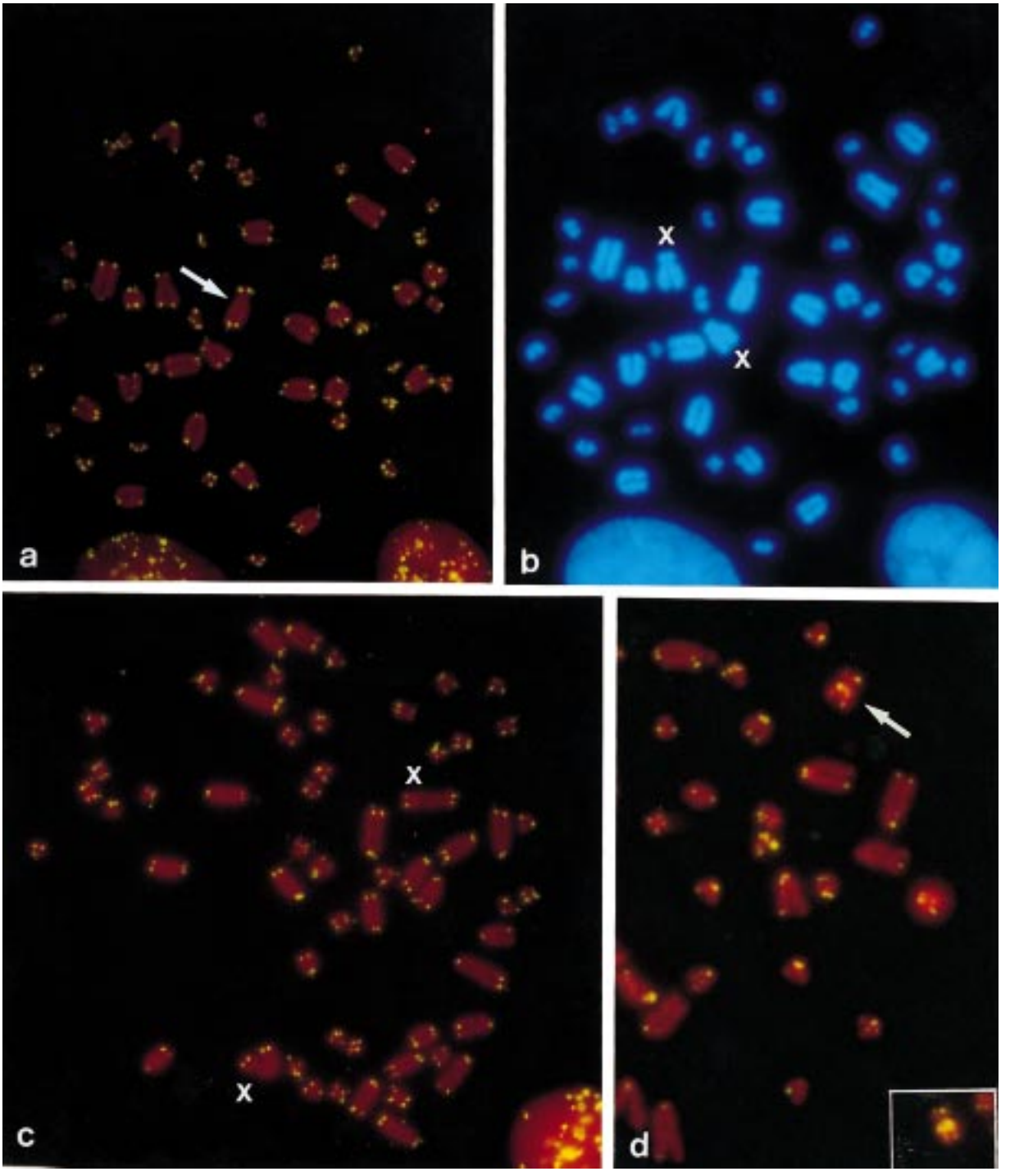

Figure 2. Metaphases of *Nectomys* after FISH of telomeric probes: **a** $2n = 53$, female XcXc, subtelocentric B (arrow); the same metaphase in **b** after DAPI staining. **c** $2n = 57$, female XaXb, acrocentric B. **d** Partial metaphase in which the submetacentric B (arrow) presents an interstitial telomeric band; inset the supernumerary from other specimen.

presenting the minimum lengths. Single axes were not observed, except for the non-homologous regions of X and Y chromosomes. Supernumerary SC did not show a distinct behaviour in relation to the autosomes, which present gradual size differences (Figure 6c). In this substage, sex chromosomes exhibited maximum pairing of the 'homologous' regions that was around one-sixth of the X and half of the Y.

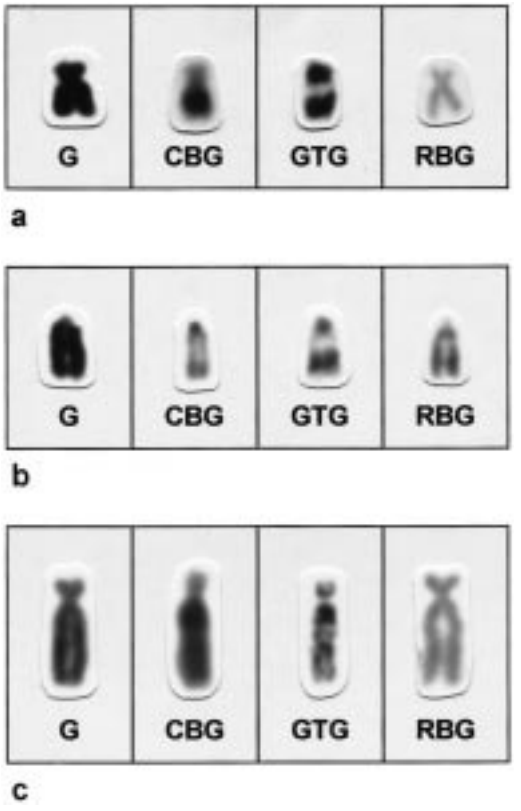

Figure 3. Three different morphologies of the supernumerary after Giemsa staining (G), CBG, GTG and RBG banding. **a** Submetacentric; **b** acrocentric; **c** subtelocentric.

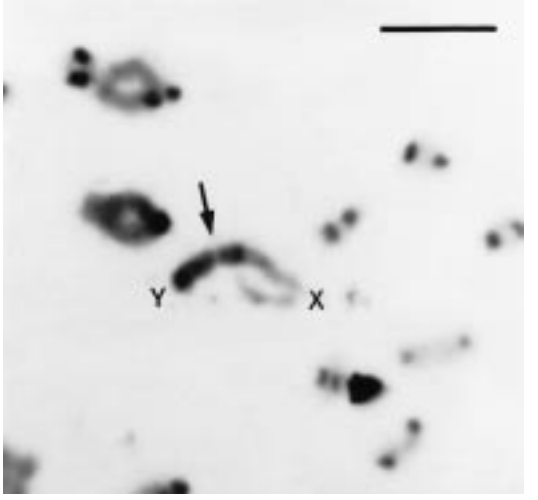

Figure 5. Partial diakinesis CBG-banded focusing pairing of short arms of Xc and Ya. Bar = 10 μm.

Discussion

Supernumeraries (B) are additional chromosomes that have been described in many species including plants and animals, being a cause of chromosomal number polymorphism in natural populations. In spite of being quite common, several aspects of their composition and function remain unknown.

Supernumeraries are also widespread among rodents and, in the present paper, we describe the heterogeneity and meiotic behaviour of B chromosomes in specimens

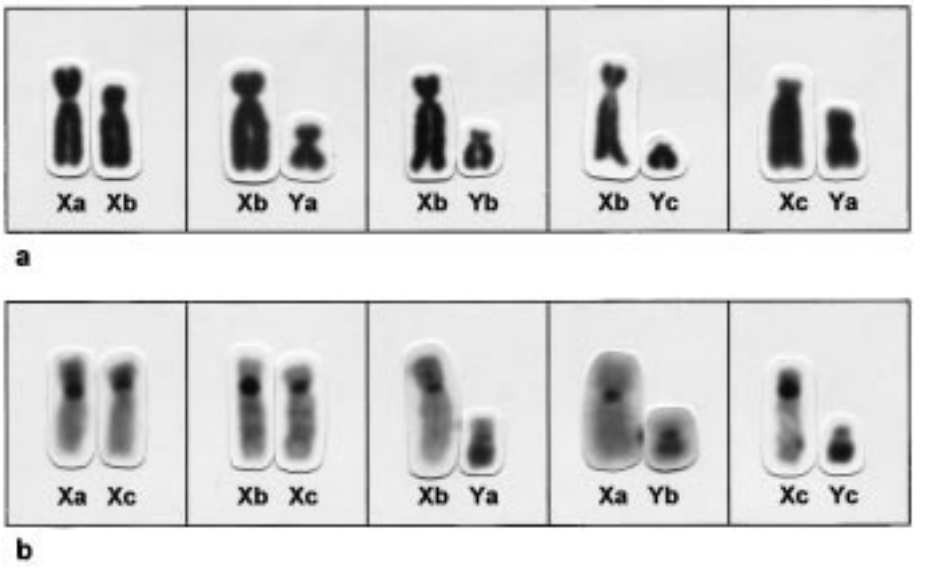

Figure 4. Sex chromosomes after Giemsa staining (**a**). **b** CBG banding.

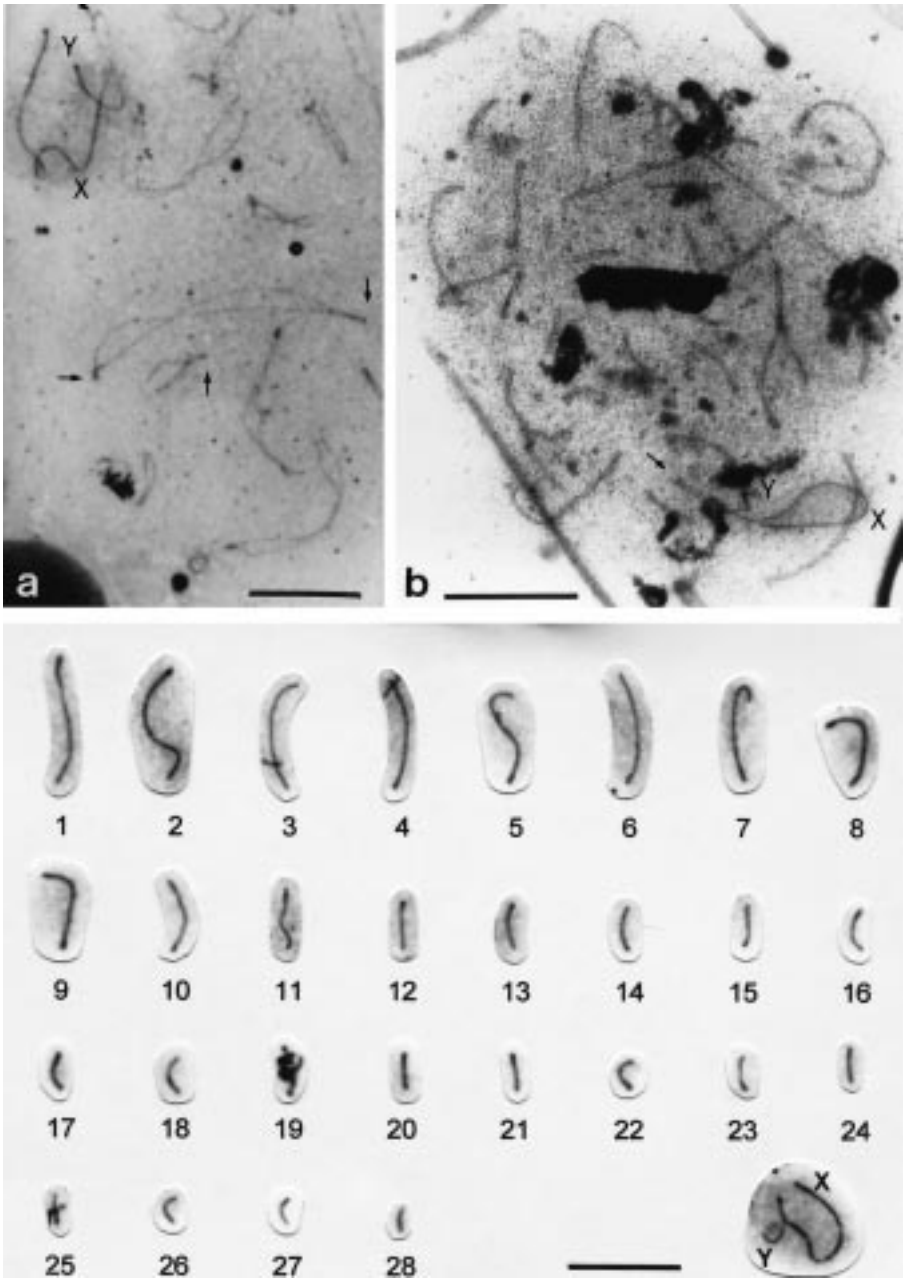

Figure 6. Synaptonemal complex of *Nectomys*, $2n = 57$: **a** Partial mid-zygonema by light microscopy; sex chromosomes (XbYa) are identified and arrows indicate the regions of synapsis initiation Bar $= 10\ \mu$m. **b** Early pachynema by electron microscopy; the SC of sex chromosomes (XcYb) and the region of synapsis are indicated Bar $= 10\ \mu$m. **c** SC karyotype at mid pachynema by light microscopy; sex chromosomes (XbYa) are indicated Bar $= 10\ \mu$m. The supernumerary was an autopaired univalent indistinguishable from the autosomal axes.

of *Nectomys* with $2n = 56$, 57 and $2n = 52$, 53 from three different localities of Brazil, using conventional cytogenetic techniques, FISH of telomeric probes and synaptonemal complex studies of meiotic cells.

Around 36% of the sample from Sáo Paulo had $2n = 57$. Within the supernumerary-bearing specimens, the submetacentric form was found in about 93% of the specimens and the acrocentric form in 7%. The unique *Nectomys* specimen collected from the state of Mato Grosso showed $2n = 53$, and the B chromosome was different from those detected in Sáo Paulo. Different morphologies and size of Bs (metacentric, submetacentric and acrocentric) were described in populations of *Nectomys*, but we detected that the medium-size

submetacentric is the most widespread and frequent form in Brazilian samples.

Our FISH results on the supernumeraries revealed a conspicuous ITB (interstitial telomeric bands), coincident with the negative G-band of the submetacentric B, in opposition to the acrocentric and subtelocentric forms which did not exhibit interstitial signals. Our present data about telomeric sequences, variability of amount and localization of constitutive heterochromatin, size and morphology indicate a remarkable heterogeneity in the composition of B chromosomes in the samples of *Nectomys* with basic karyotypes of $2n = 56$ and 52.

FISH in autosomes revealed that tandem fusions of pairs $3 + 11$ and $5 + 24$ from $2n = 56$, which gave rise to pairs 1 and 4 respectively of $2n = 52$, did not show ITBs at the chromosomes of the last one. FISH studies with telomeric probes involving chromosomal rearrangements in other species also exhibited no interstitial telomere signals (Garagna *et al.* 1995, Nanda *et al.* 1995, Silva & Yonenaga-Yassuda 1997). Absence of ITB signal in the chromosomes involved in tandem fusion can indicate that telomeric repeats were really lost during the events or, on the other hand, that these regions can be too small to be detected with this technology.

Synaptonemal complex analysis showed auto-pairing of the univalent supernumerary. Similar behaviour of autopairing of Bs was also observed in the fox, *Vulpes fulvus*, and in the grasshoppers, *Myrmeleotettix maculatus* and *Euthystira brachyptera* (Switonski *et al.* 1987, Fletcher & Hewitt 1988). The lack of similarity between B and A chromosomes suggests that significant differences exist between these two chromosome groups (Peppers *et al.* 1997) and, although they have been regarded as having originated from the A complement, it seems that they progressively become less homologous with As over time because of accumulation of mutations (Beukeboom 1994).

Nectomys, as well as a large number of other Oryzomyines, exhibits a high degree of polymorphism in sex chromosomes. Specimens investigated here presented three morphologies of the X chromosome and three morphologies of the Y, being Xb and Ya the most common forms. The heterochromatic short arms of the X are less resistant to alkali denaturation than the pericentromeric regions after $Ba(OH)_2$ treatment (probably as a result of differential condensation) and show late-replicating DNA after BrdU incorporation. Mechanisms of addition/deletion of heterochromatin could explain the different forms of the X. The variability of the Y chromosome is clearly displayed in Figure 4a. CBG banding (Figure 4b) revealed a large distal C-positive block in the long arm and a smaller one in the pericentromeric region; the other regions are C-band negative. After BrdU incorporation, we detected the Y as an entirely late-replicating DNA chromosome, however Yonenaga-Yassuda *et al.* (1988) described a single early-replicating proximal band in the long arm of the Y of *Nectomys* and suggested that this area could contain DNA with gene expression. Those data could reveal a different nature of chromatin, perhaps involving repetitive sequences not easily detected by the C-banding technique in the short arms of Y, when compared with their pericentromeric and distal regions. This would explain the variability in size observed in the short arm of the Y chromosome. Different types of repetitive sequences were observed in other rodents. Murer-Orlando & Richer (1983) described heterogeneity of heterochromatin in sex chromosomes of *Cricetulus griseus* and suggested that this fact probably would reflect differences in the DNA–protein association in these specific chromosomes. In addition, based on molecular localization of satellite DNA families, C-banding and base-specific fluorochrome staining, Modi (1993a, b) suggested that heterochromatin of sex chromosomes in species of *Microtus* possess different repetitive elements and heterogeneity in the sequence composition.

Meiotic pairing of sex chromosomes in our sample involved the short heterochromatic arm of X and the short arm of the Y and SC analysis demonstrated that the timing of synaptic initiation of this chromosome pair is delayed in relation to autosomal synapsis. Similar results were also detected in samples of *Peromyscus*, which presented variability of the heterochromatic short arm of X and size variation of Y (which was completely heterochromatic). Other mammals however have revealed considerable variability in the extension of pairing, timing of synaptic initiation and location of initiation sites (Hale *et al.* 1991).

Finally, regarding the taxonomic problem of *Nectomys* with $2n = 52$ and 56, we did not mention consciously which species we are reporting here because *Nectomys* with these different basic diploid numbers have been considered the same species. Nevertheless, based on the cytogenetical incompatibility of the two karyomorphs during the meiosis and the infertile captive-bred hybrid with $2n = 54$ (Bonvicino *et al.* 1996), C. R. Bonvicino *et al.* (in preparation) have suggested that those two basic karyotypes represent two distinct taxonomic entities: *Nectomys squamipes* with $2n = 56$ and *Nectomys rattus* with $2n = 52$.

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