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Intraspecific variation in the distribution of the interstitial telomeric $(TTAGGG)_n$ sequences in *Micoureus demerarae* (Marsupialia: Didelphidae)

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Abstract

The distribution of the telomeric sequence $(TTAGGG)_n$ was studied in chromosomes of *Micoureus demerarae* ($2n = 14$), a South American marsupial, by fluorescence *in-situ* hybridization (FISH). The telomeric repeat sequence was present at both ends of all chromosomes, but also various interstitial telomeric sequences (ITS) were detected in the pericentromeric heterochromatic regions. Intraspecific differences in the number of ITS (2 to 8) were observed without intraindividual variation. The presence of telomere-like sequences in the same regions of constitutive heterochromatin suggest that these segments are not necessarily remnants of true telomeres resulting from chromosome rearrangements but could be part of the satellite DNA.

Introduction

Telomeric DNA is widely conserved among vertebrates and consists of the sequence TTAGGG tandemly repeated (Moyzis *et al.* 1988, Meyne *et al.* 1989, Zakian 1995). These $(TTAGGG)_n$ sequences are found mainly at the ends of chromosomes, but the distribution of these sequences at non-telomeric sites was observed in a variety of vertebrate species (Meyne *et al.* 1990, Nanda & Schmid 1994, Vermeesch *et al.* 1996, Garagna *et al.* 1997, Metcalfe *et al.* 1997, Ono & Yoshida 1997, Metcalfe *et al.* 1998). The most common non-telomeric site is at the pericentromeric region, within or at the margins of blocks of constitutive heterochromatin identified by C-banding (Meyne *et al.* 1990, Nanda & Schmid 1994, Garagna *et al.* 1997). The origin and role of $(TTAGGG)_n$ repeats at non-telomeric sites have not been elucidated yet. They could be generated by mechanisms such as mutation, unequal crossing-over, transposition, or amplification of endogenous $(TTAGGG)_n$ sequences (Wiley *et al.* 1992, Vermeesch *et al.* 1996, Garagna *et al.* 1997, Sharma & Sharma 1998). Also, the presence of interstitial telomeric sequences (ITS) could be remnants of true telomeres resulting from chromosome arrangements, such as inversions,

centric or tandem fusions, that occurred during karyotype evolution (Lee *et al.* 1993, Fagundes *et al.* 1997, Pellegrino *et al.* 1999).

Marsupials of the family Didelphidae comprise the most diverse Metatheria group in America with 70 species grouped in 15 genera (Hershkovitz 1992, Gardner 1993, Mustrangi & Patton 1997, Patton & Da Silva 1997, Patton *et al.* 2000). Chromosome numbers and morphology have been determined for more than 30 species, and these data show that three distinct types of chromosome complements characterize this family: $2n = 14$, $2n = 18$ and $2n = 22$ (Hayman 1990). In both the American and Australian species, not only is $2n = 14$ the commonest number, but the morphology of the autosomal chromosomes in these karyotypes is very similar. The similarity in morphology and the widespread occurrence of this complement has been interpreted as evidence of a conserved ancestral complement (Hayman & Martin 1969, 1974). Moreover, the G-bands from 14 Australian marsupials species and one South American species, all with $2n = 14$ karyotype, were compared and a very similar pattern was observed, supporting the hypothesis of ancestrality of this complement (Rofe & Hayman 1985). Additionally, this hypothesis has been supported by many studies, ranging from comparative serology (Kirsch 1977), morphological data (Reig *et al.* 1987, Szalay 1994), DNA/DNA hybridization (Kirsch & Palma 1995), cytochrome *b* sequences (Patton *et al.* 1996), IRBP sequence (Jansa & Voss 2000), to chromosome painting (Glas *et al.* 1999, Rens *et al.* 1999).

Recently, the distribution of $(TTAGGG)_n$ sequences at non-telomeric sites of some chromosomes in two species of Didelphidae (only one specimen of *Marmosops incanus*, exceptionally with $2n = 17$, due to trisomies of pairs 2, 5, and 6 in culture cells; and two other specimens of *Monodelphis domestica*, with $2n = 18$) was reported and interpreted as evidence that stretches of telomeric sequence were retained during centric fusions (Svartman & Vianna-Morgante 1998). The authors suggest that the $2n = 14$ and the complement with $2n = 18$ evolved from a karyotype with a higher diploid number. In order to test these competing hypotheses, $(TTAGGG)_n$ sequence should be mapped in a significant number of other species and for more individuals.

In this paper, we describe the chromosomal distribution of the constitutive heterochromatin (C-bands) and of the $(TTAGGG)_n$ sequences by FISH in *Micoureus demerarae* (Gardner 1993), previously known as *Marmosa cinerea*, a South American marsupial, with $2n = 14$. We found a variable number of large blocks of the telomeric sequence at interstitial sites.

Materials and Methods

Specimens and mitotic metaphases

Seventeen wild-caught specimens (11 females and 6 males) of *Micoureus demerarae* from five localities in different states of Brazil – São Paulo (SP), Mato Grosso (MT), Bahia (BA), Ceará (CE) and Goiás (GO) – were examined cytogenetically (Table 1). The voucher specimens are deposited in the Museu de Zoologia da Universidade de São Paulo (MZUSP) collection, in the state of São Paulo, Brazil.

Mitotic metaphases were obtained from bone marrow and spleen after *in-vivo* colchicine treatment. Mitotic cells were spread onto clean glass slides, air dried and stored at $-20°C$ until use. Analyses were performed after routine

Table 1. Collection localities, number of specimens of *Micoureus demerarae* examined from a locality, and number of chromosomes with interstitial telomeric sequences (ITS).

Locality	Number of specimens	Number of ITS
Aripuanã, MT (10°10′S, 59°27′W)	3	8
Cláudia, MT	2	6
(11°35′S, 55°08′W)	1	7
	1	5
Graúcha do Norte, MT	1	5
(13°11′S, 57°23′W	1	6
Pacoti, CE	1	5
(4°13′S, 38°55′W)	1	6
São Domingos, GO (13°23′S, 46°19′W	1	6
Vila Rica, MT (10°01′S, 51°07′W)	1	6
Una, BA (15°17′S, 39°04′W)	3	4
Santa Rita do Passa Quatro, SP (21°44′S, 47°34′W)	1	2

Giemsa and CBG banding staining techniques (Sumner 1972).

Fluorescence in-situ *hybridization (FISH)*

Chromosomes of each specimen were hybridized with a commercially available telomeric probe (All Human Telomeres, digoxigenin labeled, ONCOR) according to the recommended protocol. Slides were heat denatured in 70% deionized formamide/2 × SSC for 2 min at 70°C. *In-situ* hybridization with the telomeric probe was carried out overnight in 50% formamide/2 × SSC at 37°C and then slides were washed in 30% formamide/2 × SSC for 8 min at 37°C, in 2 × SSC for 10 min at 37°C and in buffer at room temperature. Hybridization signals were detected by incubation with fluorescein isothiocyanate (FITC)-labeled antiodigoxigenin, and the slides were counterstained with propidium iodide in a fluorescence antifade solution. Chromosomes signals were detected using a Zeiss Axiophoto microscope equipped with a FITC filter and photographed using Ekatachrome 400 (Kodak) color slide film.

Results

The karyotype of *Micoureus demerarae* consists of metacentric/submetacentrics four large pairs, two smaller pairs of acrocentrics and the sex chromosomes: the X chromosome is a small acrocentric and the Y is an acrocentric smaller than the X, both perfectly distinguished morphologically (Figure 1a). C-banding patterns revealed large blocks of constitutive heterochromatin at the pericentromeric regions in all autosome pairs. The X chromosome exhibited a heterochromatic block in the pericentromeric region and a large band in the distal region of the long arm, and the Y is entirely heterochromatic (Figure 1b). No polymorphism in C-banding was observed.

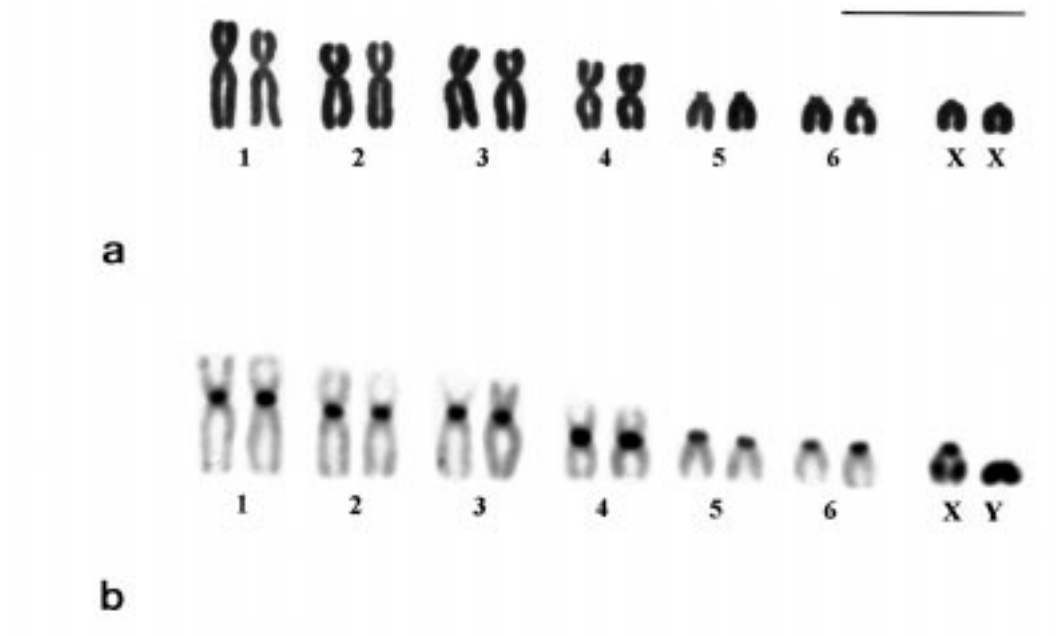

Figure 1. (**a**) Conventionally stained karyotype from *Micoureus demerarae* female. (**b**) C-banded karyotype from a male, showing the heterochromatin constitutive at the pericentromeric regions. Bar = 10 μm.

After FISH with $(TTAGGG)_n$ sequence probe, strong signals were observed at the ends of all chromosomes, including the sex chromosomes. In addition, ITS were detected in the pericentromeric heterochromatic regions of metacentric/submetacentric chromosomes in several individuals of this species. These pericentromeric ITS presented a variable number among individuals from different localities, ranging from 2 up to 8 (Figure 2 and Table 1). The specimen from Santa Rita do Passa Quatro exhibited ITS on one pair of metacentric/submetacentric chromosomes, and the specimens from Aripuanã displayed ITS in the four largest biarmed pairs. Interindividual variation in the number of ITS was also detected among specimens from the same locality, for example Cláudia, Gaúcha do Norte and Pacoti (Table 1). Within an individual, there was no variation in the ITS number. No ITS were observed in sex chromosomes. For the sake of repeatability, approximately 300 metaphases were analyzed, with a minimum number of at least 15 metaphases per specimen. Only the readily visible and consistent ITS are reported.

The presence of ITS in the pericentromeric region matched perfectly with that of constitutive heterochromatin, suggesting that repetitive DNA and $(TTAGGG)_n$ repeats collocalize in the same region.

Discussion

Chromosomes of several mammal species exhibit ITS, and the most common location is in the pericentromeric region which, in general, is a constitutive heterochromatin region (positive C-band) (Meyen *et al.* 1990, Vermeesch *et al.* 1996, Garagna *et al.* 1997, Ono & Yoshida 1997, Sharma & Sharma 1998). Constitutive heterochromatin is invariably associated with relatively short DNA sequences that are highly repeated in long tandem arrays, commonly referred to as satellite DNA (Choo 1997). It is important to note that the $(TTAGGG)_n$ sequence has been reported as a component of the satellite DNA of some vertebrate species (Southern 1970, Arnason *et al.* 1998, Adegoke *et al.* 1993, Garrido-Ramos *et al.* 1998).

In *Micoureus demerarae*, we show that the number of ITS after FISH with $(TTAGGG)_n$ sequence is variable, differing among individuals, and moreover, the localizations of these sites correspond to conspicuous block of constitutive heterochromatin. Possibly these telomeric sequences are part of the satellite DNA and, in this context, we suggest that their origin is probably not through such mechanisms as chromosomal fusion or inversion. If these ITS are remnants of telomeres resulting in this type of chromosomal rearrangement, the variability observed has to be explained by progressive loss of this sequence. However, the intraspecific variability observed seems to preclude such a hypothesis. These telomeric sequences could have emanated by illegitimate recombination or by some other process of excision and reintegration at new sites, with subsequent amplification of short $(TTAGGG)_n$ tandem repeats within the chromosome.

How telomeric sequences became part of the satellite DNA of *Micoureus demerarae* is obscure and awaits further study but, probably, what we are visualizing may be random events or a response to some unknown agent of selection (Wiley *et al.* 1992).

The exceptional hybridization pattern herein reported has never been observed in mammals, and it is tempting to relate the presence of these ITS to the process of karyotype evolution of the marsupial species. However, multidisciplinary approaches, including cytogenetic, molecular (serology, DNA–DNA hybridization or mitochondrial/nuclear sequences) and morphological (cranial, dental and postcranial traits) studies, are fully concordant with the hypothesis that a $2n = 14$ conserved karyotype is the ancestral for both Australian and American marsupials species (Kirsch 1977, Rofe & Hayman 1985, Reig *et al.* 1987, Hayman 1990, Szalay 1994, Kirsch & Palma 1995, Patton *et al.* 1996, Glas *et al.* 1999). The variable number of ITS observed suggests that this is a polymorphic character occurring in the natural population of *Micoureus demerarae*. From the results reported here, we can hypothesize that the internal telomere repeat signals detected result from the presence of such a sequence motif in the satellite DNA of *Micoureus demerarae* and probably play no role in chromosomal evolution.

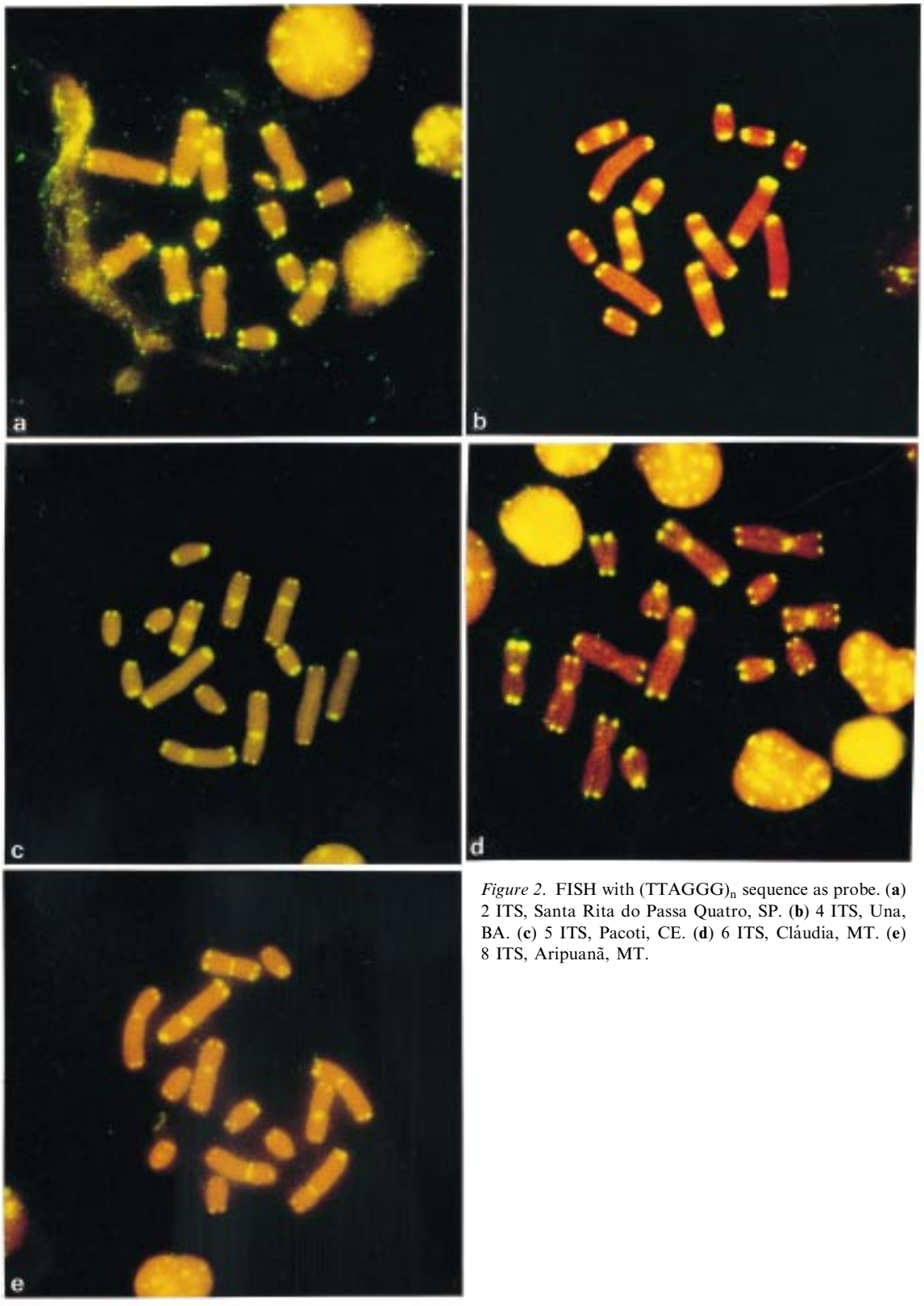

Figure 2. FISH with $(TTAGGG)_n$ sequence as probe. (**a**) 2 ITS, Santa Rita do Passa Quatro, SP. (**b**) 4 ITS, Una, BA. (**c**) 5 ITS, Pacoti, CE. (**d**) 6 ITS, Cláudia, MT. (**e**) 8 ITS, Aripuanã, MT.

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