Original Article

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Non-telomeric sites as evidence of chromosomal rearrangement and repetitive (TTAGGG)$_n$ arrays in heterochromatic and euchromatic regions in four species of *Akodon* (Rodentia, Muridae)

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Abstract. Comparative studies among four species – *Akodon azarae* (2n = 38), *A. lindberghi* (2n = 42), *A. paranaensis* (2n = 44) and *A. serrensis* (2n = 46) – employing classic cytogenetics (C- and G-bands) and fluorescence in situ hybridization with telomeric (TTAGGG)$_n$ sequences are reported here. Non-telomeric signals in addition to the regular telomeric sites were detected in three species: *A. azarae*, *A. lindberghi* and *A. serrensis*. One interstitial telomeric site (ITS) was observed proximally at the long arm of chromosome 1 of *A. azarae*. The comparison of G-banding patterns among the species indicated that the ITS was due to a tandem fusion/fission rearrangement. Non-telomeric signals of *A. lindberghi* and *A. serrensis* were not related to chromosomal rearrangements; instead, the sequences co-localized with (i) heterochromatic regions of all chromosomes in *A. serrensis*; (ii) some heterochromatic regions in *A. lindberghi*, and (iii) both euchromatic and heterochromatic regions in the metacentric pair of *A. lindberghi*. These exceptional findings revealed that ITS in *Akodon* can be related to chromosomal rearrangements and repetitive sequences in the constitutive heterochromatin and that the richness of TTAGGG-like sequences in the euchromatin could be hypothesized to be a result of amplification of the referred sequence along the chromosome arms.

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Comparative cytogenetic and homeology detection among chromosomes of related species have been attempted over decades at different levels. The early approaches hinged primarily on gross cytogenetics, based on karyotype description and comparative analyses after banding patterns that allowed detection of chromosome polymorphisms, heteromorphisms and homeologies between species (Chowdhary and Raudsepp, 2001).

More recently, fluorescent in situ hybridization (FISH) using telomeric probes has contributed to confirm the occurrence of rearrangements involved in karyotype differentiation among related species (Fagundes et al., 1997).

Telomeres are terminal structures of linear eukaryotic chromosomes, fundamental for maintaining chromosome stability (Blackburn, 1994; Zakian, 1995). The telomeric DNA, which consists of TTAGGG tandem repeats, is conserved in all vertebrate species and has been included in

Table 1. Species, diploid number (2n), fundamental number (FNa), sex (m = male; f = female) and collection locations from Brazil

Species	2n	FNa	Sex	Location
Akodon azarae	38	38	1 f	Cachoeira do Sul, Rio Grande do Sul state (52°53′S 30°02′W)
Akodon lindberghi	42	42	2 m 2 f	Serra da Canastra, Minas Gerais state (46°39′46″S 20°08′46′W) Juiz de Fora, Minas Gerais state (43°21′S 21°45′W)
Akodon paranaensis	44	44	1 f 1 f; 3 m	Venâncio Aires, Rio Grande do Sul state (52°11′S 29°36′W) Itá, Santa Catarina state (52°19′S 27°17′W)
Akodon serrensis	46	46	1 f	Piraquara, Paraná state (49°07′S 25°42′W)
Total			6 f; 5 m	

studies of chromosome evolution for many vertebrates, using FISH (Moyzis et al., 1988; Wurster-Hill et al., 1989; Meyne et al., 1990; Blackburn, 1991; Lizarralde et al., 2005).

Several reports have associated the presence of interstitial telomeric signals (ITS) with the occurrence of structural and/or numerical chromosome changes, such as Robertsonian rearrangements, pericentric inversion and tandem fusion/fission (Fagundes et al., 1997; Silva and Yonenaga-Yassuda, 1998a; Pellegrino et al., 1999; Ventura et al., 2004). Some studies described interstitial telomeric sites as a result of remaining telomeres of chromosome rearrangements in karyotypes of cervids (Lee et al., 1993; Yang et al., 1997), giraffes (Petit et al., 1994; Vermeesh et al., 1996), rodents (Fagundes et al., 1997; Silva and Yonenaga-Yassuda 1998a; Ventura et al., 2004; Vieira et al., 2004) and lizards (Pellegrino et al., 1999). On the other hand, ITS associated with non-telomeric regions of the chromosomes are common in some groups and hybridization to pericentromeric regions are also frequently observed. A variety of species showed relatively large signals associated with constitutive heterochromatin regions, which has been explained by the fact that arrays of satellite DNA are components of the heterochromatin of some mammalian species, e.g. *Eulemur macaco* (Garagna et al., 1997); *Micoureus demerarae* (Pagnozzi et al., 2000); *Carollia perspicillata* and *Ammospermophilus harrisii* (Multani et al., 2001); *Marmosops parvidens, M. incanus* and *Monodelphis domestica* (Pagnozzi et al., 2002). These data revealed that the presence of ITS is not always evidence of chromosomal rearrangements.

In the rodent *Akodon cursor* Winge, 1887 (2n = 14, 15 and 16), which is characterized by an uncommon variability in diploid number (2n) and number of autosomal arms (FNa), FISH with telomeric probes after high resolution G-banding provided evidence to assume the reduction of diploid number through centric fusion and pericentric inversions, suggesting 2n = 16 as the primitive and 2n = 14 as the derived karyotype for the species (Fagundes et al., 1997).

The karyotype of a new species of *Akodon* with 2n = 10 was compared to the *A. cursor* 2n = 16 karyotype and rearrangements such as pericentric inversions, tandem fusions, and loss of telomeres were proposed, supported by the analysis of telomeric FISH, Ag-NORs and G-banding (Silva and Yonenaga-Yassuda, 1998a; Silva et al., 2006). ITS were observed in the 2n = 10 karyotype in addition to the telomeric signals, however those ITS do not seem to correspond to the exact putative positions involved in the chromosomal rearrangements. Moreover, ITS were observed in chromosome 1, co-localized with pericentromeric heterochromatin. The authors believe that more complex rearrangements should have occurred during the evolutionary process which drove the differentiation of both, 2n = 10 and 2n = 16 karyotypes.

In this work we investigate the position of telomeric signals in four species of rodents of the genus *Akodon*, in which diploid numbers range from 2n = 38 to 2n = 46, using banding patterns and FISH with telomeric probes. We detected hybridization signals associated with either telomeric or non-telomeric segments in the genome, related or not to chromosomal rearrangements, and additionally positioned at euchromatic and heterochromatic regions.
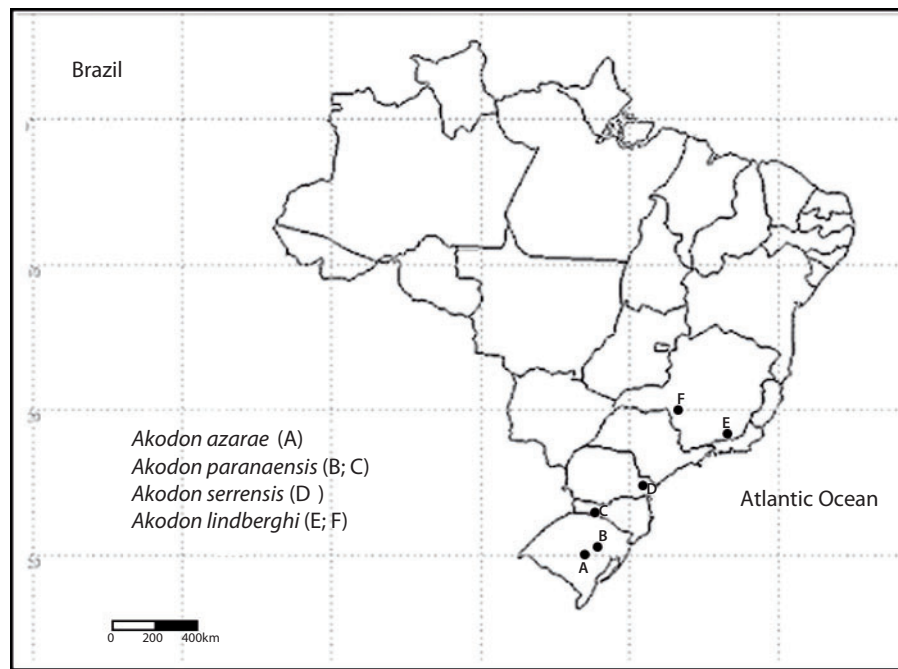
Material and methods

Chromosome preparations were obtained from bone marrow of 11 specimens of four species of the genus *Akodon*: *A. azarae* (Fischer, 1829), *A. lindberghi* (Hershkovitz, 1990), *A. paranaensis* (Christoff et al., 2000) and *A. serrensis* (Thomas, 1902). The specimens were collected from six localities in Brazil (Table 1 and Fig. 1). Cells were spread onto clean slides, air dried, and stored at –20°C until use. G- and C-banding patterns were based on routine cytogenetic procedures.

Telomeric FISH was performed using two methodologies. Chromosomes of *A. azarae,* one male of *A. lindberghi* and *A. paranaensis* were hybridized with telomeric probe (All Human Telomeres, digoxigenin labeled, ONCOR); the other three specimens (two females and one male) of *A. lindberghi*, and the single specimen of *A. serrensis*, were hybridized with the Telomere PNA FISH Kit/Cy3 (DAKO, code No. K 5326).

For the ONCOR protocol, slides were heat denatured in 70% deionized formamide, 2× SSC for 2 min at 70°C. In situ hybridization 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 anti-digoxigenin, and the slides were counterstained with propidium iodide in a fluorescence antifade solution. Chromosome signals were detected using a Zeiss Axiophot microscope

Fig. 1. Collection localities of *Akodon* species: (A) Cachoeira do Sul, Rio Grande do Sul state (52°53′S 30°02′W); (B) Venâncio Aires, Rio Grande do Sul state (52°11′S 29°36′W); (C) Itá, Santa Catarina state (52°19′S 27°17′W); (D) Piraquara, Paraná state (49°07′S 25°42′W); (E) Juiz de Fora, Minas Gerais state (43°21′S 21°45′W); (F) Serra da Canastra, Minas Gerais state (46°39′46″S 20°08′46′W).

Brazil

Akodon azarae (A)
Akodon paranaensis (B; C)
Akodon serrensis (D)
Akodon lindberghi (E; F)

Atlantic Ocean

0   200   400km

equipped with an FITC filter and photographed using Ektachrome 400 (Kodak) color slide film.

For the DAKO protocol, during the pre-treatment, slides were immersed in TBS for about 2 min, and then in 3.7% formaldehyde in TBS for exactly 2 min; the slides were washed again in TBS and immersed in a pre-treatment solution for 10 min followed by another TBS wash and then immersed in a cold ethanol series (70, 85 and 96%). For denaturation and hybridization, Telomere PNA Probe/Cy3 was added to the slides and covered with coverslips. The slides were placed in a pre-heated incubator adjusted to 80°C for 8 min and after that, placed in the dark at room temperature for 30 min. In order to remove coverslips, the slides were immersed in a rinse solution, washed in a pre-heated wash solution at 65°C, and once more immersed in the same cold ethanol series as previously. A mounting solution (Vectashield antifade supplemented with 0.1 μg/ml DAPI) was applied to each slide. FISH images were observed using a Zeiss Axiophot fluorescence microscope with RBG filter sets. Images were saved in the computer and analyzed after overlapping both chromosome and signal images.

Fig. 2. (**A**, **B**) *Akodon azarae* (2n = 38) metaphases. Arrows indicate pair 1 which bears the ITS. (**A**) C-banding pattern. (**B**) FISH with telomeric probe.

Results

After FISH employing telomeric probes in four *Akodon* species, our results showed that the signals are not exclusively located at telomeres, i.e., they were also detected interstitially in some chromosomes of *A. azarae*, *A. lindberghi* and *A. serrensis*, and the intensity of signals at both ends of all chromosomes varied among the species as well.

Akodon azarae presented 2n = 38 and FNa = 38. The karyotype has 34 acrocentric autosomes and a small pair of metacentrics (pair 18). X and Y are also acrocentric chromosomes. C-bands evidenced heterochromatic blocks at the pericentromeric regions of some autosomes (pairs 1 to 10, one homolog of pair 12 and the small metacentric pair 18) and X chromosome; in pairs 1 and 3 these blocks are heteromorphic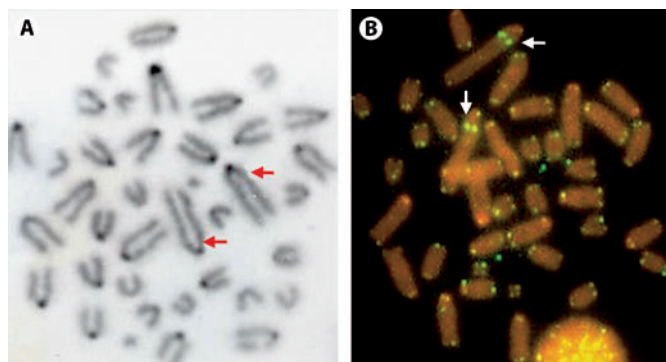 (Fig. 2A). FISH revealed weak signals at the end of all chromosomes and additionally an intense ITS proximal on chromosome 1 (Fig. 2B). No correspondence between the hybridization signals and heterochromatic regions was observed.

Akodon lindberghi presented 2n = 42 and FNa = 42 with 38 acrocentric autosomes, X and Y acrocentrics, and one small metacentric pair (pair 20). After C-banding analysis, conspicuous blocks of pericentromeric heterochromatin in all chromosomes were visualized, including the small metacentric pair (Fig. 3A). Stronger telomeric signals were observed on pericentromeric regions of some autosomes and on the X chromosome of males (Fig. 3B); in one of the two females analyzed only one X chromosome showed a stronger signal which allowed its distinction from the other X (Fig. 3C) while in the other female the signals of the X were regular and it was not possible to distinguish these chromo-
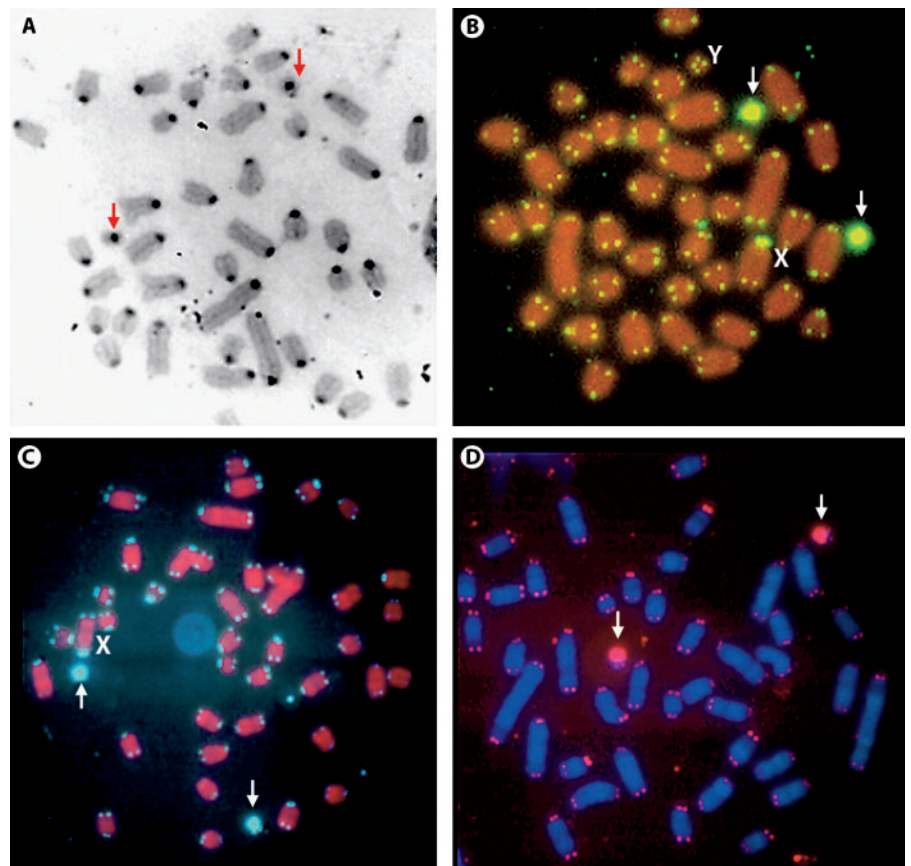
Fig. 3. Metaphases of *Akodon lindberghi* (2n = 42). (**A**) C-banding pattern. (**B**, **D**) Telomeric FISH in different specimens. (**B**) Male metaphase: the X chromosome and metacentric pair completely hybridized are indicated. (**C**) Female metaphase: one single distinguishable X and the metacentric pair completely hybridized are indicated. (**D**) Female metaphase: X chromosomes are indistinguishable and metacentric pair partially hybridized (arrows).
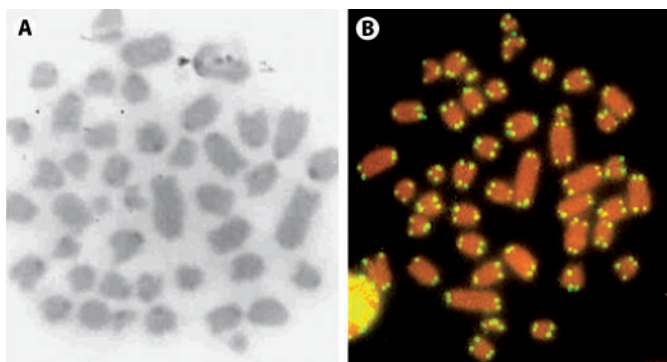

Fig. 4. (**A**, **B**) *Akodon paranaensis* (2n = 44) metaphases. (**A**) C-banding pattern. (**B**) FISH with telomeric probe, signals are exclusively telomeric.

somes from the medium autosomes based on the intensity of FISH signals (Fig. 3D).

In addition, three specimens showed the small metacentric pair hybridized throughout the extension with telomeric probes (Fig. 3B and 3C); and in the fourth specimen, the small metacentric showed signals of hybridization in only one of the arms (Fig. 3D).

Akodon paranaensis presented 2n = 44 and FNa = 44 with all the autosomes being acrocentrics except for one small metacentric pair (pair 21); the X is a medium-sized acrocentric and the Y is a small acrocentric. C-banding showed the near absence of heterochromatin in the pericentromeric regions of the autosomes (Fig. 4A). FISH showed strong telomeric signals exclusively at the ends of all chromosomes (Fig. 4B).

Akodon serrensis showed 2n = 46 and FNa = 46; the karyotype is composed of 42 acrocentrics, a small metacentric pair (pair 22) and X and Y acrocentrics. All autosomes showed conspicuous C-band blocks at pericentromeric regions (Fig. 5A). FISH showed weak telomeric signals at the end of all chromosome arms (Fig. 5B). Non-telomeric sites were observed in the pericentromeric region of all chromosomes (Fig. 5B and 5C), corresponding to the regions that present conspicuous C-band blocks.

G-banding patterns of the four different karyotypes *(A. azarae, A. lindberghi, A. serrensis* and *A. paranaensis)* were compared. Chromosome 1 is larger in the species with lower diploid number – *A. azarae* (AAZ1) and *A. lindberghi* (ALI1) – and presents an extra negative proximal G-band when compared to the chromosome 1 of species with higher diploid number – *A. paranaensis* (APA1) and *A. serrensis* (ASE1) (Fig. 6).
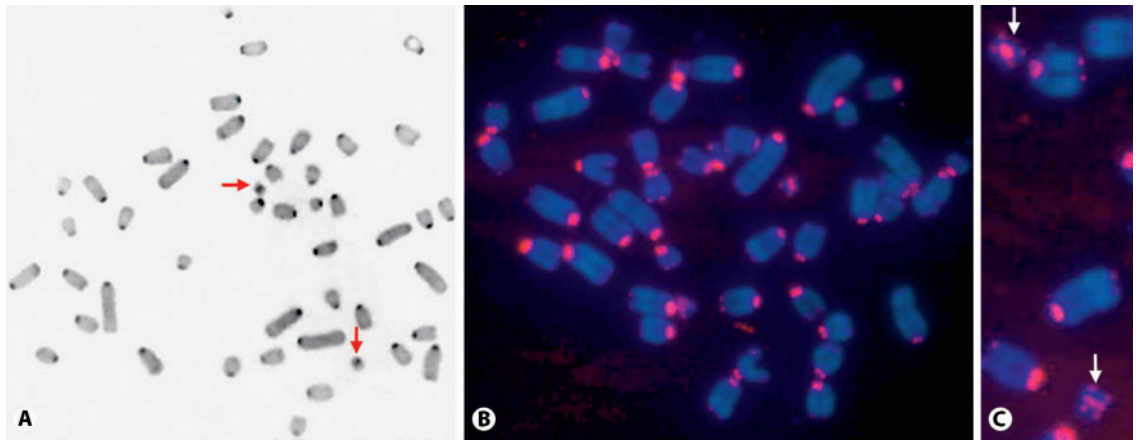
Fig. 5. *Akodon serrensis* (2n = 46) metaphases. (**A**) C-banding patterns. (**B**, **C**) FISH with telomeric probes. (**B**) Spread metaphase, with pericentromeric hybridization. (**C**) Detached to the metacentric pair (arrows).

Discussion

The karyotypes of all species reported here were similar to those previously described in the literature: *A. azarae* (Bianchi et al., 1976, 1989; Bianchi and Merani, 1984; Vitullo et al., 1986; Sbalqueiro, 1989); *Akodon lindberghi* (Svartman and Almeida, 1994; Geise et al., 1996); *A. paranaensis* (Liascovich and Reig, 1989; Sbalqueiro, 1989; Christoff et al., 2000); and *A. serrensis* (Sbalqueiro, 1989; Fagundes, 1993; Geise, 1995; Christoff et al., 2000).

The localization of telomeric sequences using FISH has been conducted in studies of chromosome evolution for many vertebrates, as numerical and structural karyotypic changes can occur in the process of diversification and speciation. Besides the hybridization of telomeric probes in the terminal region of the chromosomes, the detection of interstitial signals is relatively common in several species (Meyne et al., 1990).

In our sample composed of four species we found interstitial telomeric sequences in three of them *(A. azarae, A. lindberghi* and *A. serrensis)* associated with a chromosome rearrangement, pericentromeric heterochromatin and euchromatic regions that apparently are not related to rearrangements. Only *A. paranaensis* did not present ITS.

The proximal ITS found on chromosome 1 of *A. azarae* (AAZ1) seems to be related to the area of the chromosome rearrangement event which is a tandem fusion/fission involving chromosomes 1 and 20 of the other two species *(A. paranaensis* and *A. serrensis)* (Fig. 6).

Despite the similarity of G-banding patterns with AAZ1, chromosome 1 of *A. lindberghi* (ALI1) did not exhibit ITS at the same site. We could hypothesize that (1) if a fusion event had occurred, the interstitial telomeric sequence would have been lost in *A. lindberghi* but it would have been kept in *A. azarae*; (2) if a fission event had occurred, only *A. azarae* would be able to give rise to chromosomes 1 and 20 of the other species with higher diploid numbers. The comparative G-banding among AAZ1, ABO1 and ALI1 con-
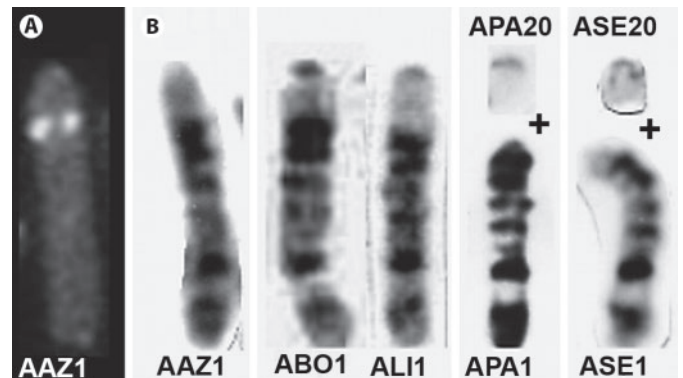

Fig. 6. (**A**) Telomeric FISH in chromosome 1 of *Akodon azarae* (AAZ1): ITS proximally at the long arm. (**B**) G-banding comparison among chromosome 1 of *A. azarae* (AAZ1), *A. boliviensis* (ABO1), from Vieira et al. (2004), *A. lindberghi* (ALI1), *A. paranaensis* (APA1) from Sbalqueiro (1989) and *A. serrensis* (ASE1). Plus signs indicate the rearrangement sites between chromosome 1 and chromosome 20 of *A. paranaensis* (APA1 and APA20) and between chromosome 1 and chromosome 20 of *A. serrensis* (ASE1 and ASE20).

firms the homeology of this chromosome and allows inferring that APA1 + APA20 and ASE1 + ASE20 are involved in the rearrangement.

Loss of telomeric sequences is supposed to occur in other rodent species (Garagna et al., 1995; Silva and Yonenaga-Yassuda, 1998b). Fagundes and Yonenaga-Yassuda (1998) proposed that recent events tend to maintain the ITS, likewise ancient rearrangements tend to lose those sequences.

The same ITS signal observed in AAZ1 was reported for specimens of *A. azarae* and *A. boliviensis* (ABO1) from Argentina. The authors suggested that the ITS detected on both species could be remainders from ancestral rearrangements, since they did not correspond to heterochromatic regions (Vieira et al., 2004). These results reinforce our

proposition that the presence of the ITS in *A. azarae* (and consequently to *A. boliviensis*) is due to a tandem fusion/fission rearrangement, responsible for part of the difference found in diploid numbers among the species herein presented.

Other kinds of not exclusively telomeric signals were observed in *A. lindberghi* and *A. serrensis*.

A. serrensis showed proximal telomeric signals larger than those observed at the distal end of the acrocentrics; the small metacentric pair exhibited weak signals at the ends of both arms and ITS on pericentromeric regions, corresponding to heterochromatic blocks. There is co-localization with the telomeric probe and pericentromeric regions, in which constitutive heterochromatin is found. This could be related to the repetitive nature of the heterochromatin.

Co-localization of heterochromatin and telomeric sequences was previously reported in the literature. According to Multani et al. (2001), at least three types of heterochromatin in mammals can be related to the presence of the $(TTAGGG)_n$ sequence: telomeric heterochromatin, non-telomeric heterochromatin and a combination of both. *A. azarae* and *A. paranaensis* presented only non-telomeric heterochromatin, while *A. serrensis* presented only telomeric heterochromatin. In *A. lindberghi* both kinds of heterochromatin were found, considering the telomeric hybridization.

In addition to the telomeric signal co-localized with pericentromeric heterochromatin, other kinds of non-telomeric signal were observed on the small metacentric pair of *A. lindberghi*: (a) the whole chromosome presented hybridization signal (including euchromatic arms and heterochromatic pericentromeric regions) or (b) only one of the arms and the pericentromeric region were labeled. C-banding patterns of the small metacentric pair were the same in all individuals. This exceptional result of co-localization of the telomeric probe with euchromatin in the whole or partially in the metacentric pair of *A. lindberghi* suggests that the eu-

chromatin of these chromosomes is composed of a highly repetitive region rich in TTAGGG-like sequences and that they are not related to rearrangement sites. Instead of this they could be associated with amplifications along the chromosome arms.

The telomeric sites present a high repetitive DNA sequence $(TTAGGG)_n$. Non-telomeric sites observed at the pericentromeric region of the chromosomes (corresponding to heterochromatic blocks) suggest that telomeric sequence is a common component of satellite DNA in some vertebrate species (Nanda and Schmid, 1994; Garagna et al., 1997; Nanda et al., 2002; Pagnozzi et al., 2002).

Karyotypic comparison using G-banding patterns among *Akodon* species *(Akodon cursor, A. montensis, A. lindberghi* and *A. serrensis)* carried out by Geise et al. (1998) showed that these species share a substantial proportion of their chromosome complements and that the metacentric pair presents the same G-banding patterns. It was suggested that the referred pair is invariant among those *Akodon* species. Our data of telomeric FISH, however, show that although these chromosomes have the same G-banding pattern, molecular cytogenetics indicated a finer resolution in which sequences of this small metacentric can be distinct among *Akodon* species.

Our results raised questions about the ordinary interpretation concerning the presence of ITS in karyotypes. *Akodon* species appear to be good examples that FISH results must be associated with G- and C-band techniques, and an accurate interpretation of the karyotype evolution of the species should be considered.

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