Localization of Telomeric Sequences in the Chromosomes of Three Species of Calomys (Rodentia, Sigmodontinae)

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Summary The distribution of telomeric sequences in 3 species of the phyllotine genus Calomys, whose members are distributed through an extensive area of South America, were analyzed by FISH with a PNA probe. C. musculinus, with a highly reordered karyotype with respect to other species of the genus, and C. venustus only showed fluorescent signals in a telomeric position. C. laucha, on the other hand, presented in addition a remarkable set of internal telomeric signals (ITS). ITS were seen constantly in the centromeric regions of biarmed pairs 1 and 2 and the X chromosome of this species, being the latter signal formed by 2 separated marks. On the basis of previous karyotypic information, these constant signals are interpreted as resulting from rearrangements occurred during karyotypic evolution. Additional signals of generally lower intensity were observed with different frequencies in up to 6 subterminal chromosomes of this species.

Key words Calomys laucha, Calomys musculinus, Calomys venustus, Telomeric sequences, PNA.

The ends of vertebrate chromosomes are characterized by the presence of tandem repeats of the canonical sequence T2AG3 (Moyzis et al. 1988, Meyne et al. 1989, 1990). Less frequently, tracts of this telomeric sequence can be detected at additional, non-telomeric locations, mainly at pericentric regions but also at interstitial sites, and the distribution of these internal telomeric signals (ITS) can vary among closely related species (Meyne et al. 1990). ITS are generally considered to be relics of chromosome rearrangements (Meyne et al. 1990, Metcalfe et al. 2002, Nanda et al. 2002) or to correspond to repetitive DNA rich in (T2AG3)n sequences (Garagna et al. 1997, Meyne et al. 1990, Multani et al. 2001, Pagnozzi et al. 2000). In addition, molecular studies have suggested that some short internal telomeric repeats might have been originated by the repair of double-stranded breaks (Azzalin et al. 2001, Faravelli et al. 2002, Nergadze et al. 2004).

The analysis of ITS in cases of polytypism and/or polymorphism for some chromosomal rearrangements has contributed to understand their mechanism of origin. For instance, in M. musculus domesticus, the paradigmatic case of centric fusion, no telomeric sequences are retained in the biarmed chromosomes (Garagna et al. 1995, 2001, Nanda et al. 1995, Kalitsis et al. 2006); no ITS are observed either in the biarmed chromosomes of the shrew Suncus murinus (Rogatcheva et al. 2000). In other cases with a high level of Rb (Robertsonian) variation, such as in the Sorex araneus complex (Zhdanova et al. 2005) and Mus minutoides (Castiglia et al. 2002, 2006), the opposite situation is the rule.

With respect to sigmodontines, a polymorphic chromosome of Akodon cursor, probably originated by pericentric inversions and centric fusion of 2 subtelocentrics, shows a centromeric signal (Fagundes et al. 1997a, b). In Akodon dolores, with Rb polymorphisms of pairs 2 to 5 and another of pair 1 that resembles that of A. cursor (Bianchi et al. 1979, Wittouck et al. 1995), centromeric ITS are observed only in pairs 4 and 5 (Viera et al. 2004). Similarly, 2 cytotypes of the Oryzomys
*subflavus* complex, related by 4 Rb rearrangements, show ITS in only 2 of the 4 biarmed pairs (Andrades-Miranda et al. 2002). The polymorphic Rb chromosome of *Bolomys lasiurus* is also characterized by an ITS (Fagundes and Yonenaga-Yassuda 1998).

ITS locations in related species have been also analyzed, although it must be considered that the older origin of the rearrangements would in principle increase the probability of sequence amplification or loss. Concerning sigmodontine rodents, no ITS reflect the remarkable rearrangements that relate *Akodon cursor* and *A. montensis* (Fagundes et al. 1997b), or this species and the more distant *Bolomys lasiurus* (Fagundes and Yonenaga-Yassuda 1998), nor show ITS the chromosomes that result from tandem fusions in *Nectomys* (Silva and Yonenaga-Yassuda 1998). On the other hand, the homeologous telocentric first pairs of *A. azarae* and *A. boliviensis*, which present a noticeable proximal IT (Viera et al. 2004, Ventura et al. 2006), correspond to pairs 1 plus 20 of *A. paranaensis* and *A. serrensis* (Ventura et al. 2006).

In this paper we study the distribution of telomeric signals in the chromosome complements of 3 species of the phyllotine genus *Calomys*: *C. laucha*, *C. musculinus* and *C. venustus*, using fluorescence *in situ* hybridization (FISH) with a telomeric peptide nucleic acid (PNA) probe. The members of this widespread and systematically complex genus (Chiappero et al. 2002, Salazar-Bravo et al. 2001, Almeida et al. 2007) are found through an extensive area of South America (Redford and Eisenberg 1992), occupying different mountain and lowland habitats. Some of its species have been identified as reservoirs of the viral agents of emerging infectious diseases (Carrol et al. 2005, Enria et al. 1998, Johnson et al. 1997, Mills et al. 1994).

**Materials and methods**

The specimens used in this study were live-trapped in several localities of the province of Córdoba, Argentina, as follows: *C. musculinus* (1 male) in the vicinity of Chucul; *C. laucha* (4 males, 2 females) in this locality and in Holmberg, and *C. venustus* (2 males, 1 female), in the neighbourhood of Río Cuarto.

Mitotic chromosome spreads from bone marrow were obtained by conventional procedures, following *in vivo* injection of colchicine. For FISH, a FITC-labeled (C3TA2)3 PNA probe was used (Applied Biosystems), following Viera et al. (2002). Briefly, slides were fixed in 4% formaldehyde, treated with pepsin, fixed again, dehydrated in an ethanol series and air-dried. After the addition of the hybridization mix, denaturation was performed and the slides were hybridized for 2 h at room temperature, then washed and counterstained with DAPI, and finally mounted in Vectashield. Observations were made under a Zeiss Axiophot microscope equipped with epifluorescence optics and the corresponding filter sets. Images were recorded with an AxioCam HRc camera (Zeiss) and the AxioVision software (Zeiss), and processed using the Adobe Photoshop 8.0.1 program.

**Results**

The specimens of *C. venustus* here analyzed showed a karyotype of 56 chromosomes, composed of 6 biarmed and 21 telocentric autosomal pairs (AFN, Autosomal Fundamental Number =66), a submetacentric X and a small subterminal Y chromosome. This karyotype conforms to that previously described in specimens from Córdoba and San Luis (Gardenal et al. 1977, Lisanti et al. 1976, 2004, Vitullo et al. 1990). Only the chromosomal ends presented the expected telomeric marks in this species; the telomeric signals at the centric end of the apparently telocentric chromosomes are clearly double (Fig. 1A).

The only specimen of *C. musculinus* here analyzed was heterozygous for a previously described polymorphism (Forcone et al. 1980, Lisanti et al. 1996), consisting in the presence of a small metacentric without centric heterochromatin in place of a small subterminal chromosome.
The species complement (2n=38) is composed of 10 meta-submetacentric and 8 subterminal autosomal pairs, a submetacentric X and a minute Y chromosome (Gardenal et al. 1977, Forcone et al. 1980, Lisanti et al. 1996). As in C. venustus, only the chromosomal ends were marked by the PNA probe in C. musculinus cells (Fig. 1D).

C. laucha specimens showed a complement of 64 chromosomes formed by 3 biarmed and 28 terminal or subterminal autosomal pairs (AFN=68), a submetacentric X and a subterminal Y chromosome, a karyotype basically corresponding to that observed in specimens from Córdoba (Gardenal et al. 1977, Lisanti et al. 2004) and several localities of Uruguay and Argentina (Brum-Zorrilla et al. 1990). The metaphase cells of this species not only presented the expected marks at the telomeres, but also showed a remarkable set of signals at non-telomeric positions. In effect, conspicuous marks were observed in the centric regions of the biarmed chromosomes of pairs 1 and 2 and of the X and, in addition, interstitial signals were present in up to 6 subterminal chromosomes per cell, probably corresponding to three pairs (Fig. 1B, C).

The ITS on the chromosomes of pairs 1 and 2 were clearly centric in position, being that on pair 1 noticeably brighter than the marks seen at telomeric sites (Fig. 1B, C). On the other hand, the signal on the X was particular: it was double, consisting of a paracentric mark on the short arm and another, brighter one on the long arm (Fig. 1B, C).

In order to estimate the frequency of the different ITS, we studied 35 complete C. laucha metaphase cells. The signals on the chromosomes of pairs 1 and 2 and on the X were present in

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**Fig. 1.** FISH with a telomeric PNA probe on three *Calomys* species. A) *C. venustus*, male cell; no ITS are seen. B) *C. laucha*, male cell with the maximum number of ITS found; notice the internal marks on pairs 1, 2 and on the X (identified), and on 6 subterminal chromosomes (arrowheads). C) Selected examples of ITS on pairs 1, 2 and X chromosome from several male and female cells of *C. laucha*. D) *C. musculinus*, male cell; no ITS are seen. The bar corresponds to 10 μm.

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every cell. So, in 32 out of the 35 cells studied (91%), the ITS on both members of pairs 1 and 2 and on the X chromosome were observed; in the remaining three cells, only one of the chromosomes of pair 2 did not bear the centric mark. The interstitial signals on the subterminal chromosomes, on the other hand, lacked this regularity. Six marked subterminal chromosomes were present in 11 cells (31.4%), whereas 2 cells (5.7%) showed ITS on 5 subterminals, 9 cells (25.7%) had 4 interstitially marked subterminals, 11 cells presented three (31.4%) and 2 (5.7%) showed only 2 subterminal chromosome bearing ITS.

Discussion

A karyotype of 68 terminal autosomes (2n=70, AFN=68) has been considered the primitive phyllotine karyotype (Pearson and Patton 1976). It has been proposed (Vitullo et al. 1990, Espinosa et al. 1997) that Calomys karyotypes have evolved from this basic karyotype chiefly through progressive steps of centric fusion, plus several pericentric inversions necessary to explain the more derived karyotypes (C. callosus and C. musculinus). Phylogenetic studies based on the mitochondrial cytb gene (Salazar-Bravo et al. 2001, Almeida et al. 2007) confirm this general trend, but indicate that chromosome number reduction has taken place independently in each main clade. In the analysis of Almeida et al. (2007), C. musculinus groups with the highland clade, and C. laucha appears as a sister taxon to the “large bodied” group of lowland species which includes C. venustus.

On this ground, it is remarkable the lack of ITS on C. venustus chromosomes, with five biarmed pairs, and especially in the highly reordered complement of C. musculinus. The only specimen of C. musculinus analyzed was heterozygous for a rearrangement interpretable as arising by a pericentric inversion of an acrocentric which has suffered a previous loss of centric heterochromatin (Forcone et al. 1980). As expected from this mechanism, no ITS was present in this chromosome.

With respect to heterochromatin content, while C-bands cannot be detected in C. venustus (Lisanti et al. 2004), conspicuous pericentric blocks are seen in C. musculinus autosomes and the X (Forcone et al. 1980, Lisanti et al. 1996, Corach et al. 1988). Moderate centric C-bands are also noticed in C. laucha karyotype (Lisanti et al. 2004). C. laucha and C. musculinus possess relatively abundant repetitive sequences with at least partial homology but different organization, which could have undergone intense amplification in the latter species (Corach et al. 1988, Corach and Semorile 1989, Corach 1990). Our results indicate that the repetitive fractions of these species heterochromatin are not enriched in telomeric-like sequences.

On the other hand, centric C-bands are also present in the chromosomes of C. tener (Fagundes et al. 2000), a species that is basal to C. laucha and to the clade of large-bodied species which includes C. venustus (Almeida et al. 2007). It seems then that a process of reduction of highly repetitive sequences has taken place during C. venustus karyotypic evolution.

In a G-band comparison (Lisanti et al. 2004), most of the autosomes of C. venustus and C. laucha could be matched, either directly (as shared chromosomes) or as implicated in rearrangements; no equivalence was found, however, for 4 autosomes of each species, comprising 5 chromosomal arms, including the biarmed pairs 1 of laucha and 5 of venustus.

Of the 5 biarmed pairs present in C. venustus and the 3 present in C. laucha, only the smallest pair (pairs 6 and 3, respectively) appears to be directly conserved. On the other hand, C. venustus first pair would have originated by a tandem fusion between the short arm of biarmed pair 2 and the centromeric end of pair 26 of C. laucha. It seems, then, that laucha pair 2 and the small conserved metacentric should have been present in a karyotype ancestral to these 2 species, before independent karyotype evolution started, a karyotype perhaps related to those of C. tener (2n=66, AFN=66) and C. expulsus (2n=66, AFN=68) (Svartman and Almeida 1992, Fagundes et al. 2000). It can be observed that these karyotypes include a metacentric pair similar in size and, at least in C. tener (Fagundes et al. 2000), also in banding pattern, to the small metacentric of laucha.
and *venustus*. The absence of the centric mark of *C. laucha* pair 2 in the fused *venustus* pair 1 probably results from subsequent loss of the telomeric sequences. The fusion point in the short arm of this chromosome is not represented either by an ITS; in this case, it is not possible to know whether the fusion process did not include telomeric sequences or these were lost after it.

The biarmed pairs 2, 3 and 4 of *C. venustus* are represented in *C. laucha* karyotype by acrocentric pairs (Lisanti et al. 2004). In this case too, the absence of pericentric ITS in the biarmed *venustus* chromosomes cannot help to decide whether telomeric sequences were not incorporated during the fusion, or were initially retained but lost during the general reduction of repetitive sequences occurred in *C. venustus*.

*C. laucha* specimens showed constant ITS on the 1st and 2nd pairs and on the X chromosome, and less frequent ITS on a variable number of subterminal chromosomes. According to the hypothesis of independent chromosome number reduction, the centric ITS on pairs 1 and 2, of which that on the former was particularly strong, would represent relics of centric fusions of telocentrics of the primitive karyotype, noticeably amplified in the case of the first pair.

The particular signal on the X chromosome of *C. laucha*, whose relative size (5.13%, Lisanti et al. 2004) conforms to the standard size of the X of eutherian mammals, deserves attention. Taking into account that the G-banding patterns of the X of *C. venustus* and *C. laucha* differ only by the position of the unique band of the short arm, it is tempting to suppose a mechanism of several successive steps to explain the origin of this double signal: 1) a paracentric inversion of the short arm, with a break in the telomeric region and the other near the centromere, 2) considerable amplification of the telomeric sequences in the new position, and 3) a pericentric inversion with a break in the region of the amplified sequences and the other in the proximity of the centromere.

The interstitial signals present in several acrocentric chromosomes of *C. laucha* remind those observed in some telocentrics of *Akodon azarae* (Viera et al. 2004). As in that case, the signals are not seen in every cell, which could indicate that the repeat length of this sequence is near the resolution limit of the technique. These ITS could result from the amplification of pre-existing T_2A_3 repeats, perhaps originated by double stranded breaks repair (Azzalin et al. 2001, Faravelli et al. 2002, Nergadze et al. 2004).

The application of these and other cytogenetic and molecular approaches to more *Calomys* species would surely contribute to a better understanding of karyotypic evolution in this systematically complex genus.

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References


2007 Telomeric sequences in three species of Calomys 171


