Chromosomal localization of the telomeric (TTAGGG)$_n$ sequence in four species of Armadillo (Dasypodidae) from Argentina: an approach to explaining karyotype evolution in the Xenarthra

M. S. Lizarralde[1,2]*, A. D. Bolzán[3], S. Poljak[1,2], M. I. Pigozzi[1], J. Bustos[1] & M. S. Merani[1]
[1]*Centro de Investigaciones en Reproducción (CIR), 2da Unidad Académica de Histología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso10, 1121 Capital Federal, Argentina; Tel: +54-11-59509612/ +55-221-4210112; Fax: +54-11-59509612/+54-221-4253320; E-mail: mlizarralde@fmed.uba.ar;* [2]*Centro Austral de Investigaciones Científicas, CADIC-CONICET, CC. 92, 9410 Ushuaia, Tierra del Fuego, Argentina;* [3]*Instituto Multidisciplinario de Biología Celular, IMBICE, CC 403, 1900 La Plata, Buenos Aires, Argentina*
*Correspondence

Abstract

The distribution of the vertebrate telomeric sequence (TTAGGG)$_n$ in four species of armadillos (Dasypodidae, Xenarthra), i.e. *Chaetophractus villosus* (2n = 60), *Chaetophractus vellerosus* (2n = 62), *Dasypus hybridus* (2n = 64) and *Zaedyus pichiy* (2n = 62) was examined by FISH with a peptide nucleic acid (PNA) probe. Besides the expected telomeric hybridization, interstitial (centromeric) locations of the (TTAGGG)$_n$ sequence were observed in one chromosome pair of *Chaetophractus vellerosus* and *Zaedyus pichiy*, suggesting chromosome fusion of ancestral chromosomes occurring during the evolution of Dasypodidae. In addition, all the species analysed showed one to four apparently telocentric chromosomes, exhibiting only two telomeric signals. However, the immunodetection study of kinetochore proteins on synaptonemal complex spreads from *C. villosus* showed that the apparently telocentric chromosomes have a tiny short arm that can be resolved only in the more elongated pachytene bivalents. This finding suggests that none of the species of armadillos possess true telocentric chromosomes. Our present results support a reduction in the diploid number by fusion of acrocentrics with loss of chromosome material as a tendency in Dasypodidae.

Introduction

The mammalian order Xenarthra is composed of 30 living species placed into two suborders: Cingulata, represented by Dasypodidae (armadillos) and Pilosa, represented by Myrmecophagidae (anteaters), Bradypodidae (three-toed sloths) and Megalonychidae (two-toed sloths) (Madsen *et al.* 2001, Delsuc *et al.* 2001, 2002, 2003, 2004). These animals are found from the south-central and southeastern United States to southern South America (Wetzel 1985). Armadillos (Dasypodidae) are the oldest and most diverse lineage of Xenarthra with 21 described species living mostly in South America, 14 of which are present in Argentina (Wetzel 1985).

Previous cytogenetic studies using conventional staining and banding techniques have shown that karyotypic evolution in Dasypodidae is characterized by a high variability in chromosome number (ranging from 2n = 38 to 2n = 64) and morphology (Jorge

et al. 1977, 1985). At present, the only data on chromosome polymorphisms described in armadillos corresponds to one pericentric inversion of par 1 in *Chaetophractus villosus*, which was described in an isolated population of this species (Lopez *et al.* 1997, Merani 2002). Despite all the studies on the cytogenetics of Dasypodidae performed so far, the precise nature of the chromosomal rearrangements involved in the karyotype evolution of this group of mammals is still unknown. This is due to the fact that all of these studies were restricted to conventional staining and banding methods, and that banding pattern comparisons are difficult in Xenarthra due to the small size of most of the chromosomes of the karyotype (Jorge *et al.* 1977, 1985).

In order to better characterize the karyological evolution of Dasypodidae, we decided to perform an analysis of the distribution of the conserved vertebrate telomeric sequence TTAGGG (Moyzis *et al.* 1988, Meyne *et al.* 1989) on the chromosomes of several species of armadillos. Telomeres are specialized nucleoproteic complexes localized at the physical ends of linear eukaryotic chromosomes that maintain their stability and integrity (McClintock 1941, Blackburn 1991). Telomeres can be lost or gained during speciation and karyotypic evolution (Meyne *et al.* 1990) and in many species telomeric repeat sequences – the DNA component of telomeres – can be present at both telomeric and non-telomeric sites of the chromosomes (Meyne *et al.* 1990, Multani *et al.* 2001). The appearance of these non-telomeric locations – mainly at the centromeric or pericentromeric region of chromosomes – is believed to result from chromosomal fusion and fission events that occurred during karyotype evolution of the species (Meyne *et al.* 1990).

In this study, we localized the telomeric repeats in the karyotypes of four species of armadillos of Argentina: *Chaetophractus villosus*, *Chaetophractus vellerosus*, *Dasypus hybridus* and *Zaedyus pichiy* to identify possible chromosomal rearrangements involving telomeres which occurred during the evolution of Dasypodidae.

Materials and methods

Ten specimens belonging to the following species: *Dasypus hybridus*, *Chaetophractus villosus*, *Chaetophractus vellerosus* and *Zaedyus pichiy* were studied.

They were collected at several locations in the provinces of Buenos Aires (one male and one female of *C. villosus*, one male and one female of *C. vellerosus*, and one male and one female of *D. hybridus*), Santa Cruz (two male of *Z. pichiy*) and Tierra del Fuego (one male and one female of *C. villosus*), Argentina. Data about locations where animals were collected are reported elsewhere (Poljak *et al.* 2004a, 2004b).

Mitotic chromosomes were prepared from lymphocyte culture using RPMI 1640 Medium supplemented with 20% FBS and directly from bone marrow cell suspension using standard techniques. Routine karyotypic analysis was performed on the preparations stained with 5% Giemsa solution. In order to identify possible chromosome rearrangements, metaphases of all the species studied were G-banded according to the method of Seabright (1971). C-banding was performed following the technique of Sumner (1972).

Moreover, 2 males of *C. villosus* were processed to obtain material for complementary meiosis studies. Synaptonemal complex spreads were prepared from seminiferous tubules from one testis of a hemi-castrated individual. Briefly, seminiferous tubules were minced in Hank's balanced salt solution at room temperature. One or two tubules were gently squeezed between frosted glasses and the cells resuspended in 100 mmol/L sucrose. About 60 μl of the cell suspension were dropped onto a glass slide covered with fixative (1% paraformaldehyde, 0.15% Triton X-100). Spreads were left in a humid chamber for several hours, washed in Photoflo and used immediately for immunostaining. The first antibody was an antibody against the synaptonemal complex protein 3 (SCP3, a gift from P. Moens, York University, Canada) that labels the lateral elements and anti-centromere human serum (a gift from W. Brinkley, Baylor College of Medicine, Texas, USA) that binds to centromeric proteins. Second antibodies were goat anti-human FITC and goat anti-rabbit Texas red (Jackson Immunoresearch). The second antibodies were diluted 1:100 in PBT (PBS, 3% BSA and 0.05% Triton X-100). Nuclei were counterstained with DAPI and mounted in glycerol containing antifade. Spreads were examined with a Leica fluorescence microscope and photographed with Kodak colour film using suitable filters for each fluorochrome. Single-colour immunodetection was used to obtain the exact position of the centromere with respect to the corresponding synaptonemal com-

plex, since some shifting may occur using super-imposed images after double-colour immunostaining.

In order to detect the presence of $(TTAGGG)_n$ repeats, fluorescence *in-situ* hybridization (FISH) was performed on the metaphase chromosomes with a Cy3-conjugated peptide nucleic acid (PNA) pan-telomeric probe obtained from DAKOCytomation (Glostrup, Denmark). FISH was performed according to the instructions provided by the supplier. Signals were observed using a Carl Zeiss (Germany) epi-fluorescence microscope equipped with an HBO 100 mercury lamp and filters for DAPI and Cy3 (Chroma Technology, USA). Ektachrome film, ASA 400 (Eastman Kodak Company, Rochester, NY) was used for photography. Images were processed using the Adobe Photoshop CS program.

Results and discussion

The cytogenetic analysis of the animals studied showed the following karyotypic constitutions: 2n = 60 in *C. villosus*, 2n = 62 in *C. vellerosus* and *Z.*

pichiy and 2n = 64 in *D. hybridus*. It has to be mentioned that the highest 2*n* number present in armadillos corresponds to the genus *Dasypus* (Leitao Barroso & Seuanez 1991). The standard karyotypes of these species have been previously described (Sáez *et al.* 1964, Meritt *et al.* 1973, Jorge *et al.* 1977, 1985, Pagliero *et al.* 1993, Rahn *et al.* 1994, Merani 2002, Poljak *et al.* 2004a).

C. villosus and *Z. pichiy* both have 15 autosomal pairs of biarmed chromosomes of large to small size, whereas *C. vellerosus* has 14 autosomal pairs of biarmed chromosomes and *D. hybridus* has the higher number of acrocentrics with only 8 biarmed chromosome pairs, all of them submetacentrics. The X chromosome is a large submetacentric in all the species analysed but the Y chromosome differs between them. In effect, whereas the Y chromosome is an acrocentric or subtelocentric of small size in *D. hybridus* and *C. villosus*, it is the smallest chromosome of the complement in *Z. pichiy* and *C. vellerosus*. Our results are in good agreement with the karyotypes of *D. hybridus*, *C. villosus* and *Z. pichiy* described by Jorge *et al.* (1977). However,
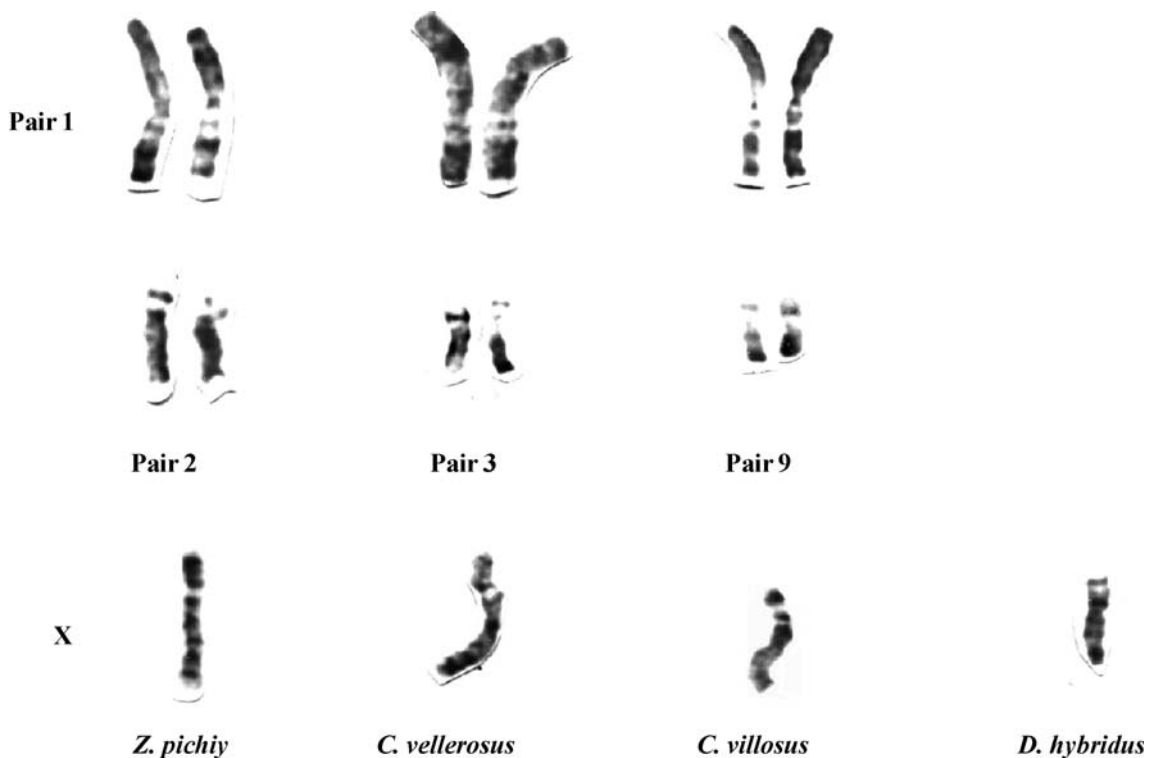

Figure 1. Interspecies chromosome homologies after G-banding in the four species of armadillos examined.

while these authors claimed that the X chromosome in *C. villosus* is acrocentric, we found that this chromosome is submetacentric (Figure 1).

The G- and C-banding patterns observed were similar to the ones previously described (Jorge *et al.* 1977, Rahn *et al.* 1994). Due to the small size of several pairs of chromosomes, G-banding pattern comparisons between the four species examined were

difficult to perform. However, it was possible to establish that chromosome 1 was conserved in *Z. pichiy*, *C. vellerosus* and *C. villosus*, and that chromosome 2 of *Z. pichiy* is completely homologous to chromosome 3 of *C. vellerosus* and partially homologous to chromosome 9 of *C. villosus* (Figure 1). In addition, the X chromosome was conserved in all of the species studied (Figure 1). The differences in
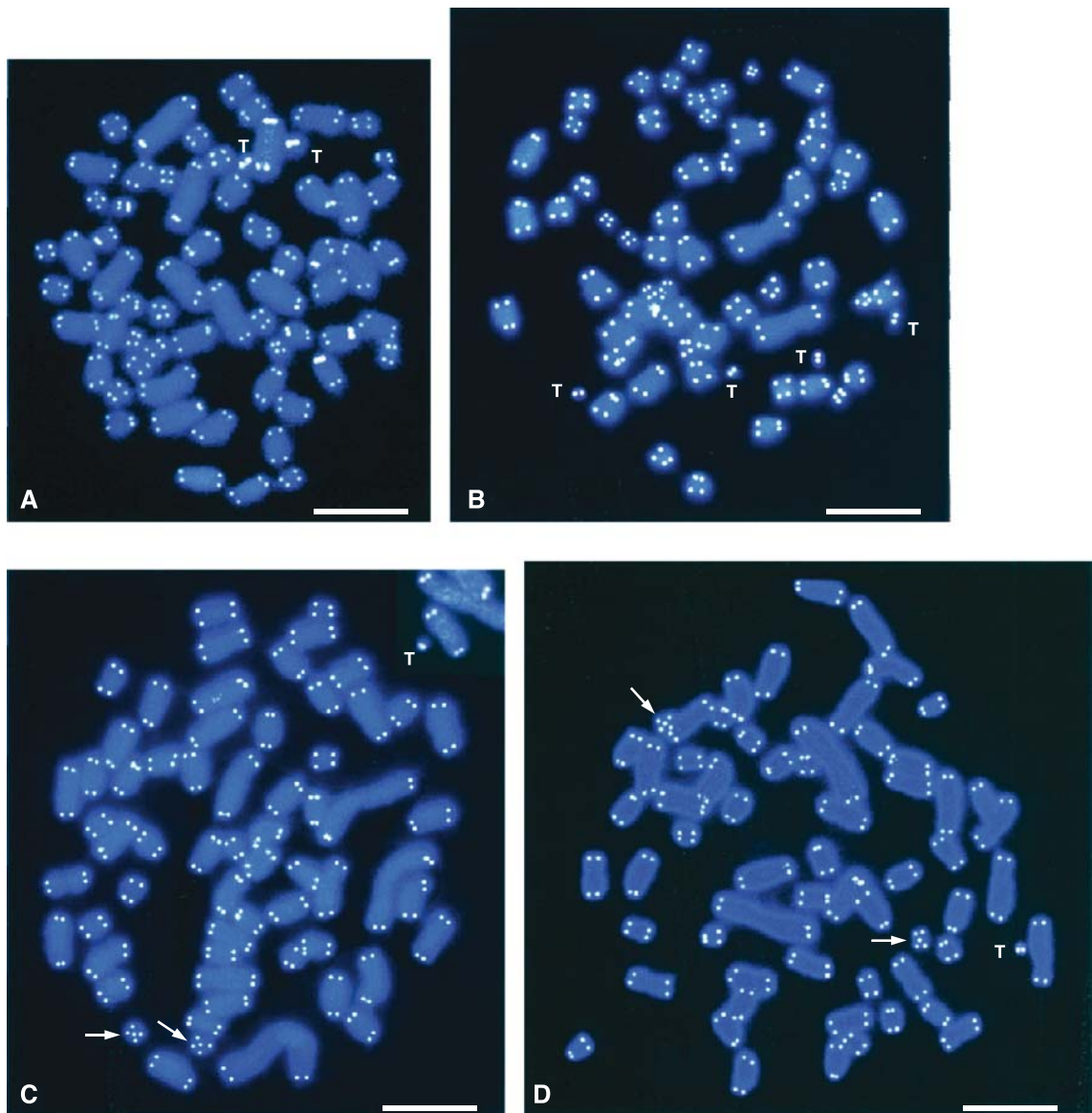

Figure 2. *In-situ* localization of the telomeric sequence (TTAGGG)$_n$ on the metaphase chromosomes of *Dasypus hybridus* (**A**, male), *Chaetophractus villosus* (**B**, male), *Chaetophractus vellerosus* (**C**, female; figure insert, male, showing the Y chromosome) and *Zaedyus pichiy* (**D**, male). Arrowheads in **C** and **D** indicate the location of ITR. Letter "T" indicates telocentric chromosomes.

G-banding pattern between the chromosome 9 of *C. villosus* and chromosomes 2 of *Z. pichiy* and 3 of *C. vellerosus* indicate that this chromosome resulted from structural rearrangements occurring in the karyotype evolution of *C. villosus*.

On the other hand, metaphases of all of the species examined showed similar C-banding patterns, with C-bands predominantly localized at the centromere (data not shown).

The four species analysed showed discrete hybridization signals on both chromatids at the telomere domains of all chromosomes, as expected (Figure 2A–D). Moreover, *C. vellerosus* and *Z. pichiy* also showed the chromosome pair 30, the smallest of the biarmed autosomes, with interstitial telomeric repeats (ITR) located at the centromeric region (Figure 2C, D). In addition, all the species analysed showed one

to four apparently telocentric chromosomes, exhibiting only two telomeric signals (Figure 2A–D). In the case of *C. vellerosus* and *Z. pichiy*, the telocentric chromosome corresponds to the Y chromosome, whereas, in *C. villosus* and *D. hybridus*, these chromosomes correspond, respectively, to the four and the two smallest autosomes of the karyotype. The centric ITR observed in the metacentric pair 30 from *C. vellerosus* and *Z. pichiy* probably represent telomeric sequences remaining after a chromosome fusion process of acrocentric chromosomes. Due to the small size of pair 30, Robertsonian fusions could not be previously detected in G-banding studies (Merani 2002). It is noteworthy to mention that the location of the observed ITR coincides with C-bands previously shown in *C. vellerosus* and *Z. pichiy* (Rahn *et al*. 1994). This is therefore another case of a
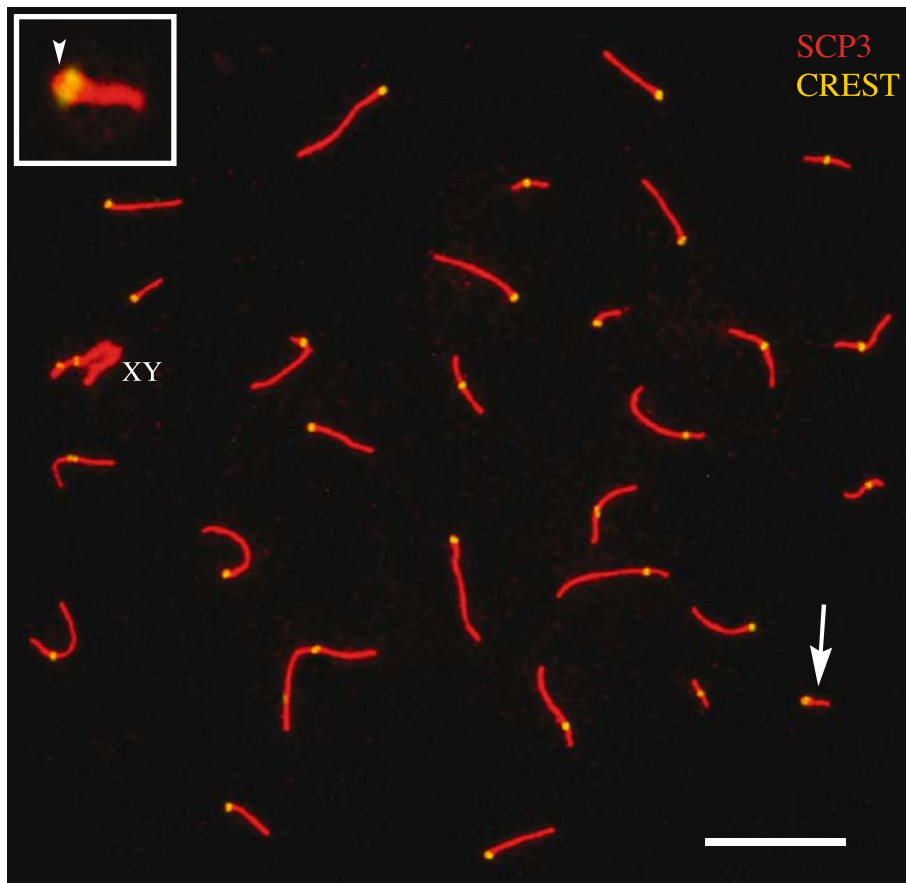

Figure 3. Centromeric location in the smallest bivalent. Synaptonemal complex spread of a pachytene spermatocyte from *Chaetophractus villosus*. Each bivalent is represented by one synaptonemal complex labelled with anti-SCP3 (red). Centromeres appear as bright signals (yellow) overlapped to the autosomal synaptonemal complexes and to the axial elements of the XY pair. Inset: detail of the shortest autosomal bivalent (arrow) showing a small short arm (arrowhead) distally located with respect to the centromeric signal.

heterochromatic region marked by telomeric probes, as found in many vertebrate species (Meyne *et al.* 1990, Multani *et al.* 2001).

The presence of only two telomeric signals in some chromosomes (see Figure 2A–D) suggests that they are of the telocentric type. However, we performed immunodetection of kinetochore proteins on synaptonemal complex spreads from *C. villosus* in order to investigate if the centromeric DNA is actually located at the telomeric position in the small chromosome pairs with only two telomeric signals (see above). Antibodies in CREST sera recognize different kinetochore proteins related to the repetitive DNA of the centromere in a number of vertebrates. Therefore, the presence of these proteins can be ascribed to the positions of centromeres. Pachytene chromosomes were used because, at this stage, their absolute axial lengths are relatively longer than those of the corresponding mitotic chromosomes and therefore give a better resolution. In double-immuno-stained spreads, the signal corresponding to the synaptonemal complex always exceeds the signal of the centromere (Figure 3). It means that the smallest chromosomes, that show only two telomeric signals with FISH, have a tiny short arm that can be resolved only in the more elongated pachytene bivalents. These results are in agreement with electron micros-copy observations of pachytene spreads in the same species that showed kinetochore plates located near – not at the end of – one telomere in the smallest bivalents (Sciurano *et al.* unpublished).

The above findings suggest that none of the species of armadillos posses true telocentric chromosomes. Therefore, the presence of only two telomeric signals in those apparently telocentric chromosomes found in the four species of armadillos studied here could be ascribed to the fact that the short arms of these chromosomes contain relatively few TTAGGG repeats not detectable by the FISH technique. Alternatively, these TTAGGG repeats could be present elsewhere in the genomes of the species analysed without being observed by FISH. Clearly, further studies will be needed to confirm this hypothesis.

Armadillos, anteaters and sloths represent the only placental group with living representatives from the initial South American mammalian stock (Patterson & Pascual 1972). The earliest records of Xenarthra in the Paleocene, about 58 million years ago (MYA), suggest that the group probably originated earlier

during the Paleocene or even possibly during the Late Cretaceous (Vizcaíno 1994, Vizcaíno *et al.* 1998, Oliveira & Bergqvist 1998, Delsuc *et al.* 2004). The species of the order Xenarthra have diploid chromosome numbers ranging from 38 to 64 and the lowest number is present in the armadillo Tolypeutes matacus (2n = 38) whereas all the others species described have chromosome numbers between 2n = 50 and 2n = 64.

Our present results allow us to hypothesize that *D. hybridus* (2n = 64 and 8 biarmed chromosomes) represents an ancestral karyotype in the evolution of armadillos (Dasypodidae), whereas *Z. pichiy* (2n = 62 and 15 biarmed chromosomes), *C. vellerosus* (2n = 62 and 14 biarmed chromosomes) and *C. villosus* (2n = 60 and 15 biarmed chromosomes) are closely related species derived from an ancestral form with 2n = 64. Very likely, the karyotype evolution of *Z. pichiy* and *C. vellerosus* involved a pair of chromosome fusions – Robertsonian translocations (Meyne *et al.* 1990, Slijepcevic 1998) without loss of telomeres (as shown by the presence of ITR). Very likely, biarmed chromosomes in *Z. pichiy* and *C. vellerosus* appeared through Robertsonian fusions and perhaps pericentric inversions occurring during the evolution of Dasypodidae. Likewise, *C. villosus* would have originated from *C. vellerosus* by a pair of chromosome fusions not involving telomeres – since the karyotype of this species shows no ITRs – followed by a loss of chromosome material (as observed by G-banding patterns comparison). Thus, our present results support a reduction of the diploid number by fusion of acrocentrics as a tendency in Dasypodidae (2n = 64 to 2n = 62 to 2n = 60) with loss of chromosome material.

The above hypothesis is supported by recent studies regarding molecular phylogeny of Xenarthrans which suggest that the genus *Dasypus* arose from a different branch from the genera Chaeto-phractus and Zaedyus during the evolution of armadillos (Delsuc *et al.* 2002, 2003, 2004), i.e. the latter genera are much more related to each other than to *Dasypus*. Moreover, a very recent study of the DNA content of several groups of mammals, including Xenarthra (Redi *et al.* 2005) showed that *Dasypus hybridus* has a larger genome size (4.89 pcg DNA) than the other species examined in our present study, i.e. *Chaetophractus vellerosus* (4.46 pcg DNA), *Zaedyus pichiy* (4.21 pcg DNA) and *Chaeto-*

phractus villosus (4.18 pcg DNA). These data shows that the evolution of Dasypodidae occurred with a loss of genetic material.

The extension of more sophisticated cytogenetic techniques, like ZOO-FISH, to the species analysed in the present work and other species of Dasypodidae will provide further insights into the karyotype evolution of Xenarthra. These studies are being planned in our laboratory.

Acknowledgements

References

Blackburn EH (1991) Structure and function of telomeres. *Nature* **350**: 569–573.

Delsuc F, Catzeflis FM, Stanhope MJ, Douzery EJP (2001) The evolution of armadillos, anteaters and sloths depicted by nuclear and mithochondrial phylogenies: implication for the status of the enigmatic fossil Eurotamandua. *Proc R Soc London B* **268**: 1605–1615.

Delsuc F, Scally M, Madsen O (2002) Molecular phylogeny of living Xenarthrans and the impact of character and taxon sampling on the placental tree rooting. *Mol Biol Evol* **19**: 1656–1671.

Delsuc F, Stanhope MJ, Douzery EJP (2003) Molecular systematics of armadillos (Xenarthra, Dasypodidae): contribution of maximum likelihood and Bayesian analyses of mithocondrial and nuclear genes. *Mol Phyl Evol* **28**: 261–275.

Delsuc F, Vizcaíno SF, Douzery EJP (2004) Influence of Tertiary paleoenvironmental changes on the diversification of South American mammals: a relaxed molecular clock study within Xenarthrans. *Evol Biol* **4**: 1–13.

Jorge W, Meritt DA, Benirschke K (1977) Chromosome studies in Edentata. *Cytobios* **18**: 157–172.

Jorge W, Orsi-Souza AT, Best R (1985) The somatic chromosome of Xenarthra. In: Montgomery G, ed. *The Evolution and Ecology of Armadillos, Sloths and Vermilinguas.* Washington, London: Smithsonian Institution Press, pp 121–129.

Leitao Barroso CM, Seuanez H (1991) Chromosome studies on *Dasypus, Euphractus* and *Cabassous* (Edentata: Dasypodidae). *Cytobios* **68**: 179–196.

Lopez MS, González Moreno MC, Casanave EB, Rahn MI, Merani

MS (1997) Estudio cromosómico y variación genética intrapoblacional en *Chaetophractus villosus* (Dasypodidae). *XII Jornadas Argentinas de Mastozoología*, Mendoza, Argentina p. 85 (Abstract).

Madsen O, Scally M, Douady CJ et al. (2001) Parallel adaptive radiation in two major clades of placental mammals. *Nature* **409**: 610–614.

McClintock B (1941) The stability of broken ends of chromosomes of *Zea mays. Genetics* **23**: 234–282.

Merani MS (2002) Variaciones citogenéticas y genéticas intra e interpoblacionales en Xenarthra. *I Congreso Osvaldo Reig de Vertebradología Básica y Evolutiva e Historia y Filosofía de la Ciencia*, p. 52 (Abstract).

Meritt DA, Low RJ, Benirschke K (1973) The chromosomes of *Zaedyus pichiy. Mammal Chrom Newslett* **14**: 108–109.

Meyne J, Ratliff RL, Moyzis RK (1989) Conservation of the human telomere sequence (TTAGGG)ₙ among vertebrates. *Proc Natl Acad Sci USA* **86**: 7049–7053.

Meyne J, Baker RJ, Hobart HH et al. (1990) Distribution of nontelomeric sites of the (TTAGGG)ₙ telomeric sequence in vertebrate chromosomes. *Chromosoma* **99**: 3–10.

Moyzis RK, Jones MD, Meyne J, Ratliff RL, Wu JR (1988) A highly conserved repetitive DNA sequence (TTAGGG)ₙ present at the telomeres of human chromosomes. *Proc Natl Acad Sci USA* **85**: 6622–6626.

Multani AS, Ozen M, Furlong CL, Zhao Y-J, Hsu TC, Pathak S (2001) Heterochromatin and interstitial telomeric DNA homology. *Chromosoma* **110**: 214–220.

Oliveira E, Bergqvist L (1998) A new Paleocene armadillo (Mammalia, Dasypodoidea) from the Itaboraí Basin, Brazil. *Paleógeno de América del Sur y de la Península Antártica, Asociación Paleontológica Argentina*, Special Publication **5, 30**: 3–13.

Pagliaro FE, Rahn MI, Sassaroli JC, Raspal D, Merani MS (1993) Cariotipo en dos géneros de la familia Dasypodidae. *VIII Jornadas Argentinas de Mastozoología*, San Carlos de Bariloche, Argentina, p 97 (Abstract).

Patterson B, Pascual R (1972) The fossil mammal fauna of South America. In: Keast A, Eark FC, Glass B, eds. *Evolution, Mammals and Southern Continents*, Albany, NY: State University of New York Press, pp 247–309.

Poljak S, González P, Deferrari G et al. (2004a) Caracterización de la población del armadillo *Chaetophractus villosus* como exótica en la Isla Grande de Tierra del Fuego. *I Congreso Nacional de Conservación de la Biodiversidad*, Escobar, Buenos, Aires Libro de Actas, p 114 (Abstract).

Poljak S, Lizarralde M, Merani M (2004b) Características ecológico poblacionales en tres especies de armadillos de la Prov. de Buenos Aires. *I Congreso Nacional de Conservación de la Biodiversidad*, Escobar, Buenos Aires Libro de Actas, p 115 (Abstract).

Rahn MI, Tiranti S, Meranis M (1994) Cariotipo en tres géneros de la familia Dasypodidae. *IX Jornadas Argentinas de Mastozoología*, Córdoba, Argentina, Libro de Actas, p 105 (Abstract).

Redi CA, Zacharias H, Merani S et al. (2005) Genome sizes in Afrotheria, Xenarthra, Euarchontoglires, and Laurasiatheria. *J Hered* **96**: 485–493.

Sáez FA, Drets ME, Brum N (1964) The chromosomes of the mulita (*Dasypus hybridus*): A mammalian edentate of South America. In: Pavan C, ed. *Mammalian Cytogenetics and Related*

Problems in Radiobiology. New York: Pergamon Press, pp 163–170.

Seabright M (1971) The use of proteolytic enzymes for the mapping of structural rearrangements in chromosomes of mammals. *Chromosoma* **36**: 201–210.

Slijepcevic P (1998) Telomeres and mechanisms of Robertsonian fusions. *Chromosoma* **107**: 136–140.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. *Exp Cell Res* **75**: 304–306.

Vizcaíno SF (1994) Sistemática y Anatomía de los Astegotheriini Ameghino, 1906 (nuevo rango) (Dasypodidae, Dasypodinae). *Ameghiniana* **30**: 35–40.

Vizcaíno SF, Reguero MA, Goin FJ, Pascual R (1998) Antarctica as background for mammalian evolution. *Paleógeno de América del Sur y de la Península Antártica*, Asociación Paleontológica Argentina, Special Publication **5, 30**: 201–211.

Wetzel RM (1985) Taxonomy and distribution of armadillos, Dasypodidae. In: Montgomery G, ed. *The Evolution and Ecology of Armadillos, Sloths and Vermilinguas*. Washington, London: Smithsonian Institution Press, pp 23–46.