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Karyotype and Chromosome Variability in the Armadillo *Chaetophractus villosus* in Argentina

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Abstract
Karyotype and cytotype variations for the large hairy armadillo *(Chaetophractus villosus)* were studied throughout the species' Argentine distribution. Peripheral blood lymphocyte cultures of 421 animals were used to obtain mitotic metaphases. Preparations were subjected to conventional staining, G- and C-banding, and FISH involving a telomeric probe. Meiotic analysis was performed on testis material from 10 adults. Spermatocytes were examined for synaptonemal complexes in microspreads. The karyotype (2n = 60 XX/XY; FN = 84 without XY) showed an autosomal complement of 6 metacentric and 7 submetacentric chromosomes; the remainder was acrocentric. The X chromosome was submetacentric and the Y acrocentric. Centromeric C+ marks were observed in all chromosomes except pair 16. Three NOR signals were detected in 6q, 12p, and 26p. Two chromosomal rearrangements were characterized in chromosome pair 1 a pericentric inversion seen in the material from Jacinto Aráuz, General Madariaga and Pellegrini and a deletion in the material from Loma Verde. Interstitial telomeric signals were observed in chromosome pairs 4, 12, 16, and 26. Pachytene spermatocyte analysis confirmed the basic chromosome number and morphologies observed in mitotic karyotypes. The evolution of *C. villosus* involved chromosomal rearrangements as recorded for other species of its superorder. The present results establish the basis for the cytogenetic characterization of this species.

The superorder Xenarthra contains armadillos, anteaters, and sloths [Engelmann, 1985; Wetzel, 1985; Nowak, 1999; Delsuc et al., 2003; Gardner, 2008; Svartman, 2012]. Alongside Euarchontoglires, Laurasiatheria, and Afrotheria, it is 1 of the 4 main supraordinal clades of Eutheria [Murphy et al., 2001]. Given its possible position at the base of the eutherian tree [McKenna, 1975; De Jong et al., 1985; Sarich, 1985; Madsen et al., 2001; Murphy et al., 2001; Delsuc et al., 2003; Svartman, 2012], it is of particular cytogenetic and phylogenetic interest. Most xenarthrans, however, have been poorly studied. Indeed, still unknown species may exist, and xenarthran chromosome variability is likely underestimated [Svartman, 2012].

Armadillos (Dasypodidae) are the oldest and most diverse lineage of Xenarthra with 21 species living mostly in South America, 14 of which are found in Argentina.

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Earlier cytogenetic studies employing conventional staining showed the karyotypes of the Dasypodidae to be characterized by a wide variability in chromosome number (2n = 38–64) and morphology [Jorge et al., 1978, 1985]. The evolution of the karyotypes of this family remains poorly understood, although chromosome rearrangements, e.g. pericentric inversions, Robertsonian fusions/fissions, and heterochromatin additions, have been proposed to be involved [Jorge et al., 1978, 1985; Barroso and Seunánez, 1991; Pereira et al., 2004]. However, these suggestions were based mostly on conventional chromosome staining and on the study of just a few individuals.

The large hairy armadillo, *Chaetophractus villosus* [Desmarest, 1804], is endemic to the Neotropics. Its geographic distribution covers most of Argentina; indeed, it is the most widely distributed member of the Dasypodidae in this country [Wetzel, 1985; Redford and Eisenberg, 1992; Wilson and Reeder, 2005]. It has a diploid number of 2n = 60 [Benirschke et al., 1969; Jorge et al., 1978], but the information available on its chromosomes is limited. Modern cytogenetic techniques and resolution banding karyotypes are needed to better understand its chromosomal complement. Such studies may reveal as yet undetected chromosomal rearrangements, provide information useful in the design of conservation strategies, and greatly contribute to the comprehension of xenarthran karyotype evolution.

Telomeric sequences are frequently involved in chromosomal rearrangements; the analysis of their distribution among karyotypes is therefore valuable when examining chromosome evolution [Slijepcevic and Bryant, 1998; Bolzán and Bianchi, 2006; Mudry et al., 2007]. The present work reports the cytogenetic characterization of *C. villosus* via mitotic studies, employing G- and C-banding, NOR staining, and FISH analyses and via meiotic studies, i.e. meiosis I (MI) and meiosis II (MII) analysis and synaptonemal complex (SC) examination.

Materials and Methods

Animals and Samples

Altogether, 421 animals (233 males, 188 females), wild, adult specimens of *C. villosus*, were manually captured from across the species' Argentine range between 1998 and 2012 (online suppl. fig. 1 and table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000357219).

All animal handling conformed to the International Council for Laboratory Animal Science standards. Blood samples were taken between the first and second ring of the tail using a sterile, disposable, heparinized 21-gauge needle [Luaces et al., 2011]. After blood sampling, animals were released at the capture locations. Testicular biopsies were taken from 10 animals from different regions. These were anaesthetized with a mixture of ketamine hydrochloride (35 mg/kg i.m.) and acepromazin maleate (0.3 mg/kg i.m.). They were released after 48 h of recovery.

Mitotic Studies

Lymphocytes were cultured for 72 h at 34°C in RPMI 1640 Medium (Gibco, Grand Island, N.Y., USA) according to Moorhead et al. [1960]. Phytohaemagglutinin M (Gibco) at a concentration of 1–2% v/v was added as a mitogen. Metaphase spreads of 194 individuals from different localities were subjected to G-banding [Wang and Fedoroff, 1972] and 200 individual to C-banding [Sumner, 1972] (online suppl. table 1). A total of 50 metaphases per animal were counted and analyzed to determine the diploid number (2n). At least 10 G- and C-banded metaphases were photographed using a Leitz DMRB microscope and a Leica DFC 300 FX digital camera (Leica Microsystems, Wetzlar, Germany).

Silver nitrate staining of mitotic and meiotic material was performed following Howell and Black [1980] and Sciurano et al. [2006], respectively.

The telomeric distribution of 2 complementary oligonucleotides (Telo1: $TTAGGG_7$, Oligo number 203006A623H01 1/2; Telo2: $GGGTTA_7$, Oligo number 20306A623H02 2/2) was analyzed by FISH in metaphase spreads using a Cy3-conjugated peptide nucleic acid pantelomeric probe (Biofab Research, Roma, Italy). FISH was performed according to the probe supplier's instructions. The hybridization of repetitive sequences was performed following standard procedures [Lichter et al., 1992]. Slides were conventionally stained with propidium iodide and embedded in Vectashield medium (Vector Laboratories, Peterborough, UK). DAPI (4′,6-diamidino-2-phenylindole) counterstaining facilitated the identification of homologues. Signals were observed at a magnification of 100× using a Leica DM epifluorescence microscope (Leica Microsystems) equipped with an HBO 50 mercury lamp and filters for DAPI and Cy3 (Chroma Technology, Bellows Falls, Vt., USA). A Leica DFC 300 FX digital camera (Leica Microsystems) was used for photography. Images were processed using Adobe Photoshop CS software (Adobe Systems Inc., San Jose, Calif., USA)

Meiotic Studies

The cytogenetic protocol followed was that of Evans et al. [1964] with modifications based on our previous experience with other xenarthrans to maximize meiotic yield.

Spermatocyte microspreads for SCs were prepared as described by Sciurano et al. [2006]. Some slides were stained with 4% phosphotungstic acid in ethanol or silver nitrate [Howell and Black, 1980], while others were kept at –70°C until used for immunofluorescence microscopy. Immunolocalization of meiotic proteins was performed following Sciurano et al. [2012]. The following primary antibodies were used: mouse anti-MLH1 (1:10) (BD Pharmingen, San Diego, Calif., USA), rabbit anti-SMC3 (1:500) (Merck Millipore, Billerica, Mass., USA), and human CREST serum (1:10) (Laboratorios IFI, Buenos Aires, Argentina). Slides were examined using a Leica DM epifluorescence microscope and photographed with a Leica DFC 300 FX digital camera (both from Leica Microsystems). Separate images were superimposed using Adobe Photoshop CS software (Adobe Systems Inc.).

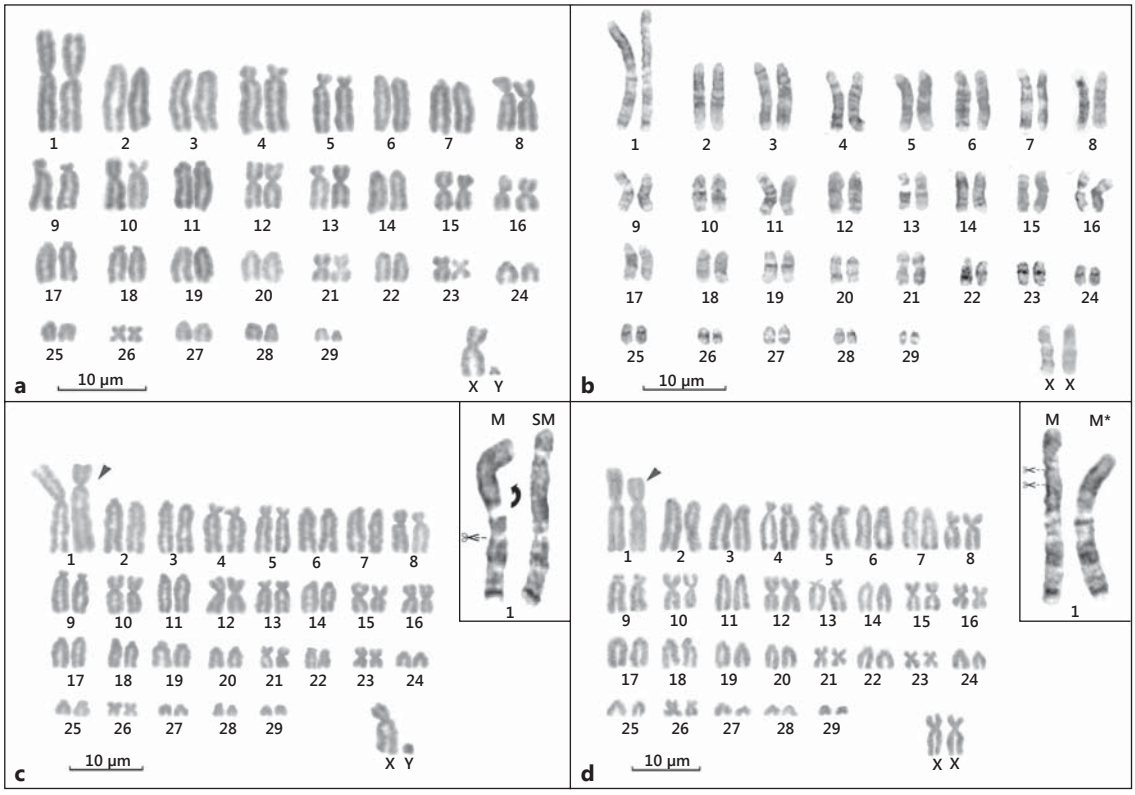

Fig. 1. a Karyotype of a male *C. villosus* from Magdalena, Province of Buenos Aires. **b** G-banded karyotype of *C. villosus*. **c** Karyotype of a male *C. villosus* with a pericentric inversion in the p arm of chromosome 1 (arrowhead); the **inset** shows G-banding of pair 1. **d** Karyotype of a female *C. villosus* with a deletion in the same arm (arrowhead); the **inset** shows G-banding of pair 1. M = Metacentric chromosome; SM = submetacentric chromosome; M* = metacentric chromosome with deletion.

Results

Mitotic Studies

All individuals showed a 2n = 60; the fundamental number (FN) was 84 (without XY). Over this large sample of individuals, the diploid number was always the same, and the sex chromosomes showed no rearrangements, neither in mitotic nor meiotic preparations (see below). Chromosome pairs 1, 15, 16, 21, 23, and 26 were metacentric (M), while 4, 5, 8, 9, 10, 12, 13, and X were submetacentric (SM); all other autosomes and the Y chromosome were acrocentric (A). Pairs 1, 4, 5, 11, 12, 13, and 26 were easily identified by their size and morphology (fig. 1a).

G-banding accurately identified the autosomal and sex chromosomes (fig. 1b). Also, the G-banding analysis revealed 2 chromosomal rearrangements: (1) a pericentric inversion of the short arm of chromosome 1 (fig. 1c) that generated a SM chromosome from a M chromosome; this was seen in animals from Jacinto Aráuz [M/M (22/34), M/SM (12/34), SM/SM (0/34)], Gral. Madariaga [M/M (2/3), M/SM (1/3), SM/SM (0/3)] and Pellegrini [M/M (6/7), M/SM (1/7), SM/SM (0/7)]; and (2) a deletion (M*, fig. 1d), also on the short arm of chromosome pair 1. This cytotype was detected in animals from Loma Verde [M/M (34/40), M/M* (6/40), M*/M* (0/40)]. The total frequency of polymorphisms, for all the studied animals was 4.82%.

The C-banding pattern was quite complex (fig. 2a) and revealed constitutive heterochromatin in the pericentric region of all chromosomes with the exception of pair 16. Remarkably, 1p was fully heterochromatic, with the pericentromeric region showing the greatest intensity (fig. 2a). Chromosome pair 16 showed no C+ bands, but interstitial telomeric signals (fig. 2b).

FISH with the peptide nucleic acid pantelomeric probe showed the expected telomeric signals in each chromosome. Prominent interstitial telomeric signals were also

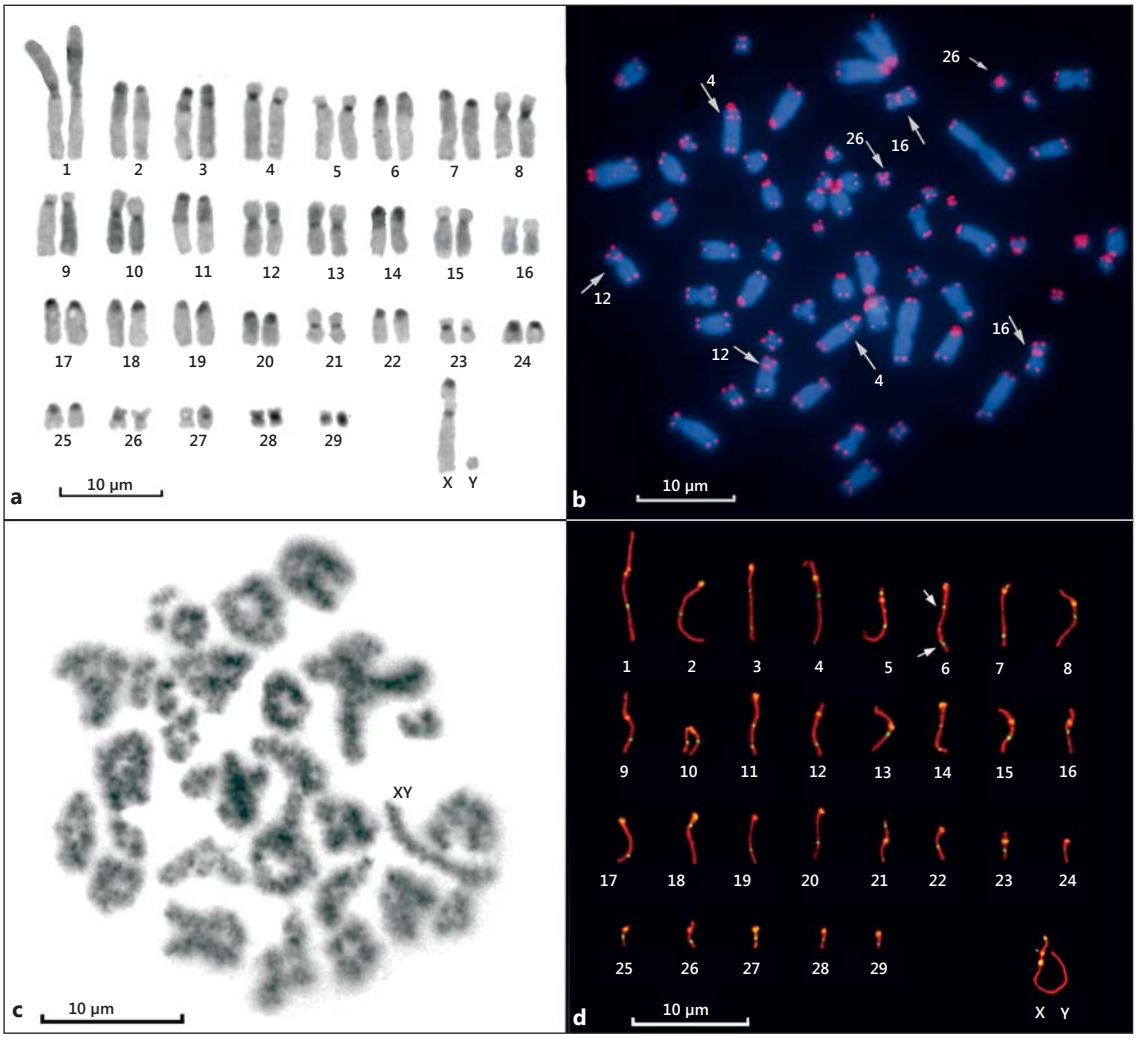

Fig. 2. a C-banded karyotype of a male *C. villosus* showing centromeric C+ heterochromatin. Note the lack of C-bands in the Y chromosome. **b** Metaphase plate showing the location of the telomeric probes. The chromosomes were counterstained with DAPI; arrows indicate interstitial telomeric signals. **c** Metaphase I preparation showing 30 bivalents. Note the XY synapsis. **d** SC karyotype for *C. villosus*. Each bivalent is represented by 1 SC immunolabeled with anti-SMC3 (red), anti-kinetochore (yellow), and anti-MLH1 (green); arrows show 2 recombination nodules in chromosome 6.

seen in the centromeric regions of chromosome pairs 4, 12, 16, and 26 (fig. 2b).

Three NOR signals were detected, 1 each in 6q, 12p and 26p, in agreement with Sciurano et al. [2006]. These were confirmed in meiotic analyses (online suppl. fig. 2a, b).

Meiotic Studies

Fifty diakinesis/metaphase I and 50 metaphase II preparations were studied. Segregation was normal, and the X and Y chromosomes were observed in the expected proportions (fig. 2c).

Twenty-nine autosomal SCs were detected. Centromere locations (kinetochores) were verified by immunolocalization, revealing 13 autosomal SCs with meta- or submetacentric components and 16 autosomal SCs with acrocentric elements. Observations on pachytene spermatocytes confirmed the chromosome morphology and FN recorded in mitotic karyotypes (fig. 2d). Chromosome pair 1 always had the longest SC (submedial kinetochore). Meiotic karyotypes obtained from microspreads of pachytene spermatocyte nuclei in meiotic prophase revealed the size and synapsis dynamics of the sex chromo-

somes. The kinetochore positions in the XY body were clearly established, with the Y chromosome being acrocentric and the X chromosome submetacentric. In most of the spermatocytes, the Y chromosome showed complete synapsis with the X chromosome.

Discussion

The present results modify the mitotic karyotype of the species described by Benirschke et al. [1969] and Jorge et al. [1985]. The paired homologous chromosomes described by these authors differ from those revealed in the present work, suggesting that their specimen showed polymorphisms, at least in chromosome pair 1. Further, some chromosome pairs in the present work did not match to those reported by Benirschke et al. [1969] and Jorge et al. [1985]. Certainly, the submetacentric X chromosome detected in the present work did not match to the X chromosome described by these authors. Indeed, the X chromosome of Benirschke et al. [1969], with its large acrocentric element, would appear to be the presently identified chromosome 2. Further, these authors reported the X chromosome to be acrocentric and to show a prominent paracentromeric C+ band [Jorge et al., 1978], while the present results show it to be submetacentric with a clearly defined short arm. This short arm pairs with the acrocentric Y chromosome, as reported by Sciurano et al. [2006, 2012]. Thus, the presently identified chromosome pair 2 appears to correspond to the erroneously identified sex chromosomes of Jorge et al. [1978].

G- and C- banding confirmed the present chromosome designations and identified those erroneously paired by the above authors. For example, the present chromosome 2 showed the same G- and C-banding patterns as the X chromosome described by Jorge et al. [1978]. Any sex chromosome designation was clearly ruled out by the meiotic and mitotic analyses. In addition, G-banding suggested that chromosome pair 1 might correspond to pair 1 of *Euphractus sexcinctus* [Liu et al., 2011]. The C-banding pattern in this chromosome (q arm) was again similar to that described for *E. sexcinctus* and corresponded to that of human chromosome 5 [Liu et al., 2011]. Interestingly, the Y chromosome of *C. villosus* is not heterochromatic as in most mammals [Sciurano et al., 2006]. The patterns of positive C-bands found for this species may shed light on the relationship between chromosome diversification and adaptation. Differences in the distribution of C-positive regions in different xenarthrans [Dobigny et al., 2005; Liu et al., 2011] may indicate variability in constitutive heterochromatin in this group.

Meiotic studies of diakinesis/metaphase I and the SCs of early prophase spermatocytes confirmed the morphology of the sex chromosomes. The X chromosome was submetacentric with a clearly defined short arm that pairs with the acrocentric Y chromosome. The Y chromosome was seen to be the smallest; this contrasts with that reported by Jorge et al. [1978, 1985], but agrees with that reported by Sciurano et al. [2006].

The chromosomal rearrangement (pericentric inversion) present in the Jacinto Aráuz, Pellegrini, and Madariaga animals was the same, perhaps reflecting a common ancestral event. The deletion in chromosome 1 identified in the Loma Verde specimens, however, must have come about through an independent event. This region of chromosome 1 might be a hotspot for change in this species.

Sciurano et al. [2006] reported *C. villosus* to have 3 NORs, as seen in the present work. Species with more primitive karyotypes [Chiarelli and Capanna, 1973], such as *Dasypus hybridus* (2n = 64, FN = 79) [Saez et al., 1964], only have 1 NOR [Sciurano et al., 2006]. The increase in the number of NORs over the evolution of different species (so-called rDNA dispersion) is well documented [Hirai et al., 1996].

Lizarralde et al. [2005] analyzed the chromosomes of 4 armadillo species by FISH, using a telomeric TTAGGG probe from DAKO Cytomation (Glostrup, Denmark) and found no interstitial signals in those of *C. villosus*. It is well known that clusters of different repetitive DNA sequences, including subtelomeric and interstitial telomeric repeats, characterize the breakpoints of recurrent chromosomal rearrangements [Azzalin et al., 2001; Nergadze et al., 2004]. However, the telomeric probes Telo1 and Telo2 used in the present work and by other authors [Gornung et al., 2011] detected interstitial telomeric sequences in chromosomes 4, 12, 16, and 26; these were interpreted as remnants of acrocentric fusions.

The present work shows that the evolution of *C. villosus* involved chromosome rearrangements. Such evolutionary events are shared by other species of the same superorder. The present results establish the basis for future comparative cytogenetic studies.

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