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CYTOGENETIC INVESTIGATIONS IN SHEEP REARED IN SOUTHERN-ITALY BY USING BOTH CHROMOSOME BANDING AND FISH-MAPPING TECHNIQUES

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SUMMARY

Several sheep breeds are raised in Southern-Italy. Two of them are "Laticauda" and "Bagnolese", which are both considered endangered breeds and inserted in projects for genetic conservation and valorization of their products. One of these projects is the PSR, Misure 214 e2 of Campania Region. In the present study we reported the preliminary results obtained on Laticauda breed and some of their hybrids by using both chromosome banding and fluorescence in situ hybridization (FISH) mapping techniques. Peripheral blood samples from 25 animals (2 males and 23 females) were cultured for 72 hours and treated for late-incorporation of 5-Bromodeoxyuridine (BrdU) to obtain R-banded chromosome preparations which were used for both karyotype construction and FISH-mapping applications.

R-banded karyotypes were performed by using the GENIKON software and following the latest international chromosome nomenclature. For FISH-mapping, both ovine and bovine BAC-clones were used, biotinilated by Nick-translation and precipitated by ethanol in presence of COT-DNA. Then they were in situ hybridized on R-banded chromosomes over night and hybridization signals were detected by using Vector chemicals. At least 30 metaphase for probe were studied by using a fluorescence microscope equipped with a digital camera and computer.

Karyotype construction revealed a normal karyotype for all studied animals except female sheep which were found to be carrier of a reciprocal translocation between chromosomes 4 and 12. Concerning the FISH-mapping, examples of locus assignments are reported in both autosomes and X-chromosome.

***Key words**: sheep, cytogenetics, endangered breed, R-banding, Fluorescence In Situ Hibridization*

INTRODUCTION

Domestic sheep (*Ovis aries*, 2n=54, OAR) represents one of the most important domestic species in the world producing milk, meat and pool. It belongs to the Bovidae family, subfamily "Caprinae".

The genus *Ovis* is the best living example of "bovid karyotype evolution using Robertsonian translocations" at the autosomal level. Indeed, starting from the closest species, the goat (*Capra hircus*, 2n=60), *Ovis* autosomal karyotype are evolved by a progressive reduction of the diploid number to 58 in the Urial (*Ovis vignei*), 56 in the Arkar-Argali (*Ovis ammon*), 54 in domestic sheep (*Ovis aries*) and other species, and 52 in the Siberian sheep (*Ovis nivicola*) by using Robertsonian translocations or centric fusion translocation (Table 1).

In domestic sheep (2n=54), the three biarmed chromosome pairs were originated by centric fusion translocation of the homologous chromosomes of cattle/goat 1/3, 2/8 and 5/11, as demonstrated by both banding technique (reviewed in **Iannuzzi and Di Meo, 1995**) and comparative FISH-mapping (**ISCNDA2000, 2001a**; **Di Meo et al., 2007**; **Goldammer et al., 2009**) techniques.

Table 1. Diploid number in the genus *Capra* (goat) and *Ovis* (sheep)

Goat	Sheep (*Ovis*)			
2n=60	2n=58	2n=56	2n=54	2n=52
Capra hircus	*Ovis vignei* (urial)	*Ovis ammon* (arkar-argali)	*Ovis aries* (domestic sheep) *Ovis canadensis* (large horn sheep) *Ovis dalli* (dall sheep) *Ovis musimon* (European muflon) *Ovis orientalis* (Asiatic muflon)	*Ovis nivicola* (siberian sheep)

Although autosomes (chromosome arms) in bovids were highly conservative, a chromosome autosomal mutation differentiated subfamily "Bovinae" from both subfamily "Caprine" and remaining bovid subfamilies (**Iannuzzi et al., 2009**). Indeed, a small pericentromeric region translocated, by a simple translocation event, from "Bovinae" chromosome 9 to "Caprinae" chromosome 14, as revealed by both linkage- (**de Gortari et al., 1998**) and FISH- (**Iannuzzi et al., 2001a, 2009**) mapping analyses.

Opposite to the autosomes, sex chromosomes of bovids were differentiated by complex chromosome rearrangements. Indeed, sex chromosomes of bovids differ in shape (due to the centromere position) and size (due to the different amount of constitutive heterochromatin). In bovids we have essentially three types of X-chromosomes: (a) submetacentric, as in cattle (genus *Bos*), (b) acrocentric, as in river buffalo and (c) acrocentric with visible p-arms in sheep/goat and other various bovid species.

Chromosome banding comparisons revealed that large chromosome regions share similar banding patterns but only after a detailed comparative FISH-mapping (gene order) analysis where it was possible to establish the exact karyotype evolution in the sex chromosomes (reviewed in **Iannuzzi et al., 2009**). Indeed, (a) cattle and river buffalo differentiated by a centromere transposition (or centromere repositioning) since they have the same gene order; (b) sheep and goat Xs (and remaining X-chromosomes of other bovid subfamilies) differentiated from "bovinae" X by at least four chromosome regions transpositions, including that of the centromere (reviewed in **Iannuzzi et al., 2009**). Also the Y-chromosome differs in shape and size as bovids being submetacentric in cattle, acrocentric in rive buffalo and a very small metacentric in sheep and goat.

Cytogenetic investigations applied to sheep with morphological abnormalities or with reproductive problems have been performed in sheep (reviewed in **Villagomez et al., 2009**), although the gap with other species, such as cattle and pig, is really enormous for the few studies performed so far in sheep clinical cytogenetics. The more common cytogenetic investigations were related to intersex animals (**Villagomez et al., 2009**).

In the present study we report the preliminary data obtained investigating sheep of Laticauda breed, one of the two breeds, together to the Bagnolese, inserted in a project of Campania region (Southern-Italy) for their genetic conservation and product valorization.

MATERIALS AND METHODS

Animals studied and cell cultures.

Twenty-five sheep, of which 20 of Laticauda breed (all females) and 5 hibrids LaticaudaxComisana breeds (2 male and 3 female) both raised in Campania region (Southern-Italy) underwent to cytogenetic investigation.

Peripheral blood samples (1 ml for each culture) were cultured for about 72 h at 38° C in RPMI medium enriched by foetal calf serum (10%), Penicillin/Streptomycin (0.1 ml), L-glutamine (0.05 ml when present in the medium, 0.1 ml when it is not), Concavalin A, as mitogen (15 µg/ml) and a drop of heparin to prevent cell agglutination.

Two types of cell cultures were performed: normal cultures (without addition of any base analog) and cultures treated for late incorporation of 5-Bromodeoxyuridine (BrdU) to obtain R-banded chromosome preparations. For the latter, thymidine (Sigma, 300 µg/ml)

block, for cell cycle synchronization, was performed after 48 h from cell culture starting time. Cell block was removed 18 h later by washing cells twice and recovering them in fresh medium containing BrdU (15 µg/ml) and Hoechst33285 (30 µg/ml) for the last 6 h, including a colcemid (0.1 µg/ml) treatment for 1 h (for both type of cell cultures). An hypotonic treatment (0.5% KCl) and three fixative treatments in methanol/acetic acid (3:1) followed. Two drops of cell suspension were then allowed in cleaned and cold slides kept in distilled water and air dried.

Slides obtained from normal cultures were processed for CBA-banding while those treated for late-BrdU incorporation were used to get both RBA-banding and Fluorescence In Situ Hybridization (FISH) techniques.

Banding techniques

CBA-banding. Slides kept at room temperature and aged one week or more, were treated as reported in **Iannuzzi and Di Berardino (2008).** Briefly, slides were treated with HCl (0.1 N) for 30 min, washed with distilled water and air dried. Then slides were dipped in a $Ba(OH)_2$ (5%) filtered solution for 20-30 min at 50 ° C, washed with tap water and distilled water and air dried. Slides were then treated with 2xSSC at 60° C for 30 min and then for 15 sec in 2xSSC at room temperature, dehydrated in alcohol series and air dried. Finally, slides were stained with acridine orange (0.1% in P-buffer, pH=7.0) for 1 h, washed with tap and distilled water, air dried and mounted in P-buffer pH=7.0 and sealed with rubber cement.

RBA-banding. Slides obtained from late BrdU incorporation were treated as earlier reported (**Iannuzzi and Di Berardino, 2008**). Briefly, slides were stained with Hoechst 33258 (25 µg/ml) for 10 min, washed with distilled water, air dried and mounted with a glass coverslip using 0.8 ml of 2xSSC avoiding any pressure on coverslip. Then slides were exposed UV light (distance from lamp 4 cm) for 30 min, washed with distilled water and air dried. Then slides were stained with acridine orange (0.01% in P-buffer pH=7.0) for 10 min, washed in tap water and distilled water, mounted in Sorensen buffer pH=7.0 and sealed with rubber cement.

Fluorescence in situ hybridization (FISH).

Ovine and bovine BAC-clones from both INRA (France) and CHORI (USA) genomic libraries were used. Biotin incorporation in the probe DNA, in situ hybridization and FITC-signal detection and RBPI-banding (R-banding using BrdU-treated cells and Propidium Iodide staining) were as reported in **Iannuzzi and Di Beradino (2008)**.

Microscope observation and karyotyping.

Three different and independent cytogenetic stations equipped with a fluorescence microscopes connected with a coupled CCD camera and PC were used. At least 30 metaphase per animal were studied. Sheep karyotypes were arranged according to the **ISCNDB2000 (2001)** using GENIKON software.

RESULTS AND DISCUSSION

Sheep Laticauda (Figure 1) is a typical breed living in Campania region (Southern-Italy), in particular in the provinces of Benevento, Avellino and Caserta. Its name is due to the shape of the tail which is very wide (Figure 1). This breed has probably been originated by crosses of the North-African Barbaric breed with local breeds. Its produce both milk and meat and it is normal that females obtain twins or multiple births. The milk is all transformed in cheese named “pecorino” and also the meat (lamb) is well appreciated.

All investigated animals showed normal karyotype (Figure 2), exception of one female which has been found to carry a new reciprocal translocation, probably involving chromosomes 4 and 12. Further studies are in progress to characterize this chromosome abnormality by using both chromosome banding and FISH-mapping with specific chromosome probes.

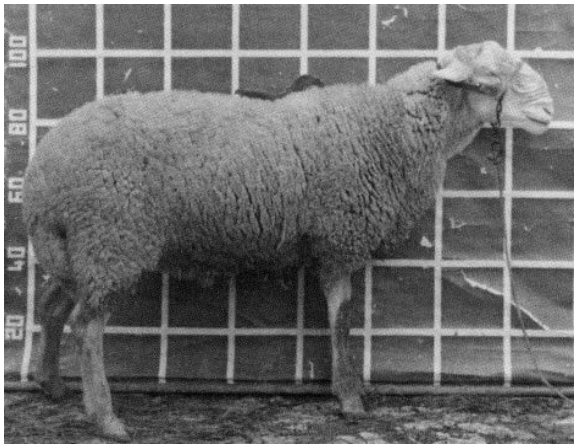

Figure 1. Laticauda sheep breed (Campania region, Southern-Italy). Note the wide tail, typical by this breed.

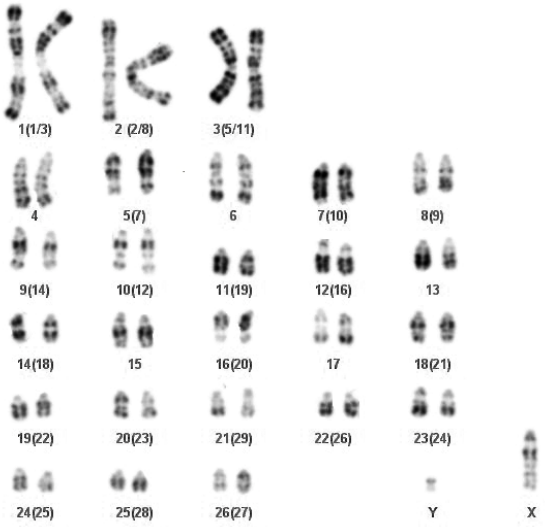

Figure 2. Reverse RBA-banded normal karyotype of a male sheep (*Ovis aries*, 2n=54). The number reported between parenthesis are the homoeologous autosomes of goat/cattle karyotypes.

Sheep karyotype is characterized by three biarmed autosome pairs (autosomes) being the remaining autosomes acrocentrics. X is acrocentric but with evident p-arms and the Y-chromosome is the smallest chromosome and metacentric (Figure 2).

The CBA-banding patterns (Figure 3) observed in all animals were in agreement with those achieved in this species being the C-bands very pronounced in the acrocentric autosomes, very small in the biarmed pairs, C-band negative in the X-chromosme and with the Y-chromosome entirely heterochromatic or with evident C-band positive in the p-arms (Figure 3).

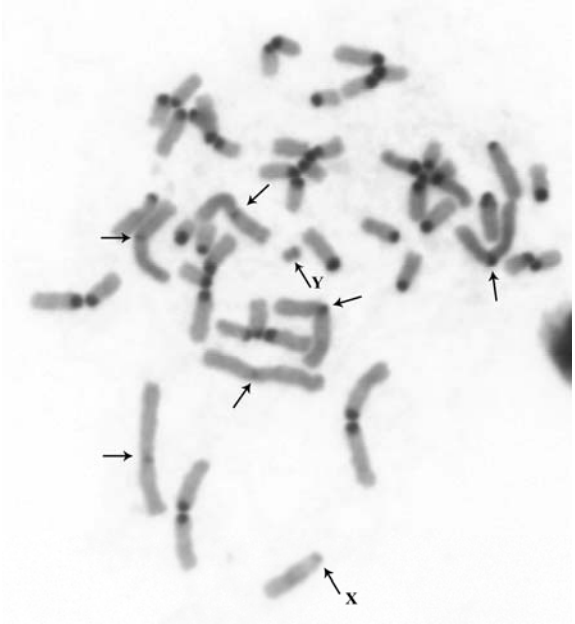

Figure 3. Reverse CBA-banding in a male sheep metaphase plate. Arrows indicate the biarmed chromosome pairs and sex chromosomes. Note the smaller C-banding patterns in the biarmed pairs, compared to the those of the acrocentic chromosomes, the C-band negative in the X-chromosome and the C-band positive in the p-arms of the Y-chromosome (arrows).

During the last years several loci containing both type one loci (expressed sequences) and type II loci (SSRs/microsatellite marker/STSs) have been mapped in sheep by FISH using specific BAC-clones (Figure 4). In this figure is shown the FISH-mapping of enJSRV27, one of the endogenous retrovirus (ERVs) family present in all mammalian genomes (including humans) for infections occurred long time ago (**Chessa et al., 2009**).

FISH-mapping technique has been revealing a powerful tool in all cytogenetic sectors. Indeed, it's the best and fast method to (a) physically map loci in specific chromosome regions (**Di Meo et al., 2007; Goldammer et al., 2009**), (b) identify correctly chromosomes and chromosome regions involved in chromosome abnormalities (**Iannuzzi et al.,**

2001b; Molteni et al. 2007), (c) anchor radiation hybrid maps to specific chromosome regions (**Perucatti et al., 2009**) and (d) clarify the chromosome evolution of species by analyzing the gene order among homologous chromosomes of species (**Iannuzzi et al., 2000, 2001, 2009**).

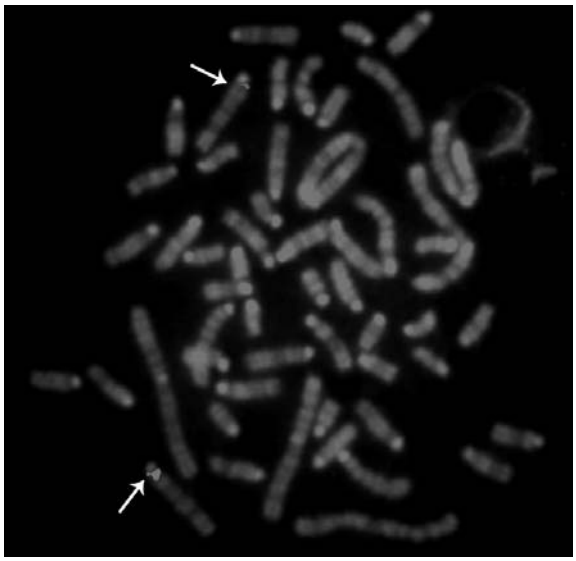

Figure 3. FISH-mapping of enJSRV27 in sheep chromosome 6q13. Note the hybridization FITC-signals in both chromosomes 6q13 (arrows). FITC-signals (green) and RBPI-banding (red) were acquired separately and then signals were superimposed on RBPI-banding (the figure reported here is in black and white).

However, cytogenetic maps still remain at relatively low density and further studies are necessary, especially for some chromosome regions, to obtain a full and dense map to be used for several applications, as reported above.

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