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Development of a sequential multicolor-FISH approach with 13 chromosome specific painting probes for the rapid identification of river buffalo (*Bubalus bubalis*, 2n=50) chromosomes

Alfredo Pauciullo[1,*], Angela Perucatti[1], Alessandra Iannuzzi[1], Domenico Incarnato[1], Viviana Genualdo[1], Dino Di Berardino[2], Leopoldo Iannuzzi[1]

[1]*ISPAAM, Laboratory of Animal Cytogenetics and Gene Mapping, National Research Council (CNR), via Argine 1085, 80147 Naples, Italy*

[2]*Department of Agriculture, University of Naples Federico II, via Università 100, 80055 Portici, Naples, Italy*

[*]Corresponding author: Alfredo Pauciullo

Phone: +390815966006

FAX: +390815965291

email: alfredo.pauciullo@cnr.it

Abstract

The development of new molecular techniques (array-CGH, M-FISH, SKY-FISH, etc…) led to great advancements in the whole field of molecular cytogenetic, however the application of these methods are still very limited in farm animals. In the present study we report -for the first time- the production of 13 river buffalo (*Bubalus bubalis*, 2n=50) chromosome-specific painting probes, generated via chromosome microdissection and DOP-PCR procedure. A sequential multicolor-FISH approach is also proposed on the same slide for the rapid identification of river buffalo chromosome/arms namely 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y, using both conventional and late replicating banded chromosome preparations counterstained by DAPI. The provided 'Bank' of chromosome-specific painting probes is useful for any further cytogenetic investigation not only for the buffalo breeds, but also for other species of the family Bovidae, such as cattle, sheep and goats, for chromosome abnormality diagnosis, and, more generally, for evolutionary studies.

Keywords: Chromosome painting probes; microdissection; DOP-PCR; River buffalo

Introduction

Cytogenetic analysis of farm animal populations has been performed -so far- by using, basically, conventional karyotyping and banding techniques (Iannuzzi & Di Berardino, 2008). Nevertheless, in the last 40 years, several official cytogenetic screening programs have been established worldwide and hundreds of original chromosomal abnormalities have been detected and characterized in livestock populations (Ducos et al. 2008).

While classical cytogenetic analysis still remains the reference method for the routine screening of numerical and structural chromosomal aberrations in domestic animals, in the recent years, the development of new molecular techniques, as the next generation sequencing (NGS) and the SNP-Chip genotyping, led to great advancements in the whole field of molecular cytogenetics. Examples are represented by the recent karyo-mapping (Handyside et al. 2010) and the array-CGH (Pinkel et al. 1998) which are applied in clinical investigations for chromosome imbalances and miscarriage detections in humans and, in some case, also in domestic animals (De Lorenzi et al. 2012 a, b). The same goal is also reached by the application of multicolor FISH (M-FISH) or multicolor spectral karyotyping (SKY) technology, which allow the visualization of each chromosome pair in a different color (Schröck et al. 1996; Speicheret al. 1996).

All the aforementioned methods are very well established in humans, whereas are still very limited -or not yet applicable- to farm animals, both for the very recent availability of array platforms (as in the case of CGH, limited only

to bovine species among the domestic ruminant) both for the absence of commercially available chromosome-specific probes (as in the case of M-FISH).

Within the family Bovidae, specific attention has been given to the *Bos taurus* species, where a complete set of whole-chromosome painting probes are -so far- available (Rubes et al. 2008, Ropiquet et al. 2010; Cernohorska et al. 2013). In order to fill the actually existing lack of chromosome specific painting probes within the family Bovidae, we decided to start producing river buffalo (*Bubalus bubalis*, river type, 2n = 50, XY) painting probes taking advantage by the fact that the first 5 autosomal biarmed pairs are composed by 10 precisely identified and standardized chromosomes of cattle (Iannuzzi, 1994). In addition, probes for chromosomes 18, X and Y are also reported, thus covering nearly a third of the river buffalo karyotype (8 pairs out of 25).

This is the first report on the production of chromosome specific painting probes from the species *Bubalus bubalis*, Mediterranean river type (2n = 50, XY). In addition, a sequential multicolor-FISH approach is presented for the rapid identification of the following chromosomes/arms, namely: 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y.

Materials and methods

Cell cultures

Peripheral blood cultures from four (two males and two females) clinically healthy adult river buffaloes belonging to the Italian Mediterranean breed, reared in southern Italy, were performed according to Iannuzzi & Di Berardino (2008). Four replicates for each sample were prepared. Two replicates followed the conventional cultures protocol and then treated for GTG-banding. The other two replicates were treated with BrdU (10 μg/ml) and H33258 (20 μg/ml) (Sigma, MO, USA) six hours before harvesting to label late replicating regions of the genome. All replicates were subjected to 20-min of colcemid (0.05 μg/ml) treatment, followed by centrifugation steps, hypotonic (KCl 75mM) and fixative methanol/glacial acetic acid (3:1) treatments.

Chromosome microdissection and painting probes preparations

For the production of probes via chromosome microdissection, the fixed lymphocyte suspension was spread onto a precleaned 24 x 60 mm coverslip, air dried and then treated for GTG-banding. Microdissection was performed by using micro-needles pulled from glass capillary G-1000 (Narishige, Japan). The probes corresponding to the biarmed pairs (from 1 to 5) were produced by dissecting out the centromeric area, to avoid in the following PCR unspecific repetitive amplification of the centromeric regions. The probe corresponding to the X chromosome was produced by dissecting the region Xq21-25, analogous to the Xcen region of the bovine chromosome (Nicodemo et al. 2009). The probes corresponding to chromosomes 18 and Y were produced by scraping the entire chromosomes.

Each micro-needle used for microdissection was broken in a 0.2 ml tube containing a collection buffer made of 5X Sequenase reaction buffer (Affimetrix, OH, USA) and water in a final volume of 3.4 µl. On average 15 copies of the same chromosome were collected in the each tube. All tubes underwent to topoisomerase I (10U/µl) treatment at 37°C for 30 min followed by enzyme inactivation at 95°C per 10 min. Highly processive chromosomal amplification was accomplished by degenerate oligonucleotide primer and sequenase ver. 2.0 DNA polymerase (Affimetrix) through a primary DOP-PCR reaction carried out at 94°C for 1 min, 30°C for 1 min and 37°C for 2 min. The enzyme was diluted according to the manufactured guidelines and added during the annealing step at every cycle of the reaction for the first 8 cycles. Further 40 cycles of PCR amplification were performed at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min in a reaction volume of 50µl made of 1X AmpliTaq buffer, 3.5mM of MgCl2, 1 pmol of primer, dNTPs each at 200 µM, 2.5 U of Ampli*Taq* DNA Polymerase (Applied Biosystem, Germany).

Each probe was labeled separately by using a secondary DOP-PCR using 2 µL of products from the first reaction as template. Labeling was performed according to the labeling scheme in table 1, with digoxigeni-11-dUTP and biotin-16-dUTP (Roche, Germany).

Fluorescent in situ Hybridization (FISH)

Six sequential rounds of FISH were performed on the same slide. Each round was realized by using two probes simultaneously hybridized on the metaphase plate according to Pauciullo et al. (2012), with the exception of the second FISH round in which 3 probes (2p, 2q and 18) were used simultaneously. The labeled probes were mixed (table 1), and each precipitated in absolute ethanol together with 10 µg salmon sperm DNA and 10 µg calf thymus DNA (both from Sigma). The pellets were vacuum-dried and then resuspended in 15 µl hybridization solution (50% formamide in 2X SSC + 10% dextran sulfate) for 1 h at 37°C. The probe solutions were denatured for 10 min at 75°C and pre-hybridized for 60 min at 37°C.

Metaphase preparations were denatured for 3 min in a solution of 70% formamide in 2X SSC (pH 7.0) at 75°C. The slides were hybridized in a moist chamber at 37°C overnight. After hybridization, coverslips were removed by a gentle washing step in 2X SCC. The slides were then washed 2 x 5 min in 0.1X SSC at 60°C. The biotin-labeled probe was revealed using a fluorescein isothiocyanate (FITC) fluorochrome conjugated to avidin (Vector Laboratories, California, USA), and the digoxigenin-labeled probe using a rhodamine fluorochrome conjugated to an anti-digoxigenin antibody from sheep (Roche, Germany). Slides were counterstained with DAPI (4,6-diamidino-2-phenylindole) solution (0.24 µg/ml; Sigma) in Antifade (Vector Laboratories).

The slides were observed at 100x magnification with a Leica DM5500 fluorescence microscope equipped with DAPI, FITC, Spectrum orange specific filters, the FITC/Spectrum orange double filter, and provided with a Cytovision MB 8 image-analysis system (Leica Microsystems, Wetzlar, Germany). Digital images were captured in gray-scale, whereas false colors were created by the image-analyzing system for a reliable evaluation of the painting probes. 30 metaphases were acquired for each slide.

At the end of each round of FISH, the oil for microscope observation was removed from the coverslips and the slides were washed 2 x 15 min in PBST in a gently shacking, then air dried and immediately reused in the denaturation step for the next round of FISH.

Results and discussion

In the present study we report -for the first time- the production of 13 river buffalo (*Bubalus bubalis*, 2n=50) chromosome-specific painting probes, generated via chromosome microdissection and DOP-PCR procedure. In addition, a sequential multicolor-FISH approach is proposed, for the first time, for the rapid identification of the following chromosomes/arms, namely: 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y in this species.

To make sure that the produced DNA probes were chromosome specific, they were sequentially hybridized to replicating banded (by late BrdU incorporation and H33258) (Figure 1a) and to conventional (Figure 1b) river buffalo metaphases both stained with DAPI. The first chromosome preparations gave a strong QF-banding due to the combined affinity of the H33258 and DAPI for AT rich regions. In both metaphase chromosomes, the FISH painting signals were very clearly represented. Furthermore, the proposed sequential procedure has the main advantage that the hybridization can be repeated on the same metaphase plate up to six times, provided that chromosomes are stained with DAPI. To our experience, in fact, other stains to reveal banding such as acridine orange or Hoechst 33258 have been found to damage the chromatin, thus making impossible the sequential hybridization steps.

In the present case, the FISH signals appeared to be somewhat negatively affected by the BrdU/H33258 incorporation to induce late replicating banding, since the probe signals appeared fragmented and less intense when compared to those observed in the conventional metaphases. This was especially evident in the long arms of chromosomes 1, 2 and 4, whereas it was less pronounced for the other two sub-metacentric chromosomes (3 and 5), whose probes covered approximately the same region in both mitotic preparations.

The precise localization of the FISH signals on each individual river buffalo chromosome is illustrated in Figures 2a and 2b, according to the standardized river buffalo GTG-banded ideogram (Iannuzzi, 1994).

Since members of the family Bovidae are characterized by a remarkable degree of chromosome banding homology (Evans et al. 1973; ISCNDA, 1990; Iannuzzi, 1994; ISCNDB, 2001; Iannuzzi & Di Berardino, 2008), it is

likely that the river buffalo painting probes presented herein might be utilized for cross-species hybridization experiments within the family. For this purpose, the table 2 shows the 13 chromosome/arms of river buffalo and the corresponding homologous chromosomes of cattle, sheep and goat (from ISCNDB, 2001), whose painting probes are - at the present- available at the ISPAAM Laboratory for any cytogeneticist who wishes to use them. Further work is going on to produce additional probes for the remaining autosomes of the river buffalo karyotype with the aim to provide a complete 'Bank' of species-specific and chromosome-specific paintings useful for any cytogenetic investigation in bovids.

In summary 13 river buffalo (*Bubalus bubalis*, 2n=50) chromosome-specific painting probes (1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y), generated via chromosome microdissection and DOP-PCR procedure were hybridized in sequential multicolor-FISH experiments for the rapid identification of river buffalo chromosome/arms. This probe collection covers nearly half of the bovine and goats karyotypes (13 out 30 chromosome pairs), and 40% of the sheep karyotype (11 out of 27 chromosome pairs), therefore it might be utilized also for cross-species hybridization experiments within the family Bovidae for chromosome abnormality diagnosis, and, more generally, for evolutionary studies.

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Table 1. Labeling scheme for the 13 chromosome/arm specific river buffalo painting probes and super imposed color

Round of FISH	Chromosome	Biotin-FITC	DIG-Rodham.	Imposed color
1	1p			
	1q			
2	2p			
	2q			
	18			
3	3p			
	3q			
4	4p			
	4q			
5	5p			
	5q			
6	X			
	Y			

Table 2 - Corresponding homologous chromosomes in river buffalo, cattle, sheep and goat (from ISCNDB, 2001).

River buffalo (2n=50)	Cattle (2n=60)	Sheep (2n=54)	Goat (2n=60)
1p	27	26	27
1q	1	1q	1
2p	23	20	23
2q	2	2q	2
3p	19	11	19
3q	8	2p	8
4p	28	25	28
4q	5	3q	5
5p	29	21	29
5q	16	12	16
18	18	14	18
X	X	X	X
Y	Y	Y	Y

Figure legend

Fig. 1 FISH obtained by using 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y chromosome specific painting probes on: (a) late replicating-banded chromosome preparations counterstained with DAPI showing a Q-banding pattern; (b) conventional river buffalo (2n=50, XY) metaphases counterstained by DAPI.

Fig. 2 Details of river buffalo chromosomes 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X and Y. (a) G-banded diagrammatic representation; (b) GTG-banding for the corresponding chromosomes; (c) late replicating banded chromosomes counterstained with DAPI showing a QF-banding; (d) specific FISH signals on R-banded chromosomes; (e) DAPI banding from conventional mitotic chromosomes; (f) specific hybridization signals on conventional chromosomes.