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ANIMAL GENETICS · SHORT COMMUNICATION

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

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Abstract The development of new molecular techniques (array CGH, M-FISH, SKY-FISH, etc.) has led to great advancements in the entire field of molecular cytogenetics. However, the application of these methods is 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 the 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

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Introduction

The cytogenetic analysis of farm animal populations has been performed, so far, by using, basically, conventional karyotyping and banding techniques (Iannuzzi and 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 recent years, the development of new molecular techniques, such as next-generation sequencing (NGS) and SNP-chip genotyping, has led to great advancements in the entire field of molecular cytogenetics. Examples are represented by the recent karyo mapping (Handyside et al. 2010) and array CGH (Pinkel et al. 1998) methods, 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. 2012a, 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; Speicher et al. 1996).

All the aforementioned methods are very well established in humans, whereas they 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) as well as for the absence of commercially available chromosome-specific probes (as in the case of M-FISH).

Within the family Bovidae, specific attention has been paid to the *Bos taurus* species, where a complete set of whole-

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chromosome painting probes are, so far, available (Rubes et al. 2008; Ropiquet et al. 2010; Cernohorska et al. 2013). In order to fill the 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 by taking advantage of the fact that the first five autosomal biarmed pairs are composed of ten 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 and Di Berardino (2008). Four replicates for each sample were prepared. Two replicates followed the conventional cultures protocol and were then treated for GTG banding. The other two replicates were treated with BrdU (10 μg/ml) and H33258 (20 μg/ml) (Sigma, MO, USA) 6 h 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 75 mM), 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 × 60-mm coverslip, air-dried, and then treated for GTG banding. Microdissection was performed by using microneedles pulled from glass capillary G-1000 (Narishige, Japan). The probes corresponding to the biarmed pairs (from 1 to 5) were produced by dissecting the centromeric area, to avoid unspecific repetitive amplification of the centromeric regions in the following polymerase chain reaction (PCR) assay. 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 microneedle used for microdissection was broken in a 0.2-ml tube containing a collection buffer composed of 5× Sequenase reaction buffer (Affymetrix, OH, USA) and water in a final volume of 3.4 μl. On average, 15 copies of the same chromosome were collected in each tube. All tubes underwent topoisomerase I (10 U/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 (Affymetrix) 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 eight cycles. A 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 50 μl composed of 1× AmpliTaq buffer, 3.5 mM of MgCl2, 1 pmol of primer, dNTPs each at 200 μM, and 2.5 U of Ampli*Taq* DNA Polymerase (Applied Biosystems, Germany).

Each probe was labeled separately by using a secondary DOP-PCR using 2 μL of products from the first reaction as the template. Labeling was performed according to the labeling scheme in Table 1, with digoxigenin-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 three probes (2p, 2q, and 18) were used simultaneously. The labeled probes were mixed (Table 1), and each of the probes was 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 of hybridization solution (50 % formamide in 2× SSC+10 % dextran sulfate) for 1 h at 37 °C. The probe solutions were denatured for 10 min at 75 °C and prehybridized for 60 min at 37 °C.

Metaphase preparations were denatured for 3 min in a solution of 70 % formamide in 2× 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 2× SCC. The slides were then washed 2 × 5 min in 0.1× SSC at 60 °C. The biotin-labeled probe was revealed using a fluorescein isothiocyanate (FITC) fluorochrome conjugated to avidin (Vector Laboratories, CA, USA), and the digoxigenin-labeled probe was revealed using a rhodamine fluorochrome conjugated to an anti-digoxigenin

Table 1 Labeling scheme for the 13 chromosome/arm-specific river buffalo painting probes and superimposed 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			

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 100× magnification with a Leica DM5500 fluorescence microscope equipped with DAPI, FITC, SpectrumOrange-specific filters, the FITC/SpectrumOrange double filter, and provided with a CytoVision MB 8 image analysis system (Leica Microsystems, Wetzlar, Germany). Digital images were captured in grayscale, whereas false colors were created by the image analysis system for a reliable evaluation of the painting probes. Thirty 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 × 15 min in PBST with gentle shaking, then air-dried and immediately reused in the denaturation step for the next round of FISH.

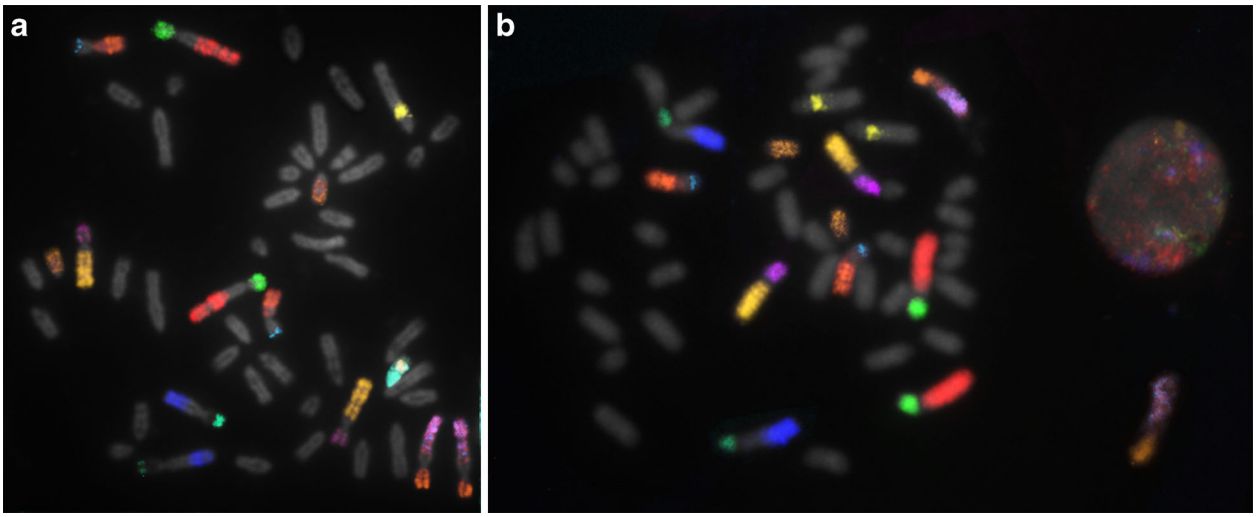
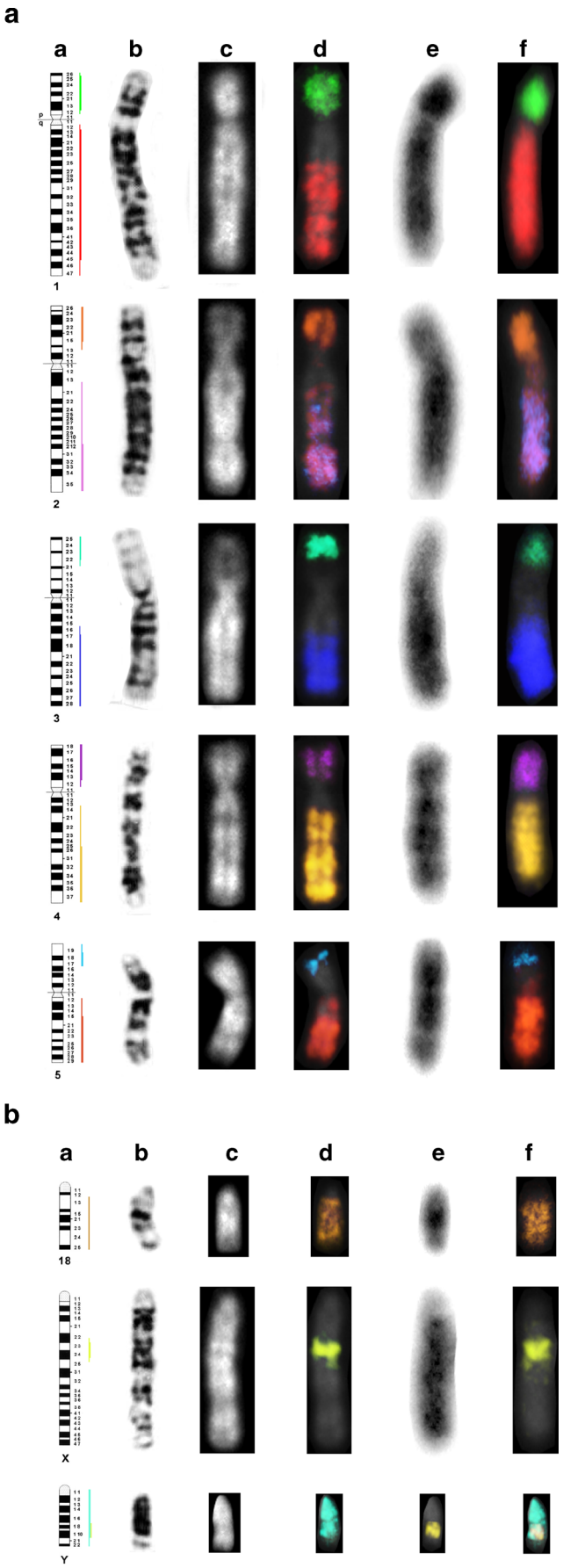


Fig. 1 FISH obtained by using 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X, and Y chromosome-specific painting probes on: (**a**) late-replicating banded chromosome preparations counterstained with DAPI showing a Q-banding pattern; (**b**) conventional river buffalo (2n=50, XY) metaphases counterstained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

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 the 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 in this species, namely: 1p-1q, 2p-2q, 3p-3q, 4p-4q, 5p-5q, 18, X, and Y.

To make sure that the produced DNA probes were chromosome-specific, they were sequentially hybridized to replicating banded (by late BrdU incorporation and H33258) (Fig. 1a) and to conventional (Fig. 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. In our experience, in fact, other stains that reveal banding such as acridine orange or Hoechst 33258 have been found to damage the chromatin, thus making the sequential hybridization steps impossible.

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 submetacentric 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 Fig. 2a, b, 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; Di Berardino et al. 1990; Iannuzzi 1994; Cribiu et al. 2001; Iannuzzi and Di Berardino 2008), it is likely that the river buffalo painting probes presented herein might be utilized for cross-species hybridization experiments within

Table 2 Corresponding homologous chromosomes in river buffalo, cattle, sheep, and goat (from Cribiu et al. 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

the family. For this purpose, Table 2 shows the 13 chromosomes/arms of river buffalo and the corresponding homologous chromosomes of cattle, sheep, and goat (from Cribiu et al. 2001), whose painting probes are, at the present, available at the ISPAAM laboratory for any cytogeneticist who wishes to use them. Further work is ongoing 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 the 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 goat karyotypes (13 out 30 chromosome pairs), and 40 % of the sheep karyotype (11 out of 27 chromosome pairs); therefore, it might also be utilized for cross-species hybridization experiments within the family Bovidae for chromosome abnormality diagnosis, and, more generally, for evolutionary studies.

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