The water buffalo: evolutionary, clinical and molecular cytogenetics

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ABSTRACT: Although buffalo population is about 1/10 of that of cattle, buffaloes interest a larger human population, especially in the east countries. For this reason, this species is of great economic importance. Two main species of buffalo are found in the world: the Asiatic (water) buffalo (Bubalus bubalis) and the African buffalo (Syncerus caffer). These two different species have both two different sub-species differing in diploid number but interbreeding within the same genus. The water buffalo, especially the river type (2n=50), is the most important one and a summary of the most important cytogenetic findings obtained until now in this species is reported in this paper.

Key words: River buffalo, Evolution, Chromosome abnormality, Fertility

INTRODUCTION - Two main species of buffalo are found in the world: the Asiatic (water) buffalo (Bubalus bubalis) and the African buffalo (Syncerus caffer). Two types of African buffalo are known: the large black savannah or Cape buffalo, Syncerus caffer caffer (2n=52) and the small reddish forest buffalo, Syncerus caffer nanus (2n=54)). The Asiatic buffalo (Bubalus bubalis) has also two main subspecies: the river buffalo with a karyotype 2n=50 and FN=60 and the swamp buffalo (2n=48, FN=58). These two subspecies differ in a chromosome pair (and FN), but they are inter-breed. The reason is that a tandem fusion translocation between river buffalo (BBU) chromosomes 4 and 9 (telomere of BBU4p and centromere of BBU9) originated swamp buffalo chromosome 1 (the largest one) (Di Berardino and Iannuzzi, 1981). Thus, all chromosome arms (and biarmed pairs) were conserved between the two Asiatic species. This also explains the crosses between the two species, although the hybrid has 49 chromosomes and may have a lower reproductive value due to the formation of unbalanced gametes and subsequent abnormal embryos which can die in early embryonic life. Standard karyotypes (CSKBB, 1994) and a cytogenetic map at low resolution (Iannuzzi et al., 2003a) are available for river buffalo, but both linkage and radiation hybrid (RH) maps are still lacking in this species. In this paper, a synthesis of the main studies performed in this species in evolutionary, clinical and molecular cytogenetics is reported.

MATERIAL AND METHODS - Peripheral blood cultures are generally preferred to obtain chromosome preparations. Various cell culture techniques are available, depending from the type of banding patterns to be obtained. Replicating G and R-banding have been proven to offer the highest resolution allowing a detailed description of chromosomes of
domestic animals, including those of river buffalo (Iannuzzi et al., 1990; CSKBB, 1994; ISCNDB, 2001). These techniques require early- or late-incorporation of a thymine base analog (i.e. 5-Bromodeoxyuridine =BrdU) during the last S-phase. Furthermore, improved cell cultures can be obtained by cell synchronization in S-phase using blocking agents such as the metotrexate or thymidine as follows. 

**Replicating G-bands.** Methotrexate (0.5 µg/ml) and BrdU (10 µg/ml) were added after 48 h of cell culture to get partial cell block and early-incorporation of BrdU, as earlier reported (Iannuzzi et al., 1989) and used to get high resolution replicating G-banding patterns in bovid chromosomes (Iannuzzi et al., 1990; Iannuzzi and Di Meo, 1995; CSKBB, 1994; ISCNDB, 2001). Cell block was removed 17 h later by washing cells twice with Puck’s saline solution and recovering them in fresh medium containing tymidine (10µg/ml) for 5.5 h including a colcemid treatment (0.05 µg/ml) for 30 min.

**Replicating R-bands.** Thymidine (300 µg/ml) was added after 48 h of cell culture as S-phase blocking agent, and removed 17 h later by washing cells twice with Puck’s saline solution and recovering them in fresh medium containing BrdU (15µg/ml) and H-33258 (20 µg/ml) for 6.0 h including a colcemid treatment (0.1 µg/ml) for 1 h. An hypotonic treatment and three fixations with methanol/acetic acid (3:1) followed.

**Chromosome banding techniques**

**CBA+GBA- and RBA-banding.** Slides from both types of cell cultures were stained with H-33258 (25 µg/ml in distilled water) for 10 min, washed with distilled water, mounted in 2xSSC (pH=7.0) using a glass coverslip and exposed to UV light for 30 min. Then slides were washed with distilled water, air dried, stained with acridine orange (0.01 % in P-buffer pH=7.0) for 10 min, washed with tap and distilled water, air dried and finally mounted in P-buffer (pH=7.0) by using a glass coverslip (by eliminating the excess of buffer using a filter paper) and sealing it with rubber cement. Slides were observed a day later or more (until 7-10 days).

**CBA-banding** was performed as earlier reported (Di Meo et al., 1995) as follows. Slides one week old (room temperature) were immerse in HCl 0.1 N for 30 min, washed with distilled water and treated with Ba(OH)₂ at 55 °C for 20-30 min (chromosome denaturizing) being sure to cover completely slides allocated in the coplin-jar. Then slides were washed with tap and distilled water directly in the coplin-jar and transferred in a coplin containing 2xSSC at 60 °C for 30 min. Slides were then transferred in a coplin containing 2xSSC at room temperature for few second, dehydrate in 75% and 95% alcohol series (3 min each) and air dried. Slides are then stained with acridine orange (0.1% in P-buffer, pH=7.0) for 1 h, washed in tap and distilled water and air dried. Then slides were mounted and observed as reported for CBA+GBA and RBA-banding.

Chromosome identification and banding followed the river buffalo standard karyotype (CSKBB, 1994) which was later correlated with the latest chromosome nomenclature of bovids (ISCNDB, 2001) on the basis of chromosome specific molecular marker assignments.

**RESULTS AND CONCLUSIONS - Evolutionary cytogenetics.** Cytogenetic studies have revealed that the *Syncerus caffer caffer* has 2n=52 and a fundamental number (FN) equal to 60, while the *Syncerus caffer nanus* has 2n=54 and FN=60. The main karyological differences are due to the presence of 4 (*Syncerus caffer caffer*) and 3 (*Syncerus caffer na-
(nus) biarmed autosome pairs, the remaining chromosomes being acrocentric, including X (the largest acrocentric) and Y (a small acrocentric) chromosomes. These biarmed pairs originated from centric fusion translocations of the following cattle (ancestral bovid) homologous chromosomes in Syncerus caffer caffer (1;13, 2;3, 5;20, 11;29) (Gallagher and Womack, 1992). Since this species is crossed with the Syncerus caffer nanus, it is presumed that the 3 biarmed chromosome pairs of this species are the same as those (3/4) of Syncerus c. c.

The river buffalo has five biarmed autosomes, the remaining ones being all acrocentric, including both X (the largest acrocentric) and Y (small acrocentric) chromosomes (Figure 1). The five biarmed river buffalo chromosomes (BBU) originated from five centric fusion translocations involving the following ten cattle (ancestral bovid) homologous chromosomes (BTA) and bovine syntenic groups (U) according to both CSKBB (1994) and ISCNDB (2001):

- **BBU1** (BTA1;27-U10/U25), **BBU2** (BTA2;23-U17/U20), **BBU3** (BTA8;19-U18/U21), **BBU4** (BTA5;28-U3/U29), **BBU5** (BTA16;29-U1/U7).

Loss of heterochromatin, mainly constitutive heterochromatin (HC) accompanied the formation of these biarmed chromosomes (Iannuzzi et al., 1987; Di Meo et al., 1995), since very small C-bands can be seen at the centromeres of the biarmed pairs, compared to the large ones observed at the centromeres of all acrocentric chromosomes, including X which shows the largest centromeric HC-block with a proximal C-band positive (Figure 2). The Y chromosome shows variable C-banding patterns depending on the degree of chromosome denaturation. Indeed, it appears completely heterochromatic (Figure 2) or with a strong C-band positive, only distally located (with the centromere C-band negative) (Di Meo et al., 1995). Thus, the C-banding technique (especially using CBA-banding) is the simplest banding technique to distinguish sex chromosomes easily (especially the Y-chromosome) from the autosomes in buffaloes.

Since BBU4 originated from centric fusion translocation of cattle homologous chromosomes (BTA) 5 and 28 (CSKBB, 1994), and river buffalo chromosome 9 is homologous to cattle chromosome 7 (CSKBB, 1994), the following three cattle homologous chromosomes (and bovine syntenic groups = U) are present in swamp buffalo chromosome 1: BTA5 (U3), BTA28 (U29) and BTA7 (U22) (Figure 3). However, it is possible that river and swamp buffaloes diverged by an ancestral water buffalo (probably the Bubalus arnee), reported to be present in Assam, Nepal and Indocina.

Figure 1. Normal GBG-banded karyotype (2n=50, XY) in the river buffalo bull “Bellemamma”. The karyotype has been constructed from a single cell.
(Mason 1974), although genetic and cytogenetic data to support this hypothesis are still lacking (Lau et al. 1998). During the tandem fusion translocation event, the centromere of BBU9 was apparently deleted or inactivated while the nucleolus organizer regions (NORs) present at the telomeres of BBU4p (BTA28) (Iannuzzi et al., 1996) were lost (Di Berardino and Iannuzzi, 1981). Indeed, BBU has six nucleolus organizer (NO) chromosomes, all located at the telomeres of the arms 3p, 4p, 6, 21, 23, 24 (Iannuzzi et al., 1996) while the swamp buffalo has five NO-chromosomes (Di Berardino and Iannuzzi, 1991).

Since no biarmed chromosome pair is shared between the genus Syncerus and Bubalus (CSKBB, 1994; Gallagher and Womack, 1992), no crosses between them are possible since the hybrids would have unbalanced chromosome sets. Thus, their division into two different genera is also supported from the chromosomal point of view.

Comparative mapping studies have been performed between river buffalo and other related species (cattle, sheep and goat), as well as between river buffalo and humans to detect conserved chromosome segments and syntenies. These studies revealed a high autosome (chromosome arms) homology among bovids. Indeed, the same chromosome banding patterns and gene order among all autosomes (or chromosome arms) of cattle, river buffalo, sheep and goat have so far been found (reviewed in Iannuzzi and Di Meo, 1995; Iannuzzi et al., 2001a). The only exception was found between “bovine” (cattle and river buffalo) and “caprine” (sheep and goat) chromosomes 9 and 14. Indeed, a simple translocation (with inversion) of a pericentromeric chromosome segment from “bovine” chromosome 9 to “caprine” chromosome 14 differentiated these two autosomes as revealed by both linkage (de Gortari et al., 1998) and FISH-mapping (Iannuzzi et al., 2001a) analyses.

In spite of the high autosome similarity among bovids, sex chromosomes diverged by more complex chromosome rearrangements. X-chromosome of bovids can mainly be found in three different types: the submetacentric cattle type, the acrocentric eland (or river buffalo) type and the acrocentric sheep (and goat) type with small and visible p-arms. Chromosome banding comparisons demonstrated that large portions of these chromosomes were conserved (reviewed in Iannuzzi and Di Meo, 1995), the presence of large blocks of constitutive heterochromatin (HC) in BBU-X (Di Meo et al., 1995) and their absence in both BTA-X and
OAR/CHI-X. (Iannuzzi and Di Meo, 1995). When detailed comparative cytogenetic maps showing the order of loci along sex chromosomes of cattle, river buffalo and sheep/goat X-chromosomes were performed, complex chromosome rearrangements were demonstrated to have occurred during the karyotype evolution of these species (reviewed in Iannuzzi et al. 2000a). In particular, BTA-X and BBU-X share the same gene order but a different centromere position, hence a centromere transposition (or centromere repositioning) with loss of constitutive heterochromatin (HC) differentiated BTA-X from BBU-X (Iannuzzi et al., 2000a).

The same occurred on the Y-chromosome of bovids. Indeed, comparative FISH-mapping analyses performed among the Y-chromosomes of cattle (Bos taurus), zebu (Bos indicus, BIN), river buffalo and sheep/goat also revealed complex chromosome rearrangements. In particular, BTA-Y and BBU-Y differ in a pericentric inversion with loss (from BBU-Y to BTA-Y) or gain (from BTA-Y to BBU-Y) of HC, BBU-Y being larger than BTA-Y (Di Meo et al., 2005a).

Another important parameter from an evolutionary point of view is represented by the nucleolus organizer regions (NORs) which are all located at the telomeres of five (cattle, sheep, goat, swamp buffalo) and 6 (river buffalo) autosomes in domestic bovids. Indeed, considering the high degree of autosome conservation among bovids (with only centromeric regions affected by chromosome rearrangements – CF), we expected to find the same nucleolus organizer chromosomes (NOCs) in bovids, but it was not so. Indeed, due to simple NOR-translocations, only some NORs were conserved in homologous chromosomes (or chromosome arms) (Iannuzzi et al., 1996). In particular, only two homologous NOCs were common to river buffalo and cattle (BBU6 and BBU24, homologous to BTA3 and BTA25, respectively) (Iannuzzi et al., 1996).

**Clinical cytogenetics** is only recently developed in river buffalo, especially in Italy where both bulls used for reproduction and many females with reproductive problems have cytogenetically been investigated by using both banding and FISH-mapping techniques. Autosome abnormalities have rarely been reported in river buffalo. A translocation (probably a reciprocal translocation) has been reported to involve BBU3 and BBU6 (Vijh et al., 1994) in a male river buffalo with normal body conformation but no indications were given on fertility.
Sex chromosome abnormalities are much more frequent in river buffalo, especially in the females. They are the following.

**X-trisomy.** Only two cases have been found so far in river buffalo, one in India (Murrah breed) (Prakash et al., 1994) and another in Italy (Razza Bufala Mediterranea Italiana) (Iannuzzi et al., 2004). Both these females were sterile for damages to internal sex adducts. The damage due to this syndrome is essentially due to the presence and action of all three Xs before their inactivation (2/3) and/or to genes which escape gene inactivation. In humans this syndrome induces taller stature in most cases and ovarian dysfunctions in a reduced number of women.

**X-monosomy.** This syndrome causes serious damage to the carriers. Indeed, in humans, about 90% of 2n=45,X conceptuses die within the first three months, while short stature, normal intelligence and primary amenorrhea are the most important features in living women. This syndrome is rare in domestic animals, although several cases have been reported in horses. Generally, animal carriers are sterile due to serious damage to internal sex adducts as reported in the four cases found so far in Indian (Yadav et al., 1990; Prakash, 1992) and Mediterranean Italian (Iannuzzi et al. 2000) buffaloes (Figure 4).

**Sex-reversal syndrome.** This syndrome occurs in both males and females which show a karyotype which is XX and XY, respectively. This syndrome is rare in humans (1 case each 25,000 births). The origin of XX males is generally due to errors during meiosis with translocations of small chromosome regions (containing genes involved in sex differentiation) from the Y-chromosome to the X-chromosome. XY females originate from deletion or mutations of genes involved in sex determination, especially the SRY-gene. This syndrome is rare in domestic animals even if it seems to be much more frequent in domestic animals than in humans, especially in horses. The carriers are generally affected by gonadal dysgenesis. In river buffalo this syndrome has been found in two sterile females of “razza Mediterranea Italiana”. One female was lacking of internal sex adducts and the other one showed pronounced atrophy of Muller’s ducts (Iannuzzi et al., 2001, 2004).

**XXY-syndrome.** This syndrome is very rare in domestic animals, compared to humans where it is known as Klinefelter’s syndrome. Only one case has recently been reported in male river buffalo calf (Patel et al., 2006). It was an usual case because the carrier had 2n=50, Y,t(X;X), since the two Xs were fused by their centromeres. Correlations between this abnormality and reproductive parameters will be performed when the animal will reach the puberty (Patel et al., 2006).

**XX/XY mosaicism (free-martin)** is the most common sex chromosome abnormality in cattle and river buffalo. The frequency of this abnormality depends on the frequency of births with twins. The carriers, especially the females, are generally sterile due to serious damage to the internal sex adducts which vary between the atrophy of Muller ducts to the complete lack of internal sex adducts (reviewed in Iannuzzi et al., 2005). This damage originates from the presence (and action) of the Y-chromosome. Indeed, placental anastomosis occurs much earlier (20-25 days) than sex differentiation (40-45 days) during the embryonic life. In addition, male differentiation occurs earlier (one week) in males than in females (Ruvinsky and Spicer, 1999). This means that even when few male cells are present, serious damage can be done in female twin. In river buffalo almost all examined cases were from single births. This means that the male co-twin dies during early embryonic life and a single female birth arises. In these conditions the breder may not realize that it is a possible case of freemartinism and
Figure 4. Abnormal RBG-banded karyotype (2n=49,X) from a female river buffalo affected by X-monosomy.

treats the female as though normal until it reaches reproductive age (or more), thereby incurring serious economic damage since the female cannot calve (or produce). In river buffalo this syndrome has been the most common chromosome abnormality found in both Indian (Balakrishnan et al., 1981) and Mediterranean Italian (bufala mediterranea italiana) (Iannuzzi et al., 2005) breeds. Actually, by summing all chromosome abnormalities found in river buffalo, about 20% of females with reproductive problems and investigated in our laboratory were found to carry sex chromosome aberrations and all of them resulted sterile for serious damages to the internal reproductive sex adducts. These results underline the necessity to extend cytogenetic analyses to all females with reproductive problems. Furthermore, since many females showed clear male traits (head, horns, prominent withers, tight pelvis) earlier analyses on these females could noticeably reduce the time of raising in the farm by a prompt elimination of carrier saving a lot of time and money.

Molecular cytogenetics Assignments of both type I (known genes) and type II loci (generally microsatellites) have been performed in river buffalo by both somatic cell hybrid cells (El Nahas et al. 1996) and FISH-mapping (Iannuzzi, 1998; Iannuzzi et al., 2003) techniques. The most recent cytogenetic map (Iannuzzi et al., 2003) shows 293 loci of which 171 of type I loci (known genes) and 122 type II (generally microsatellites). Of the total assigned loci (293), 247 were assigned by FISH by our laboratory (Iannuzzi et al. 2003). Other and more recent assignments were performed by FISH-mapping for the following loci: SLC26A2 to BBU9q26 (Kierstein et al., 2003), SMN to BBU19q13 (Iannuzzi et al., 2003b) (Figure 4), LEP to BBU8q32 (Vallinoto et al., 2004); FHT to BBU21q24 (Di Meo et al., 2005a), GDF8, TTN, GCG, NEB, CXCR4, MYL1, ACADL, IGFBP2 and FN1 to BBU2q (Di Meo et al., 2006), MUC1 to BBU6q13 (Perucatti et al., 2006), TRG2 to BBU8q17 (Antonacci et al., 2007), UMN0301, UMN0504 to BBUY (Di Meo et al., 2005b). Moreover, 40 loci more were assigned in a recent study (see poster by Di Meo et al.) bringing the total assigned loci in river buffalo to 349, noticeably extending the river buffalo cytogenetic map.
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