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Abstract Based on a recently generated comprehensive gene map for *Ovis aries* chromosome X (OARX) with an approximately even locus distribution, we assigned selected bacterial artificial chromosome (BAC) probes corresponding to these OARX loci to *Bubalus bubalis* (BBU) and *Bos taurus* (BTA) by comparative fluorescence in-situ hybridization (FISH) to improve cytogenetically the X chromosome maps in these species. Twenty-five added loci in BBUX and BTAX, respectively, contribute to a more detailed description of the cytogenetic organization of these chromosomes. Further seven loci were identified in OARX and two DNA probes were assigned to X and Y chromosomes in river buffalo, cattle, and sheep, respectively, and thus identified loci in the pseudoautosomal region. The additional assignments double the number of cytogenetic loci in BBUX and increase their number in BTAX and OARX. The larger quantity of cytogenetic anchors allows a more precise morphological comparison of bovid X chromosomes among each other and with the *Homo sapiens* (HSA) X chromosome. The anchor loci confirm and refine syntenic fragments in HSAX and identify several evolutionary breakpoints between the compared chromosomes. The cytogenetic assignments in BBUX, BTAX, and OARX represent useable anchors for the ongoing genome sequence assembly in Bovidae.

Keywords cattle · river buffalo · sheep · human · X chromosome · FISH · comparative mapping

Abbreviations

BAC	Bacterial artificial chromosome
BBU	*Bubalus bubalis*
BES	BAC end sequence
BLAST	Basic local alignment search tool
BTA	*Bos taurus*
CSKBB	Committee for Standardized Karyotype of *Bubalus bubalis*
DABCO	1,4-Diazabicyclo[2.2.2]octan

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FISH	Fluorescence in-situ hybridization
FITC	Fluorescein isothiocyanate
HSA	*Homo sapiens*
ISCNDB	International System for Chromosome Nomenclature of Domestic Bovids
ISCN	International System for Human Cytogenetic Nomenclature
OAR	*Ovis aries*
OGA	Ovine Genome Assembly
PAB	Pseudoautosomal boundary
PAR	Pseudoautosomal region
PI	Propidium iodide
RH	Radiation hybrid

Introduction

That the chromosome morphology in Bovidae farm animals, such as river buffalo, cattle, and sheep, is evolutionarily conserved was described in detail within the last years. Syntenic chromosome segments represent very similar banding patterns and gene order in these species. Evolutionary rearrangements in autosomes are rare, except from few bovid ancestral chromosome fusions, resulting in the submetacentric chromosomes OAR1 to OAR3 or BBU1 to BBU5, respectively. The translocation of a small proximal segment from OAR8 to OAR9 describes another rearrangement, which corresponds to conserved segments of the homolog chromosomes BTA9 in cattle and BBU10 in river buffalo (de Gortari et al. 1998; Iannuzzi et al. 2000; Cribiu et al. 2001). Unlike autosomes, the sex chromosomes in Bovidae differentiate by more complex chromosome rearrangements (Iannuzzi et al. 2009) and the analysis of evolutionary conservation of sex chromosomes in Bovidae remains to a certain extent unsolved in several breakpoints regions. The X chromosome is in focus of this study. It represents about 5 % of the genome in mammals, is submetacentric in cattle and sheep, and acrocentric in river buffalo (Cribiu et al. 2001; Iannuzzi 1994). It is assumed that the submetacentric morphology of BTAX is the result of a centromere transposition (Robinson et al. 1998), and it is suggested that the observed morphological size difference between BBUX and BTAX metaphase chromosomes is a consequence of the additional constitutive heterochromatin fragment q11.2 present in BBUX (Iannuzzi et al. 2000, 2009). The known cytogenetic and radiation hybrid loci indicate a similar gene order between BBUX and BTAX (e.g., Iannuzzi et al. 2000; El Nahas et al. 2001; Di Meo et al. 2002; Amaral et al. 2008; Ianella et al. 2008;). In contrast, Ponce de Leon et al. (1996) and Iannuzzi et al. (2000, 2009) demonstrated by X chromosome painting and comparative FISH that the sheep X chromosome differs from BBUX and BTAX by several rearrangements including a centromere transposition. Although numerous genetic and radiation hybrid maps have been released in recent years (e.g., Amaral et al. 2002; Everts-van der Wind et al. 2004; Maddox et al. 2001; Ihara et al. 2004; Goldammer et al. 2009a), which improved the comparative X chromosome analysis in Bovidae, these maps are not sufficiently anchored to the X chromosome by cytogenetic loci. The morphological comparison of syntenic chromosome fragments among the bovid X chromosomes and with the corresponding chromosome sequence assemblies in DNA sequence databases is still complicated due to the low number of comparable loci. Another specific of the X chromosome is that as consequence of gene dosage compensation, one of the X chromosomes in females is randomly inactivated and only a small chromosome fragment, including some genes, defined as pseudoautosomal region (PAR) escapes this inactivation (Helena Mangs and Morris 2007; Das et al. 2009). Whereas the PAR of OARX and BTAX has been analyzed in detail by Das et al. (2009), the PAR in river buffalo is defined by the gene *SLC25A6 and two sequence tagged sites (STS)*.

To improve the knowledge about bovid X chromosome conservation, to expand especially the cytogenetic maps of BBUX and BTAX, and to increase the number of comparable anchor loci of both maps with OARX, HSAX, and other vertebrates, we primarily assigned selected ovine BACs to BBUX and BTAX in a comparative FISH approach. The almost evenly in OARX distributed ovine BAC loci comprise verified sequence information from BAC end sequences (BES), genes, and STS (Dalrymple et al. 2007; Goldammer et al. 2009a, b). A comparison of the bovid X chromosome maps with HSAX reveals evolutionary breakpoints and rearrangements. We repeated the cytogenetically assignment of gene *F8* (alias *F8C;* OARXq24-q33) and of genes *GLRA2* (BTAXp22) and *F9* (BTAXp21 versus BTAXq33). The cytogenetic loci of the mentioned genes differ significant from corresponding X chromosome sequence assemblies or between previous FISH investigations (locus.jouy.inra.fr; thearkdb.org; Piumi et al. 1998; Goldammer et al. 2003).

Material and methods

Selection of BAC probes for FISH

First, all 22 BACs from a recently presented cytogenetic OARX map (Goldammer et al. 2009a) were selected as probes for comparative FISH in BBUX and BTAX. The OARX BACs comprise informative DNA sequences, which could serve as comparative anchors between bovid X chromosomes. Primers derived from these DNA sequences were used for PCR with sheep BAC DNA as target. DNA sequencing of amplification products confirmed predicted gene coding regions, STS, and BES, respectively, as already described by Dalrymple et al. (2007) and Goldammer et al. (2009a). However, additional six BACs were included in the comparative mapping approach. Each of the two ovine BACs CH243-445G24 and CH243-82B6, respectively, was identified to comprise virtually the genes *F8*, *FUNDC2*, and the STS BMS911. The bovine BACs CH240-211G2 and CH240-159O16 were both predicted to harbor the genes *F9* and *MCF2*, and the two sheep BACs CH243-480O12 and CH243-63 H19 consisted in silico the gene *GLRA2* (livestockgenomics.csiro.au/perl/gbrowse.cgi/vsheep2/; ncbi.nlm.nih.gov). PCR and DNA sequencing was performed to confirm the in silico predicted genes and STS within BACs.

BAC DNA isolation, primer design, PCR, and DNA sequencing

Genomic DNA clones for FISH were taken from the CHORI BAC libraries 243 (sheep) or 240 (cattle) (bacpac.chori.org). DNA was prepared from all BAC clones selected for FISH using the Qiagen Large-Construct Kit or the Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany). Oligonucleotide primers specific for genes, STS, and BES were designed for PCR with genomic BAC DNA using the software program Primer3 (Rozen and Skaletzky 2000). Amplification of primer specific DNA sequences was done with HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) following manufacturer's standard protocols. PCR product size was determined by agarose gel electrophoresis. Verification of appropriate DNA sequences amplification was performed by Taq cycle sequencing with the automated 48-capillary 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). BLAST in genome databases verified the identity of sequenced PCR products (Altschul et al. 1990). Tables 1 and 2 summarize information concerning all DNA sequences used as FISH probes including comparative cytogenetic loci and DNA sequence position. The reported gene names follow the standards of the human gene nomenclature HGNC (genenames.org; Seal et al. 2011).

Metaphase chromosomes

Peripheral blood samples from cattle (Agerolese breed, Southern-Italy), sheep (Laticauda breed, Southern-Italy), and river buffalo (Mediterranean Italian breed, Southern-Italy) were cultured in RPMI medium enriched with fetal calf serum (10 %), antibiotic–antimicotic mixture (1 %), L-glutamine (1 %), and Concanavalin A (15 μg/ml). After 48 h of cell culture, thymidine (300 μg/ml) was added to block cells in S-phase. Seventeen hours later, cells were washed twice and recovered in fresh medium containing BrdU (15 μg/ml) and Hoechst 33258 (30 μg/ml) for 6 h before harvesting. A colcemid treatment (0.1 μg/ml) enriched the number of metaphase cells during the last hour. Cell residues were removed by a hypotonic treatment and three fixations in methanol/acetic acid (3:1). Two drops of the remaining nuclear suspension including metaphase spreads were fixed on wet and cold slides, air dried for 1 day, and then kept in slide boxes at −20 °C until use. Morphological identification of chromosome bands for locus assignment followed international chromosome standard nomenclatures, such as ISCNDB 2000 for cattle and sheep (Cribiu et al. 2001), CSKBB 1994 for river buffalo (Iannuzzi 1994), and ISCN 2009 for human (Brothman et al. 2009).

FISH mapping

FISH mapping followed a protocol earlier reported (Iannuzzi and Di Berardino 2008). Briefly, slides were stored at 50 °C overnight and then stained for 10 min with Hoechst33258 (25 μg/ml in distilled water), mounted in 2× SSC with a coverslip, and exposed for 30 min to UV light at a distance of 4 cm from the lamp. Directly afterwards, slides were washed in distilled water, air dried, and denatured in formamide/2× SSC 70 % at 73 °C for 2.5 min. Following this, slides were rinsed in −20 °C cold alcohol series (96 %, 70 %, and 40 %) and air-dried. For later probe detection, biotin-16-dUTP was incorporated in BAC DNA

Table 1 Summarized information for the genes *F9*, *MCF2*, *F8*, *FUNDC2*, and *GLRA2* defined in bovid BACs (information for all other DNA sequences is published in Goldammer et al. (2009a and b))

BAC symbol	Gene symbol	Gene name	Bovine sequence used for primer design Accession number	Forward primer 5′-3′ Reverse primer 5′-3′	T (°C)	PCR product size (bp)
CH240-159O16	*F9*	Coagulation factor IX	NM_001103220	F: aacccctatttgcattgctgc R: ctcatggtagccagcacaga	60	211
CH240-159O16	*MCF2*	MCF2 cell line derived transforming sequence	ENSBTAT_00000005233	F: tgggcaagatgataatgcag R:tggtatcgatcaccacctt	60	179
CH243-82B6	*F8*	Coagulation factor VIII, procoagulant component	NM_001145508	F: caggaacgttggcgattatt R: cactgtgatgcagctctggt	60	395
CH243-82B6	*FUNDC2*	FUN14 domain containing 2	NM_174338	F: actttgagggaaagtttgagtcac R: accatccagtgacacctcca	60	150
CH243-63H19	*GLRA2*	Glycine receptor alpha 2 subunit B,	NM_001192914	F: acgttggcatatagcgagt R: ttgaaatgcgaagtaatttgttg	60	152

probes by nick translation using Biotin-Nick Translation Mix (Roche Diagnostic). The biotinylated probe material was ethanol precipitated in presence of bovine C_0t1-DNA and sonicated salmon sperm DNA and dissolved in the hybridization solution. After DNA denaturation in formamide/2× SSC at 73 °C for 10 min, a pre-hybridization reaction was performed at 37 °C for 1 h to suppress repetitive DNA sequence motifs within the probe DNA. The mixture was then added to prepared slides and hybridized at the same temperature overnight. In a post-hybridization step, the fluorescein isothiocyanate (FITC) avidin and anti-avidin antibody system (Vector laboratories) amplified the fluorescence signals. After this, slides were stained for 10 min with propidium iodide (PI; 25 μg/ml) and finally mounted in DABCO antifade solution to preserve fluorescence. A Nikon E-1000 fluorescence microscope connected with a coupled CCD camera served for the analysis of slides. To obtain a precise locus assignment, 30 metaphase plates for each probe were studied by overlay of digitized images from the same metaphase; one with FITC signals and another one with R-banding by using PI staining (RBPI).

Results

An improved cytogenetic map for BBUX in river buffalo and BTAX in cattle was constructed. Twenty-six ovine and two bovine BACs were included in the study. Of these, 21 BACs were assigned to BBUX and BTAX, respectively. Out of these, the BACs CH243-82B6 (*F8*, *FUNDC2*), CH240-159O16 (*F9*, *MCF2*), CH243-63H19 (*GLRA2*), CH243-347F23 (*ASMTL*), and CH243-501I14 (DU171056) have also been assigned to OARX. Examples for FISH with all 21 BAC probes on RBPI-banded chromosomes were shown in Figs. 1, 2 and 3. Seven of the initially included 28 BACs have no FISH assignments in BBUX and BTAX. Four of these BACs have been mapped earlier to OARX (Goldammer et al. 2009a) but showed nonspecific FITC signals in different BBU and BTA chromosomes after FISH. The remaining three BACs were excluded from FISH mapping. While PCR and DNA sequencing confirmed virtually predicted DNA sequences in most BACs, DNA sequencing did not approve gene *F9* in bovine BAC CH240211G2 (virtual prediction: *F9*, *MCF2*) and PCR did not amplify gene *F8* and STS BMS911 in ovine BAC CH243-445G24 (virtual prediction: *F8*, *FUNDC2*,

Table 2 Comparative mapping data for HSAX, OARX, BTAX, and BBUX

BAC FISH probe	Locus symbol (HGNC)	Identified DNA sequence within BAC	Cytogenetic localization on G or R-bands				Hsap Build 37.3 M bp	OGA Version 2.0 M bp	Btau Build 4.6.1 M bp
			HSAX	OARX	BTAX	BBUX	HSAX	OARX	BTAX
347F23[§]	*ASMTL*	Acetylserotonin O-methyltransferase-like	p22.3	p12	q43	q46	1.57	-	148.81
			Yp11.3	Yp12-p13	Yp12.2-p13	Yq21-q22	Y: 1.52	-	-
501I14[§]	DU171056	BES	p22.3[#]	p12	q43.1	q46	5.18	1.73	148.27
			Yq11.2[#]	Yp12-p13	Yp12.2-p13	Yq21-q22	Y: 18.42	-	-
57B9	DU344283	BES *(homolog to AMELX in HSAX)*	p22.31-p22.1[#]	p11-q11	q41	q44	11.31	62.44	137.55
			Yp11.2[#]	-	-	-	Y: 6.74	-	-
116D23	*CTPS2*	CTP synthase II	p22	q11-q12	q41	q44	16.73	OAR2	134.05
63H19	*GLRA2*	Glycine receptor, alpha 2	p22.1-p21.3	q12	q41	q44	14.58	9.69	136.24
372K8	*NHS*	Nance-Horan syndrome (congenital cataracts and dental anomalies)	p22.13	q12	q35	q42	17.39	12.36	133.34
376P14	*CNKSR2*	Connector enhancer of kinase suppressor of Ras 2	p22.12	q12	q35	q42	21.39	16.36	129.18
413F22	*DDX53*	DEAD (Asp-Glu-Ala-Asp) box polypeptide 53	p22.11	q12	q35	q42	23.02	-	127.36
232A8	*IL1RAPL1*	Interleukin 1 receptor accessory protein-like 1	p22.1-p21.3	q12-q21	q35	q42	28.61	24.65	121.14
32G13	*CXorf59*	Chromosome X open reading frame 59	p21.1	q21	q31	q34	36.06	-	112.36
450P3	*MED14*	Mediator complex subunit 14	p11.4	q22	q33	q38	40.59	-	108.24
501E21	*KDM5C*	Lysine (K)-specific demethylase 5C	p11.22-p11.21	q23	q31	q34	53.25	47.51	96.07
443C21	*PHF8*	PHD finger protein 8	p11.22	q23	q31	q36	54.07	46.22	96.90
444J2	*ARHGEF9*	Cdc42 guanine nucleotide exchange factor (GEF) 9	q11.1	q22	q31	q36	63.00	41.23	101.74
465D8	*DGAT2L6*	Diacylglycerol O-acyltransferase 2-like 6	q13.1	q25-q26	q25	q32	69.40	56.64	85.58
493E12	*CXorf26*	Chromosome X open reading frame 26	q13.3	q25-q26	q25	q32	75.39	61.37	80.58
511H13	DU527259	BES	q21[#]	q45	q12	q24	85.23	119.22	47.18
395L21	*LRCH2*	Leucine-rich repeats and calponin homology (CH) domain containing 2	q23	q36-q42	q23	q31	114.49	101.35	72.56
79E19	*NXT2*	Nuclear transport factor 2-like export factor 2	q23	q43	q21	q25	108.78	106.97	62.39
79E19	BMS1820	DNA segment	q23[#]	q43	q21	q25	108.87	-	62.46
79E19	*ACSL4*	Acyl-CoA synthetase long-chain family member 4	q22.3-q23	q43	q21	q25	108.88	106.84	62.55
159O16*	*F9*	Coagulation factor IX	q27.1-q27.2	q36	p22	q21	138.61	-	23.05
159O16*	*MCF2*	MCF.2 cell line derived transforming sequence	q27	q36	p22	q21	138.66	-	23.07
82B6	*FUNDC2*	FUN14 domain containing 2	q28	q34	p12.2	q23	154.25	66.31	38.83
82B6	*F8*	Coagultion factor VIII, procoagulant component	q28	q34	p12.2	q23	154.25	66.17	38.84

Bold displayed chromosome bands represent new assignments. [*] BAC from bovine CHORI BAC library CH240. [§] CH243-347F23 and CH243-501I14 have also been mapped in BBUY, BTAY, and OARY. *#In silico* FISH assignments (NCBI: BLAST and MapViewer)

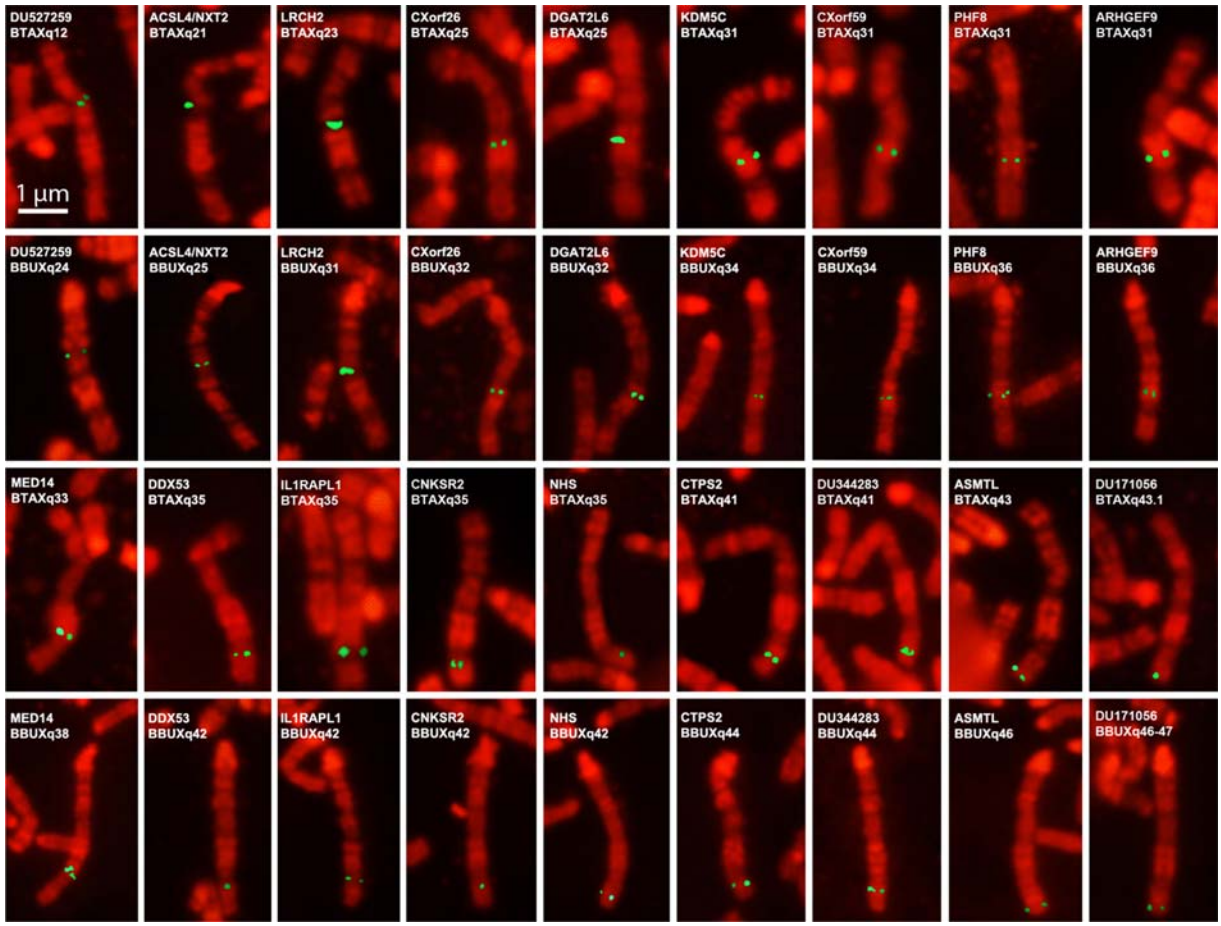


Fig. 1 Summary of FISH mapping in BBUX and BTAX. Image details show superimposed FITC signals from hybridized BAC probes on RBPI-banded BTAX and BBUX chromosomes

BMS911). The predicted STS BMS911 was also not detected in BAC CH243-82B6; however, *F8* and *FUNDC2* were confirmed and the BAC was used as probe for FISH (Fig. 2). DNA sequencing approved a 152-bp DNA fragment specific for the gene *GLRA2* in BACs CH243-480O12 and CH243-63H19, respectively, and the second one was chosen for FISH (Fig. 2). Information referring to the confirmation of the genes *F9*, *MCF2*, *F8*, *FUNDC2*, and *GLRA2* within bovid BACs were summarized in Table 1. The two BACs, CH243-347F23 (*ASMTL*) and CH243-501I14 (DU171056), both previously located in OARXp12 and OARXp11-p12, respectively (Goldammer et al. 2009a), were mapped in river buffalo, cattle, and sheep. Available comparative mapping data suggested a position of the two BACs in the PAR. The assignment of both BAC probes to BBUX/ BBUY, BTAX/ BTAY, and OARX/ OARY validated the prediction (Fig. 3). The FISH results were summarized in Table 2 and the presented loci were evaluated by comparison with available DNA sequence information. For better clarity, order of loci in OARX, BTAX, and BBUX follows the human sequence annotation *Hsap build 37.3* of HSAX. We added 25 loci to the cytogenetic maps of BBUX and BTAX, respectively. Thus, the total number of loci doubled in BBUX, whereas the number of cytogenetic anchors useful for comparison of chromosome conservation between BBUX and HSAX was almost trebled. The number of cytogenetic assignments in BTAX increased significantly by about 25 %. Seven loci were added to the cytogenetic map of OARX, including the confirmation of *ASMTL* and DU171056. The mapping of *ASMTL* and DU171056 in BBUY, BTAY, and OARY, respectively, adds another two cytogenetic anchors to each of the corresponding bovid Y chromosomes, raising the total number of identified loci to 63. The species-specific comparison of the

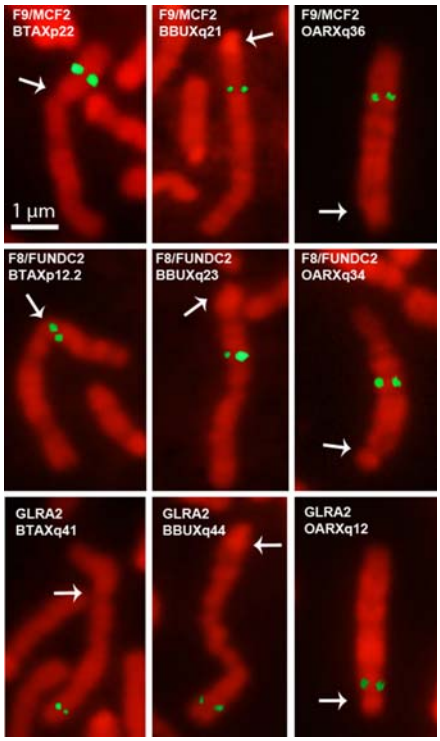


Fig. 2 FISH assignment of BAC probes CH240-O16 (*F9*, *MCF2*), CH243-82B6 (*F8*, *FUNDC2*) and CH243-63H19 (*GLRA2*) to BBUX, BTAX, and OARX, respectively. *Arrows* indicate the centromere position

cytogenetic X chromosome assignments with corresponding annotations in genome sequence assemblies or loci in the BBUX $RH_{5,000}$ map resulted largely in validation of a similar locus order between maps (Fig. 4). The loci for STS BMS182 and gene *ASMTL* represent two additional anchors between cytogenetic and $RH_{5,000}$ maps of BBUX. In BTAX, larger discrepancies in locus order occur in the regions q11–q24 versus 40 M to 80 Mbp (XBM111—DXS67) and in the regions BTAXq25-q34 versus about 80 M to 140 Mbp (*PGK1*—DXS30). Out of the seven cytogenetic assignments in OARX, the three loci DU171056, *F8*, and *FUNDC2* correspond to known positions in the OARX DNA sequence, whereas the cytogenetic loci *ASMTL*, *GLRA2*, *F9*, and *MCF2* have no annotation in the current ovine genome assembly OGA v.2.0. Numerous other small differences occur between maps within a species, which need further attention and clarification.

Based on the cytogenetic map and DNA sequence of human chromosome X, a comparative analysis between HSAX, OARX, BTAX, and BBUX was performed (Fig. 4). Whereas most of the added loci in bovid X chromosomes describe and confirm known evolutionarily conserved segments in more detail, several breakpoints have been identified within these segments. The comparison of chromosomes suggests that, in general, the proximal half of HSAX (segment p22-q13) corresponds to the proximal half of OARX (segment p12-q26) and to the inverted distal halves of BTAX (segment q25-q43), and BBUX (q32-q47), respectively. The distal half of HSAX (segment q21.33-q28) is almost syntenic

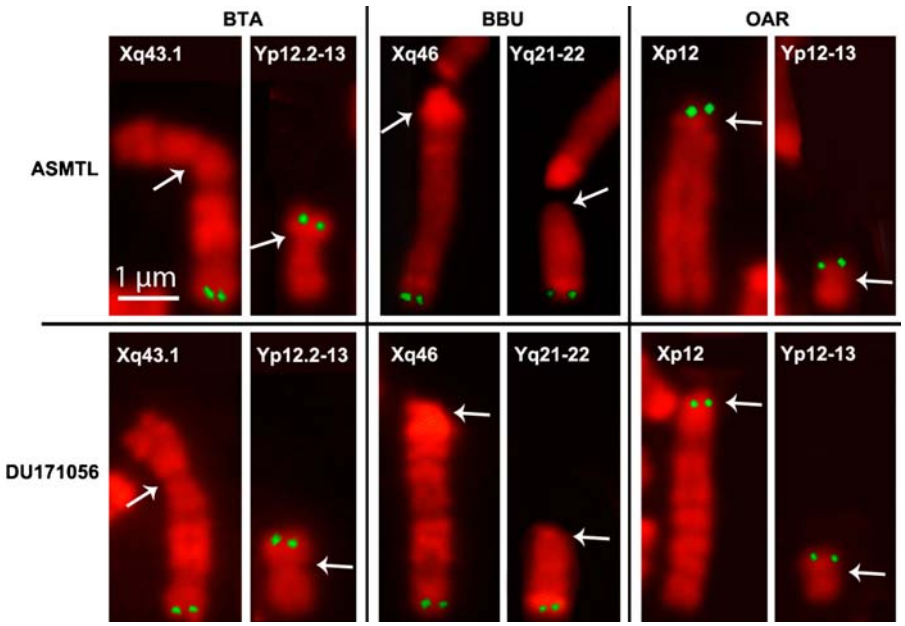


Fig. 3 FISH mapping of BAC probes CH243-347F23 (*ASMTL*) and CH243-501I14 (DU171056) to the pseudo-autosomal region in X and Y chromosomes of river buffalo, cattle, and sheep. *Arrows* indicate the centromere position

to the inverted distal half of OARX (region q32-q45). In contrast, the syntenic segments BTAXp24-q24 and BBUXq11.3-q31 show additional intra-chromosomal rearrangements in comparison to HSAXq21.33-q28 and OARXq32-q45. Furthermore, the results suggest collinearity in segment orientation and almost in gene order for the segments HSAXq24-q28, BTAp24-q11, and BBUq12-q23. The segments BTAXq12-q24 and BBUXq24-q31 are collinear with HSAXq21.33-q23. However, the position and order of the neighboring proximal and distal linked bovid segments was founds inverted to HSAX. Follow-up of the novel cytogenetic assignments of DU527259, *NXT2, ASCL4, F8,* and *FUNDC2* and the previously identified locus *LAMP2* in the displayed X chromosomes simplifies the comparison (Fig. 4). Another result of this study is that the X chromosome segment characterized by the cytogenetic loci *LRCH2*, *ACSL4*, *NXT2*, and BMS1820 represents a previously not mentioned syntenic region between HSAXq22.3-q23, OARXq42-q43, BTAXq21 and q23, and BBUXq25-q31.

Discussion

This comparative mapping study was performed to improve primarily the cytogenetic maps of BBUX and BTAX and to provide cytogenetic anchor loci that allow a more comprehensive analysis of evolutionary conservation of chromosome morphology between Bovinae X chromosomes with other X chromosomes, such as OARX or HSAX. To achieve this goal, ovine BACs with an approximately even locus distribution in OARX were selected for FISH mapping. Except for the bands OARXq31 and Xq35, the ovine loci cover all other OARX chromosome bands (Goldammer et al. 2009a). All BACs have been characterized for gene, STS, and BES content (Goldammer et al. 2009a) and the position was identified in the sheep genome assembly (Dalrymple et al. 2007; Ratnakumar et al. 2010) or in the OARX $RH_{5,000}$ map (Goldammer et al. 2009b). We recruited further BACs for FISH mapping harboring the genes *F8* and *FUNDC2*, *F9* and *MCF2*, and *GLRA2*; although cytogenetic loci for *F8*, *F9*, and *GLRA2* have been reported earlier (locus.jouy.inra.fr; thearkdb.org; Piumi et al. 1998; Goldammer et al. 2003). The loci for *F9* in BTAXp22, BBUXq21, and OARXq36, for *F8* in BTAXp12.2, BBUXq23, and OARXq36, and for *GLRA2* in BTAXq41, BBUXq44 and OARXq12 identified in this investigation correspond to homolog assignments in HSAX and to bovid DNA sequence positions if available.

The number of cytogenetic loci was doubled in BBUX. The loci order is almost collinear between BBUX and BTAX chromosomes, although chromosome banding structure and centromere position differ. Thus, the mapping in river buffalo supports the assumption of a high evolutionary conservation of DNA sequences between BBUX and BTAX in contrast to the modified chromosome morphology. The cytogenetic loci represent useful anchors for the upcoming BBUX DNA sequence. However, the approximate assignments revealed by the FISH mapping approach cannot exclude small intra-chromosomal rearrangements in gene order between BBUX and BTAX. The majority of added loci were found in the distal halves of BBUX and BTAX. This is because four ovine BAC probes characterized by the ovine BES loci DU238864, DU421846, DU359754, and DU475655 and with predicted comparative assignments in proximal regions of BBUX and BTAX were not X chromosome specific in BBUX and BTAX after FISH. Furthermore, previous comparative mapping data suggested that the bovid X chromosomes share a similar relative metaphase chromosome length (Cribiu et al. 2001; Iannuzzi 1994) and have consequently a comparable DNA sequence length. However, the presented data suggest that the proximal fragment BBUXq12-q23 (*LAMP—FUNDC2*) corresponding to BTAp24-p12.2 (*LAMP2—FUNDC2*; about 35 Mbp—as in HSAX) is 13 Mbp larger than the syntenic segment OARXq32-q41 (*LAMP2—FUNDC2*; about 22 Mbp). At this time point, it can only be hypothesized if the differences are due to intra-chromosomal rearrangements or were caused by DNA sequence annotation. Another possibility is that via FISH, detected morphological distances between assignments in the considered fragments in BTAX and OARX may be the result of species-specific condensation grades in metaphase chromosomes (e.g., Hudson et al. 2003; Ono et al. 2003). DNA sequence completion and targeted FISH mapping in both species will prove these assumptions. Considering that we used OARX specific BACs for comparative FISH in BBUX, the number of anchors only slightly increased between cytogenetic map and $RH_{5,000}$ map of BBUX; however, the locus order was confirmed between maps.

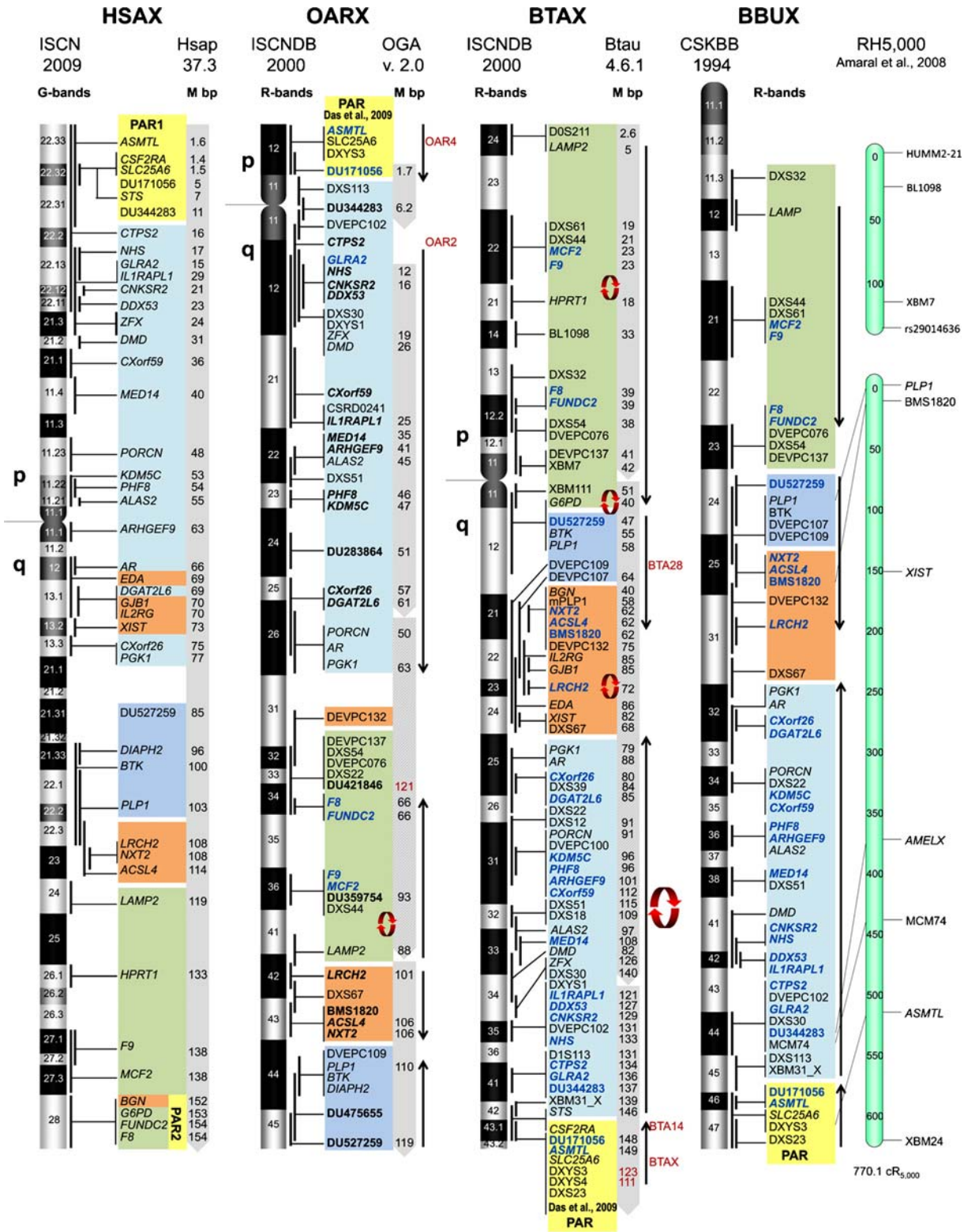
Except for the cytogenetic assignments of *ASMTL* and DU171056 to BTAY, all other cytogenetic loci in cattle anchored corresponding DNA sequence motifs

and linked genomic DNA contigs to specific R-bands in BTAX. Nonetheless, the comparison of cytogenetic and corresponding loci in the cattle DNA sequence assembly revealed also differences in locus positions. The assignments of *HPRT1*, *G6PD*, *LRCH2*, or DXS67 identified such dissimilarities. In BTAX fragment, q25-q34 occur several smaller divergences between maps. Although the addressed cytogenetic assignments provide only approximated loci compared to those in the BTAX sequence assembly, the cytogenetic order can contribute to find their correct position within the sequence. The cytogenetic loci might also display small intra-chromosomal rearrangements, which are not detectable by the BTAX sequence annotation process that includes information from comparative genomes, such as human or mice (Everts-van der Wind et al. 2005; The Bovine Genome Sequencing and Analysis Consortium et al. 2009). The cytogenetic map of BTAX comprises furthermore the loci DVEPC109 (q21-q24) and *CSF2RA* (q43.1; PAR). DVEPC109 was assigned to homologous segments in OARX and BBUX and *CSF2RA* to a corresponding region in HSAX. In the cattle DNA sequence assembly, DVEPC109 is arranged in BTA28 and *CSF2RA* in BTA14. The sequence motifs *SLC25A6*, DXYS3, and DXYS4 have imprecise annotated loci in the PAR of BTAX. A reconfirmation of loci is suggested by a targeted DNA sequence analysis in the named regions.

We mapped the sequences DU171056 and *ASMTL* to the PAR in OARX, BTAX, and BBUX. The data confirm previous assignments in BTAXqter (Das et al. 2009) and OARXp (Goldammer et al. 2009a; Das et al. 2009), respectively, and contribute furthermore to the elucidation of the PAR in BBUXq46. However, the detailed cytogenetic and BAC contig analyses of the PAR in OARX and BTAX by Das et al. (2009) suggests a collinear locus order in the PAR of BBUX. Genome database BLAST of ovine BES DU344283 (OARXp11-q11) identified high similarity of a 62-bp fragment with the gene *AMELX* (acc.# NM_001142, exon 2, 92 % identity) in HSAX. *AMELX* was RH mapped in BBUX (Amaral et al. 2008) and therefore, the BES DU344283 is suggested as additional cytogenetic anchor for the $RH_{5,000}$ map in BBUX. Das and co-workers proved that *AMELX* demarcates the putative ancestral eutherian pseudoautosomal boundary (PAB) (Iwase et al. 2003; Das et al. 2009). Thus, we suggest this locus for the PAB boundary in BBUXq44, BTAq41, and OARp11-q11.

Essentially, the comparative X chromosome analysis confirmed the complex rearrangements, which differentiate the X chromosomes in the studied Bovidae (Iannuzzi et al. 2000, 2009). The data approved and specified both previous cytogenetic data in bovid X chromosomes (e.g., Piumi et al. 1998; Iannuzzi et al. 2000, 2003, 2009; Di Meo et al. 2002; Goldammer et al. 2003, 2009a, c; Rubes et al. 2005) and to a certain amount earlier RH mapping results (e.g., Amaral et al. 2002; Ianella et al. 2008; Goldammer et al. 2009b, c). Furthermore, the data provide physical anchors for the ongoing sequencing project in sheep (livestockgenomics.csiro.au/sheep/; The International Sheep Genomics Consortium et al. 2010) and support the assembly of the current sequence version Btau 4.6.1 in cattle (ncbi.nlm.nih.gov; The Bovine Genome Sequencing and Analysis Consortium et al. 2009). The comparative X chromosome maps in Fig. 4 illustrate numerous evolutionary breakpoints between human and bovid X chromosomes. This is in contrast to other distantly related mammals, such as dog, cat, pig, and horse. In these species, the X chromosomes are evolutionarily conserved and largely collinear with HSAX (e.g., Murphy et al. 2005; Raudsepp et al. 2008). In conclusion, the interspecies-specific locus prediction or the direct transfer of cytogenetic loci or assembled sequence contigs between evolved X chromosome maps in other species and bovid X chromosome maps remains difficult.

In more detail, the PAR1 segment in HSAX and the adjacent segment of the p-arm until the chromosome band HSAXq12 correspond to continuous segments in OARX, BTAX, and BBUX, although the segments in BTAX and BBUX are inverted. We identified in this region two thirds of the added loci in BBUX and BTAX. Whereas the cytogenetic loci in the proximal half of OARX correspond well to the order in HSAX, the comparison with syntenic regions of BTAX and BBUX identified several discrepancies, which need additional validation. The next distal segment in human HSAXq12-q13.2 (*EDA*, *GJB1*, *IL2RG*, and *XIST*) and the two segments HSAq22.3-q23 (*LRCH2*, *NXT2*, *ASCL4*) and HSAXq28 (*BGN*) correspond to the continuous segment in BTAXq21-q24 in cattle. Since not all of the named loci have been identified in OARX and BBUX, it can only be assumed, if the segment BBUXq25-q31 corresponds entirely to BTAXq21-q24. Otherwise, the STS DEVPC132 identified in OARXq31-q32 an additional segment discontinuous



◀ **Fig. 4** Comparative chromosome maps of HSAX, OARX, BTAX, and BBUX. Cytogenetic loci, DNA sequence loci, in case of BBUX $RH_{5,000}$ loci, and conserved segments between chromosomes are displayed. New assignments are displayed in *blue*. OARX loci displayed in *bold black* indicate the initially used mapping resource. Same *colored blocks* of loci represent evolutionarily conserved segments between species. Loci in *yellow colored blocks* correspond to the PAR. *Gray arrows* inform about the DNA sequence position of cytogenetic loci. If no position is given, the corresponding cytogentic locus is unplaced. Diagonal *striped gray arrows* indicate intra-chromosomal contradictions between DNA sequence locus order and cytogenetic loci. *Black arrows* demonstrate the orientation of syntenic bovid X chromosome segments in relation to corresponding segments in HSAX. *Circular arrows* specify small evolutionary rearrangements in ?otherwise syntenic segments between HSAX and bovid X chromosomes or indicate intra-chromosomal differences between cytogenetic and sequence assignments. Only RH markers with a cytogenetic assignment to BBUX or BTAX are shown and in addition RH markers that represent start and end of a RH linkage group. DNA sequence positions are displayed in *red* if significantly different from the cytogenetic position in the genome. The comprehensive mapping data in the PAR of OARXp and BTAXq (Das et al. 2009) were not included in the drawing

from OARXq42-q32. According to other complex rearrangements in the distal half of OARX compared to HSAX and a lack of cytogenetic loci, it remains unclear if the two identified OARX segments or even more segments are representative to the corresponding BTAXq21-q24 segment. BLAST of correlated sequences *EDA*, *GJB1*, *IL2RG*, *XIST*, *BGN* in the ovine genome assembly OGA v2.0 (The International Sheep Genomics Consortium et al. 2010) proposed a separate segment comprising *EDA* (56 Mbp), *GJB1* (57 Mbp) *IL2RG* (57 Mbp), and *XIST* (59 Mbp). *BGN* (68 Mbp) was annotated close to *F8* (66 Mbp) and *FUNDC2* (66 Mbp) in a third segment compared to HSAX, although in a different chromosome position. The comparison of the cytogenetic loci *BGN* (BTAXq21) and *G6PD* (BTAXq11-q12), both with a DNA sequence annotation of 40 Mbp in BTAX, close to *F8* (39 Mbp) and *FUNDC2* (39 Mbp) suggested that these loci belong to segment BTAXp12 other than demonstrated by the cytogenetic map. Following the BTAX sequence assembly, the evolutionary breakpoint arisen by the locus *BGN* in HSAX and BTAX could be solved. However, the DNA sequence assembly of BTAX is not complete and the associated DNA contig (Acc# NW_033104686) contains numerous gaps around the *BGN* locus.

No comparative cytogenetic loci describe the homology between chromosome fragment HSAXq21.1-q21.32 and bovid X chromosomes. A DNA sequence BLAST with ten randomly selected genes out of this region in the sheep genome (OGA v2.0) revealed hits in different sheep autosomes. However, the same ten genes are located in a range of 15 Mbp in BTAX region 60 to 75 Mbp (Btau 4.6.1; data not shown). Selected assignments of FISH probes specific for genes in region HSAXq21.1-q21.32 in OARX, BBUX, and BTAX could force the validation of sequence annotation data.

The next two segments HSAXq21.33-q22.3 and q22.3-q23 correspond to similar segments in OARXq44-q45 and q42-q43. Whereas the order of these segments is inverted in OARX, the segment orientation of OARXq42-q43 itself agrees with the syntenic HSAX segment. The corresponding regions in BTAX and BBUX are almost collinear. Only the locus *LRCH2* in BTAX and HSAX indicates a larger discrepancy in gene order probably caused by segment BTAXq22, which corresponds to HSAXq12-q13.1. The segment HSAq24-q28 is evolutionarily conserved to proximal regions in BTAX and BBUX. The segment order there is equal, but in OARX, the syntenic segment to HSAXq24-q28 is inverted and intra-chromosomal discrepancies exist between cytogenetic and sequence locus of *LAMP2* compared to neighboring sequence assignments.

In sum, the extended cytogenetic maps for BBUX, BTAX, and OARX confirm that complex chromosome rearrangements differentiated the ancestral X chromosome in *Bovinae* in contrast to other distantly related mammals. Several new breakpoints were identified, and some previous assignments were approved. The comparative gene maps show a more detailed comparison between bovid and human X chromosomes and provide new physical anchors. This will further improve our knowledge about sex chromosome evolution and can be very useful in clinical cytogenetics (Villagomez et al. 2009), while the verified DNA sequences in mapped BAC probes will contribute to the evaluation of the bovid genome sequences.

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NCBI genome database: www.ncbi.nlm.nih.gov

Sheep database: www.thearkdb.org

Sheep Genome Sequences and Genome Browsers: www.livestockgenomics.csiro.au/sheep/