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Isolation and physical localization of new chromosome-specific centromeric repeats in farm animals

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ABSTRACT: In this study, new classes of tandemly repeated DNA sequences in the centromeric regions of three farm animal species are reported. Pericentromeric regions of bovine chromosome X, porcine chromosome 14 and equine chromosome 1 were microdissected by laser and amplified by DOP-PCR. Painting probes showed strong hybridization signals on their corresponding centromeres. Specific DNA fragments were isolated by cloning and sequenced. For each species, high homology level was found among the sequences. The presence of specific repeats within each sequence allows us to consider them as monomeric units or parts of longer tandemly repeated monomeric units that generate a specific higher-order repeat. In this respect, the isolation of new centromeric sequences enriches the genetic map of these three species and represents a useful tool for FISH analysis, thus expanding our knowledge on centromeres of farm animal species.

Keywords: *Bos taurus*; *Sus scrofa*; *Equus caballus*; cattle; pig; horse; centromeres; tandem repeats; chromosomes; FISH

Centromeres are an essential structural domain of chromosomes and play an essential role accurate chromosomal segregation during mitosis and meiosis. In humans, centromeric regions of all chromosomes contain a huge repetitive sequence, known as alphoid DNA that constitutes as much as 3–4% of chromosomal DNA (Mitchell et al., 1985; Alexandrov et al., 2001). Alphoid sequences consist of tandem repeats of an AT-rich 171-bp alphoid monomer unit, organized into higher order repeats (HORs). These monomers appear to be conserved among all human chromosomes, but the nucleotide sequence and the organization of the monomeric units are known to vary from one chromosome to another, showing substantial chromosome-specific variation which can be used as target for the construction of chromosome-specific probes (Willard, 1985; Willard and Waye, 1987).

The isolation and the analysis of satellite DNA sequences and their hybridization on individual chromosomes have opened new diagnostic possibilities in the cytogenetic fields, especially in humans. Alphoid probes which hybridize to repetitive DNA sequences on specific chromosomes have been used to identify chromosome abnormalities in interphase nuclei of tumoral cells and tissues including cells of breast (Devilee et al., 1988), bladder and buccal mucosa (Moore et al., 1993), bone marrow (Kolluri et al., 1990) and ovarian epithelium (Huang et al., 2002).

Specific alphoid DNA probes have been developed for most of the human chromosomes (Burke et al., 1985; Moyzis et al., 1987; Willard and Waye, 1987; Pellestor et al., 1996), but little information is available on high-order specific repeats in non-human species, because they have not been adequately investigated yet. At present, alpha satellite DNA probe, specific for the centromere of chimpanzee chromosome 4, has been obtained by using oligonucleotide primers specific for a conserved region of human satellite DNA (Haaf and Willard, 1997). High-order repeats probes that hybridize

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specifically to the centromere of metaphase chromosome 9 (Janzen et al., 1999), chromosome 11 (Riquet et al., 1996), and chromosome 1 (Jantsch et al., 1990) have been produced in pigs.

On the contrary, unspecific repetitive probes have been obtained for the centromeric regions of many chromosomes of species belonging to Bovidae (Nijman and Lenstra, 2001) and Caprinae subfamilies (Chaves et al., 2000). Most of the pig acrocentric and metacentric chromosomes have been hybridized by using centromeric DNA probes (Rogel-Gaillard et al., 1997a, b). In the horse chromosomes, tandem repeat elements have been localized only in the centromeres of the acrocentrics, not in the metacentrics (Broad et al., 1995). Centromeric satellite probes have been obtained for many other species including rabbit (Ekes et al., 2004), roe deer (Buntjer et al., 1998) and caribou (Lee et al., 1994).

In order to extend our knowledge on the centromeric region of farm animals, we detected and characterized new chromosome-specific repeats for cattle (*Bos taurus*), pig (*Sus scrofa domestica*) and horse (*Equus caballus*) by using chromosome microdissection of the pericentromeric area of chromosomes X, 14 and 1, respectively, FISH and sequencing.

MATERIAL AND METHODS

Chromosome preparation and PCR amplification. Metaphase spreads were prepared from bovine, porcine and equine lymphocytes by standard cytogenetic techniques. To identify the chromosome of interest, GTG-banding was performed.

The PALM® MicroLaser system (P.A.L.M. GmbH, Bernried, Germany) was used for dissection and collection of pericentromeric part of chromosomes (Bos X, Sus 14 and Equus 1). The microdissected DNA was amplified as described earlier (Kubickova et al., 2002). Briefly, after collection, parts of chromosomes were transferred to 20 µl of 10mM Tris-HCl pH 8.8 and the DOP-PCR was performed without any pretreatment in a PTC-200 thermocycler (MJ Research, Watertown, USA). The reaction mixture contained 60mM Tris-HCl pH 8.8, 15mM $(NH4)_2SO_4$, 3.5mM $MgCl_2$, 0.2mM each dNTPs, 1.6µM DOP primer (5`-CCG ACT CGA GNN NNN NAT GTG G-3`, Telenius, 1992), 0.05% W-1 (Invitrogen Life Technologies, Carlsbad, CA, USA), 2U Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA , USA) in final volume of 40 µl. After the initial denaturation step at 96°C for 3 min, eight cycles were performed at 96°C for 1 min, at 30°C for 1 min with a 2 min transition from 30°C to 72°C, and at 72°C for 2 min. This was followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. The final extension was carried out at 72°C for 5 min. PCR products (5µl) were analyzed by electrophoresis using 1% agarose gel.

Cloning and hybridization analysis. The remaining primary DOP-PCR products were reamplified under the same conditions and then purified using the High Pure PCR Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany). The pDrive vector (QIAGEN) was used for cloning. The ligation products were transformed into QIAGEN EZ Competent cells following the manufacturers' guidelines (QIAGEN PCR Cloning *plus* Kit). Screening of the recombinant clones was performed by PCR using the vector primers M 13. A small portion of the product was run on a 1% TBE agarose gel to determine the size of the insert.

Amplification products (1.5 µl) were spotted onto two copies of nylon filters and hybridized with digoxigenin-11-dUTP probes (Roche Diagnostics GmbH, Mannheim, Germany) under standard conditions (Maniatis et al., 1982). One filter was hybridized with specific primary DOP-PCR and the second one with genomic DNA at 42°C for 16 hours. Anti-DIG antibody coupled to alkaline phosphatase (anti-DIG-AP; Roche Diagnostics GmbH, Mannheim, Germany) and BM purple AP substrate (Roche Diagnostics GmbH, Mannheim, Germany) were used for DIG signal detection. In order to estimate the amount of specific repeats present in each clone, a comparison of the intensity of the hybridization signals was performed. The clones showing a higher hybridization signal with specific probe were considered positive. Chosen Dot Blot positive clones were labelled by PCR with Spectrum Orange-dUTP (Vysis, Richmond, UK) and used as probes in FISH.

Fluorescence *in situ* hybridization. Metaphase spreads for FISH were prepared from lymphocytes using standard methanol:acetic acid (3:1) fixation.

For hybridization with a single painting probe, 1 µl of probe was placed in 10 µl of a hybridization mixture containing 50% formamide, 2× SSC, 10% dextran sulphate, and 7 µg of salmon sperm DNA. The hybridization mixture was denatured at 72°C for 10 min. Slides were denatured in 70% formamide, 2× SSC (pH 7.0) at 72°C for 2 min,

dehydrated and hybridized overnight in a moist chamber at 37°C with the probe. Slides were then washed twice in 50% formamide 2× SSC (pH 7.0) at 42°C for 5 min, in 0.1× SSC (pH 7.0) at 42°C for 5 min, followed by one wash in Tris-NaCl-Tween 20 buffer at 42°C for 5 min. Slides were counterstained with 4, 6-diamino-2-phenolindole (DAPI) and mounted in vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Probe signals on metaphase spreads were visualized under an Olympus BX60 fluorescent microscope. Digital images were captured using the ISIS 3 software (MetaSystems, Altlussheim, Germany).

Sequencing. Plasmid DNA from FISH positive clones was isolated using Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Medison WI, USA) and sequenced using an ALFexpress Sequencing System (Pharmacia, Biotech). Sequences of the inserts have been deposited in EMBL database. DNAsis-Pro software (Hitachi) was used to analyze the global structure of DNA sequences and create Dot Plot graphs useful to show the degree of similarity, deletions, insertions, direct and indirect repeats. For Dot Plot analysis, a window of 8 bases was used with a stringency of 6.

RESULTS

Isolation and physical localization of bovine X, porcine 14 and equine 1 chromosome-specific repeats

DNA specific probes, generated by laser microdissection, targeted recombinant clones containing repetitive DNA sequences from the centromeric regions of bovine X, porcine 14 and equine 1 chromosomes. Most of the recombinant clones contained inserts ranging approximately from 300 bp to 500 bp. To examine the insert of the recombinant clones contained chromosome-specific repeats, a Dot Blot assay was performed on the corresponding PCR products. Clones that showed a higher hybridization signal with specific probe were considered positive. Fluorescence *in situ* hybridization was performed in order to confirm their position on species specific metaphase spreads. The G-like pattern generated by DAPI staining was used for chromosome identification.

In cattle, four clones showed strong fluorescent signal on metaphase chromosomes, specifically located in the centromeric region of the X chromosome. The signal was also clearly visible in interphase nuclei (Figure 1a), showing the ability of these clones to detect specific chromosomal domains. The same clones revealed positive hybridization signals on mitotic chromosomes of river buffalo, sheep and goat as demonstrated in Figure 2. In the pig, specific hybridization signals were generated from 11 single probes on the centromeric region of chromosomes 14; however, the signal intensity of the single clone was not satisfactory in interphase nuclei (Figure 1b). On the contrary, when the probes were mixed, a higher intensity of the hybridization signal was observed also in interphase nuclei (Figure 1c). In the horse, two probes showed a discrete fluorescent signal specifically located on the centromeric region of chromosome 1, but the intensity of hybridization was not high to show the localization of chromosomes in interphase nuclei (Figure 1d).

DNA sequencing and Dot Plot analysis

DNA sequencing was performed on two bovine recombinant clones with the strongest FISH signal and on all specific positive FISH inserts from the porcine and equine clones. In cattle, a significant homology (98.3%) was found among the insert sequences. The centromeric sequence corresponding to the highest molecular weight (without vector- and DOP-primer sequences) was deposited in the EMBL database under accession number AJ884576. In the pig, sequence analysis also indicated high homology level among the 11 clones examined. The molecular data allowed a subdivision of the clones in 2 groups: the first one, composed 6 clones with average homology of 95.6% (Accession numbers AJ889249, AJ937272-AJ937276); the second, composed 2 clones with a similarity of 99.6% (AJ920051-AJ920052). No homology was found among these two groups and the remaining 3 clones (Accession numbers AJ920048-AJ920050). BLAST analysis confirmed that the isolated DNA inserts did not have any homology with other centromeric repeated regions. In the horse, the nucleotide sequence of both investigated clones showed 98.3% homology. The sequences were deposited in the EMBL database under accession numbers AJ889250 and AJ937277. A summary of microdissected chromosomal regions and positive clones subjected to different assays is presented in Table 1. Dot Plot

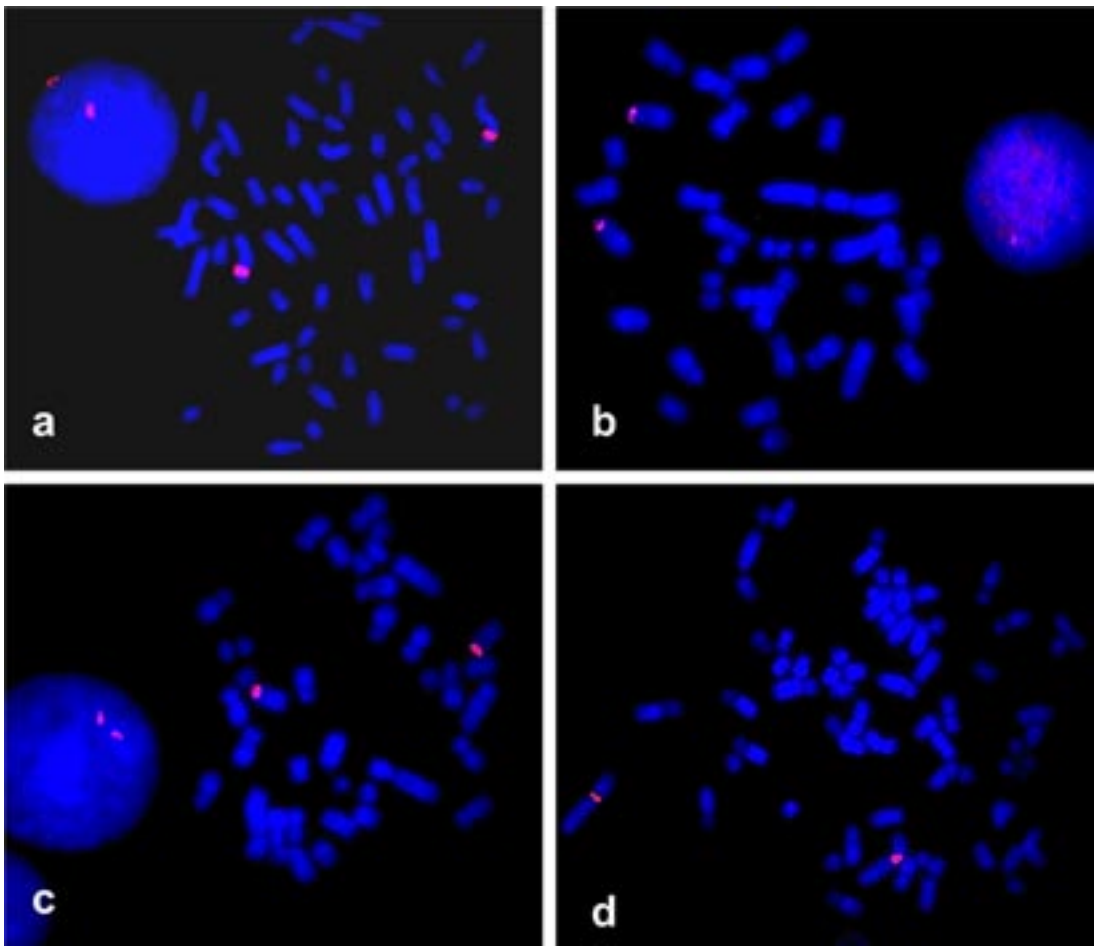

Figure 1. (**a**) Hybridization signals shown from four mixed bovine probes specifically linked just above the centromere of chromosome X (counterstained blue with DAPI). The signal is clearly visible in interphase nuclei, too. (**b**) *In situ* hybridization signal obtained from a single porcine clone to the centromeric region of pig metaphase chromosomes 14. (**c**) A higher intensity of signal was observed when FISH was performed with mix of all porcine clones. (**d**) Results of fluorescence *in situ* hybridization experiment with two probes generated from equine positive clones. A discrete fluorescent signal was specifically located on the centromeric region of equine chromosome 1

analysis showed presence of various short repeated elements in the investigated clones. The results are summarized in Table 2. In the pig, a consensus sequence was generated by multiple alignments of the inserts (Figure 3) in order to facilitate the Dot Plot analysis of the clones belonging to the first homology group.

DISCUSSION

In this study we report on new classes of tandemly repeated DNA sequences in the centromeric regions of three species of farm animals detected by laser microdissection and DOP-PCR procedures.

Table 1. Summary of positive clones subjected to different assays and homology shown from sequenced probes

Species and chromosome		Dot Blot	FISH	Sequencing		Clone names	Homology (%)
Bos taurus	X	9	4	2		B3, B11	98.3
					6	S35, S37, S50, S69, S74, S76	95.6
Sus scofa	14	34	11	11	2	S26, S38	99.6
					3	S16, S45, S46	0
Equus caballus	1	19	5	2		H2, H12	98.3

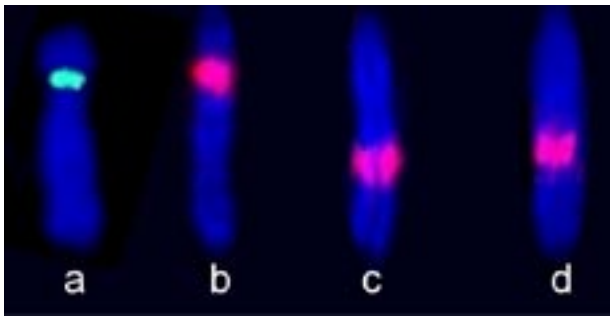

Figure 2. FISH signals on chromosome X obtained from mixed bovine clones hybridized on: (**a**) cattle (*Bos taurus*), (**b**) water buffalo (*Bubalus bubalis*), (**c**) sheep (*Ovis aries*), and (**d**) goat (*Capra hircus*) chromosomes

Table 2. Results from the Dot Plot analysis of the investigated clones

Species	Sequence	Lenght (pb)	Motif
Bos taurus	AJ884576	478	GAAAG(4)* TCAAGGGTGA(2)
Equus caballus	AJ889250	177	ACTTG(4) GGGAGCCAC(2)
	AJ937277	177	ACTTG(4) GGGAGCCAC(2)
Sus scofa	consensus	355	TGTGT(11) TGCTGG(3) GGTCCTAGG(2) GTGTTTCTACAGG(2)
	AJ920051	236	ATCAAA (2) AAACACCC(2)
	AJ920052	236	ATCAAA (2) AAACACCC(2)
	AJ920049	463	GCCCATGGC(2)

*number of repeats

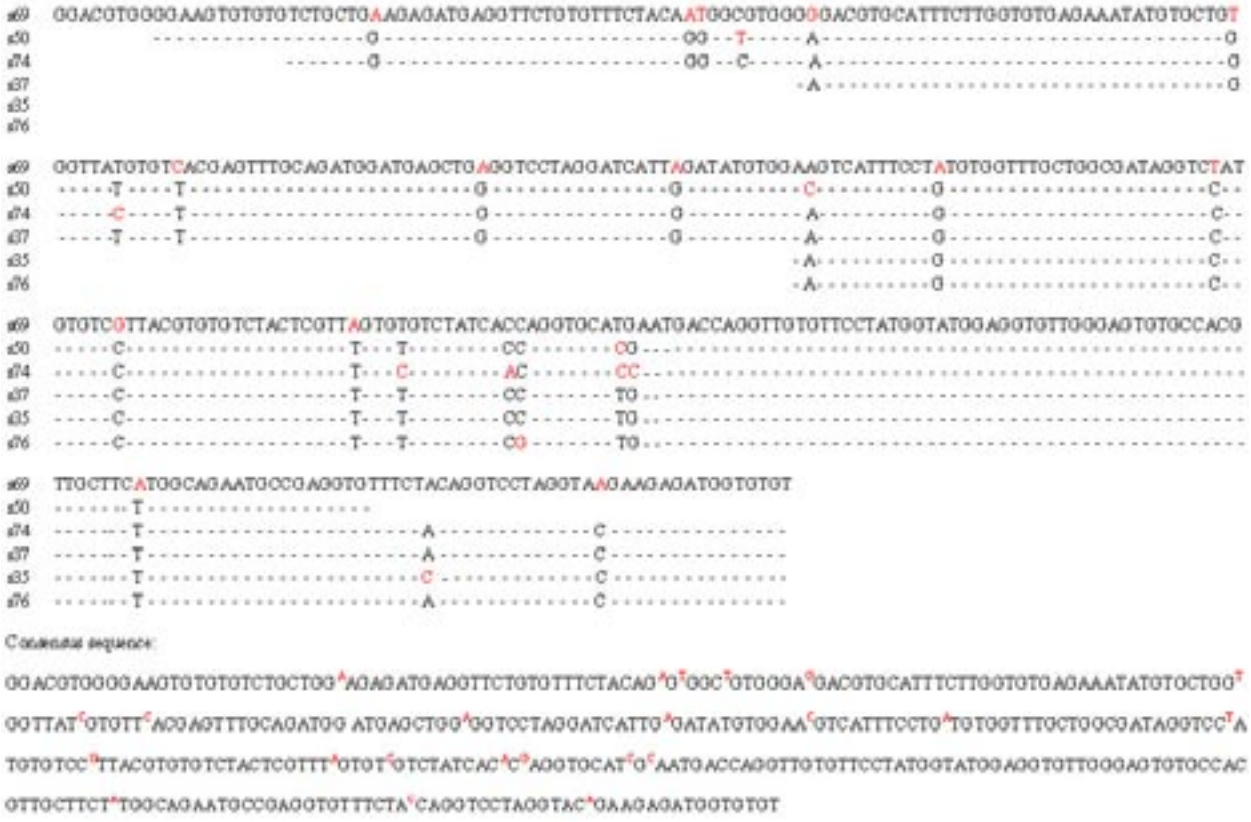

Figure 3. Consensus sequence generated by multiple alignments of porcine clones which belonging to the first homology group

In cattle FISH experiments, strong hybridization signals were shown from four bovine probes isolated from the pericentromeric region of chromosome X and specifically linked just above the centromere of the same chromosome. The signal obtained from the single clone appeared like an extensive spot, thus confirming the presence at least one specific repeated region. When the probes were mixed and hybridized together, the signal was also clearly visible in interphase nuclei showing the ability of these clones to detect specific chromosomal domains. When the isolated clones were hybridized to the acrocentric X chromosomes of water buffalo (*Bubalus bubalis*), sheep (*Ovis aries*) and goat (*Capra hircus*), strong signals were clearly detected below the centromere (water buffalo) and in the middle of the chromosome (sheep and goat) (Figure 2), confirming that the cross-hybridization of bovine probes to other species is possible (Di Berardino et al., 2004). As demonstrated by comparative FISH-mapping (Iannuzzi et al., 2000) with using 17 markers Bovinae (cattle and river buffalo) and Caprinae (goat and sheep) X-chromosomes were complex rebuilt. These rearrangements required at least four transpositions because at least 5 chromosome regions with the same gene order within each segment were found. While, cattle and river buffalo X-chromosomes differ only for one centromeric transposition with loss (cattle) or acquisition (river buffalo) of constitutive heterochromatin because the two chromosomes showed the same gene order. The signals found in the present study in the pericentromeric region of cattle p arms, proximal part of river buffalo q arms and distal part of ovine q arms of chromosomes X (Figure 2) are in agreement with the study (Iannuzzi et al., 2000).

DNA sequencing, performed on two bovine recombinant clones with the highest FISH signal, revealed high level of homology (98.3%) between the two; so, we assume that both sequences could be considered as the same monomeric unit, or a part of a longer tandemly repeated monomeric unit, that generates a specific higher-order repeat. BLAST analysis confirmed that the isolated DNA inserts did not reveal any homology with the known bovine satellites (Pluciennicza et al., 1982; Buckland, 1985; Jobse et al., 1995), and no information about satellite DNA in sub-metacentric chromosome X is available at present (Chaves et al., 2003). We believe that these sequences can be considered as a new class of specific satellite DNA located on the bovine X chromosome. The porcine and horse clones were also tested on bovine metaphases to establish whether the FISH signal is visible in species belonging to different families. However, no hybridization signal was detected, indicating that these clones could be species-specific.

All the positive porcine clones were subject to sequencing. The molecular data revealed a strong similarity level, allowing subdivision of the clones into 2 groups. The first one includes 6 clones (S35; S37; S50; S69; S74 and S76) with an average homology of 95.6%. In particular, taking the longest sequence (S69 clone) as reference, the comparison showed similarities of 96.4%; 96.3% and 94.9% for S35; S37 and S50, respectively. The last two clones of the first group showed homology of 94.0% and 96.4%, respectively, with complementary sequence of the reference clone. The second group includes 2 clones (S26 and S38), with a similarity of 99.6%. No homology was found for these clones either within these groups or between these groups and the remaining 3 clones. The analysis of our sequences showed that the clones are characterized by a variable AT content, from 42.5% (clone S45) to 55.5% (clone S38), with an average AT content of 50.4%. The results confirm that the repeated regions of porcine chromosome 14 are characterized by equilibrium in the base content, according to the data already shown Jantsch et al. (1990) on Ac2 porcine centromeric repeated family. On the contrary, the metacentric repeated regions are characterized by a lower AT content: 36.6%, 42.1% and 31.7% for chromosome 1 (Jantsch et al., 1990), chromosome 9 (Janzen et al., 1999) and chromosome 11 (Riquet et al., 1996), thus suggesting a different structure and organization of the centromeres in the metacentric and acrocentric chromosomes of this species.

BLAST analysis of all the sequences indicated that the isolated DNA inserts did not share any homology with the already known family (Mc1, Ac2 and Mc2) of porcine centromeric satellite DNA. At present, only unspecific repetitive sequences are known for all the porcine acrocentric chromosomes (Jantsch et al., 1990; Rogel-Gaillard et al., 1997a,b). On the basis of these observations, we suggest the existence of new classes of specific satellite DNA located on the centromere of porcine chromosome 14.

The same analysis was applied to the horse positive clones. Two probes showed a discrete fluorescent signal specifically hybridized on the centromeric region of chromosome 1, as shown in Figure 1d. The same probes were also hybridized on donkey (*Equus asinus*) and zebra (*Equus zebra*) metaphase

chromosomes. Although these two species are taxonomically close to the investigated one, no FISH signal was detected, thus indicating that these sequences represent a species-specific target for the centromeric region of equine chromosome 1. DNA sequencing performed on both positive probes revealed 98.3% of similarity. So far, Sakagami et al. (1994) found satellite sequences localized on centromeres of all chromosomes, except for chromosomes 2, 9 and 11. Broad et al. (1995) reported the existence of two DNA repetitive elements, known as Sau-like families, frequently present in tandem and unspecifically hybridized to acrocentric horse chromosomes. Recently, Gallagher et al. (2000) submitted unspecific centromeric sequence for many horse acrocentric, metacentric and submetacentric chromosomes (Accession number: AF324422), but at present no information is available on specific probes for this species. Here we report on the first class of specific satellite DNA localized on the centromere of equine submetacentric chromosome 1. BLAST analysis of the new sequences confirmed FISH hybridization data. No homologies were found to the known equine satellites.

Presence of several types of short repetitive elements was detected by Dot Plot analysis; however, specific for a particular species. All of the detected repeated elements are extremely short and none of the clones in our study is formed by complete or incomplete repeats of any longer basic sequence. Accordingly, we can suppose that none of the detected short repeated elements is essential for the structure of our probes.

At present, very little is known about the organization of centromeric DNA repeats in farm animals. The isolation and identification of new sequences suggest that these repeats are present in a relatively small proportion of the genome and each of these is specific for only one pair of chromosomes. According to Janzen et al. (1999), a great benefit can result from the construction of a catalog of species-specific centromeric probes and in that respect our findings extend knowledge on farm animal centromeres. Moreover, the specific hybridization of these probes on metaphases of different species could represent a powerful tool for Zoo-FISH analysis and could facilitate routine cytogenetic analysis in farm animals.

In conclusion, the characteristic repeated sequences found in the centromeric region of bovine chromosome X, porcine chromosome 14 and equine chromosome 1 might enrich the genetic map of these three species in a region that has not received adequate attention.

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