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Molecular characterization of a bovine Y-specific DNA sequence conserved in taurine and zebu breeds

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Abstract
The identification of new bovine male-specific DNA sequences is of great interest because the bovine Y chromosome remains poorly characterized in terms of physical and genetic maps. Since taurine and zebu Y chromosomes are structurally different, the identification of Y-specific sequences present in both sub-species is particularly important: these sequences are of evolutionary significance and can be broadly used for embryo sexing. In this work, we initially used the random amplified polymorphic DNA (RAPD) technique to search for male-specific sequences present as monomorphic markers in genomic DNA from zebu and taurine bulls. A male-specific RAPD band was found to be present and highly conserved in both sub-species, as demonstrated by Southern blotting, fluorescent *in situ* hybridization (FISH) and DNA sequencing. In a previous work, a pair of primers derived from this marker was successfully used in taurine and zebu embryo sexing.

Keywords: *RAPD, bovine Y-chromosome, male-specific marker, breeds*

Introduction

During several decades, the mammalian Y chromosome was considered a "genetic desert", the testis-determining factor being considered the only survivor of the sex chromosomes evolutionary process. Regardless of the mapping of spermatogenesis-associated regions on the human Y chromosome (Tiepolo and Zuffardi 1976), this vision persisted until the mid-1980s, when the development of genomic analysis techniques started.

Presently, more than 30 coding genes or gene families have been identified on the human Y chromosome. It was found that these sequences have an important role not only in sex determination, but also in the process of spermatogenesis, male fertility and growth control (Lahn and Page 1997). Recent work on the human Y chromosome complete sequencing has revealed more than 156 transcription units (Skaletsky et al. 2003). The number of such sequences mapped so far in the bovine Y chromosome is much smaller, few of them corresponding to coding genes (BOVMAP Database, Institut National de la Recherche Agronomique, France, available from http://locus.jouy.inra.fr/cgibin/bovmap/intro.pl). Therefore, the identification of new bovine male-specific DNA sequences remains of great interest since the Y chromosome exerts a pivotal role in sexual development and fertility.

A technique of enormous usage in the identification of sex-specific markers is random amplified polymorphic DNA (RAPD, Williams et al. 1990). RAPD sex-specific markers have already been identified in

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birds (Bello and Sanchez 1999) and in several species of mammals, including bovine (Xiong et al. 1995). Antoniou and Skidmore (1995) described a 3.1 Kb bovine male-specific marker detected by the random primer OPA-06 (Operon Technologies, Alameda, CA). According to these authors, the marker—designated OPA.06.3100—was present only in taurine bulls. In this work, we describe the detection of the OPA.06.3100 in zebu breeds and its molecular characterization.

Materials and methods

Animals

The search for a male-specific marker was conducted in DNA samples obtained from a group of 55 animals encompassing: 30 zebu bulls (5 Gir, 5 Guzerá, 15 Nelore and 5 Tabapuã), 15 taurine bulls (5 Holstein, 5 Jersey and 5 Simmental), and 5 Nelore females and 5 Holstein females.

DNA isolation

Male and female high molecular weight template DNA was obtained from peripheral blood, as described by Miller et al. (1988).

RAPD-PCR

The conditions for detecting the OPA-06 random primer (5′GGTCCCTGAC 3′, Operon Technologies, Alameda, CA, USA) amplification product were based on Williams et al. (1990) with minor modifications: reactions were performed in a 20 μl reaction mixture containing 20 mM of Tris–HCl, pH 8.4, 50 mM of KCl, 2.5 mM of MgC_{l2}, 200 μM of each dNTP, 0.4 μM of primer, 1.5 unit of Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA, cat. no. 11615-010) and 20 ng of genomic DNA.

Cloning and sequencing of PCR product

The PCR products amplified from DNA samples from a Nelore and a Holstein bull were purified from a 1.0% low melting point agarose gel using the CONCERTTM rapid gel extraction system (Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA, cat. no. 11456-019) and cloned using the SureClone™ Ligation Kit (Pharmacia Biotech, cat. no. 27-9300-01) according to the manufacturer's instructions.

Both strands of five selected clones (containing zebu and taurine fragment) were sequenced (ALFexpress DNA Sequencer, Pharmacia Biotech) using commercially available kits (ALFexpress AutoRead Sequencing Kit, Pharmacia Biotech 27-2690-02 and Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit with 7-deaza-dGTP, Amershan Life Science, Buckinghamshire, England, RPN 2438).

Southern blotting

Southern blotting experiments were performed according to Sambrook et al. (1989) in order to compare zebu and taurine hybridization patterns and to confirm the male and bovine-specific nature of the OPA-06 marker. Blotting was carried out after digesting male and female samples of bovine (zebu and taurine), murine and human genomic DNA with *Pst* I. The probe (purified male-specific insert) was labelled (horseradish peroxidase), hybridized and detected using the ECL™ (Direct Nucleic Acid Labelling and Detection Systems, Amersham Life Science, Buckinghamshire, England, RPN 3000) kit.

Fluorescence in situ *hybridization*

Fluorescence *in situ* hybridization (FISH) was used for determining the chromosomal location of the zebu male-specific OPA-06 derived sequence and its presence in the taurine Y chromosome. Metaphases were obtained from colchicine-treated cultured peripheral lymphocytes of a Holstein bull by means of standard cytogenetic techniques. The plasmid containing the insert–an OPA-06 male-specific fragment amplified from Nelore bull DNA—was labelled with biotin (BIONICKTM Labelling System - Invitrogen Life Technologies, Carlsbad, CA, USA, cat. no. 18247-015). Detection was performed as described by Leite-Silva et al. 2003.

Results

The OPA-06 random primer detected a male-specific marker in zebu (Nelore, Gir, Guzerá and Tabapuã breeds) as well as in taurine (Holstein, Jersey and Simmental breeds) bulls (Figure 1). This sex-linked marker was present in all males from both sub-species and absent in all taurine and zebu females here tested. Since the marker was amplified from zebu and taurine animals, PCR products from both origins (amplified from a Nelore and a Holstein bulls DNA samples) were sequenced in order to estimate their degree of conservation. It was found that both PCR products were 3216 bp long and their similarity was 98%. The Nelore derived sequence was submitted to GenBank (accession number AY374307) and thereafter designated OPA.06.3216.

The presence of the male-specific OPA.06.3126 marker in taurine and zebu was confirmed by Southern blot and FISH experiments. The OPA.06.3126 sequence used as a probe in Southern blot analysis has shown a strong and similar

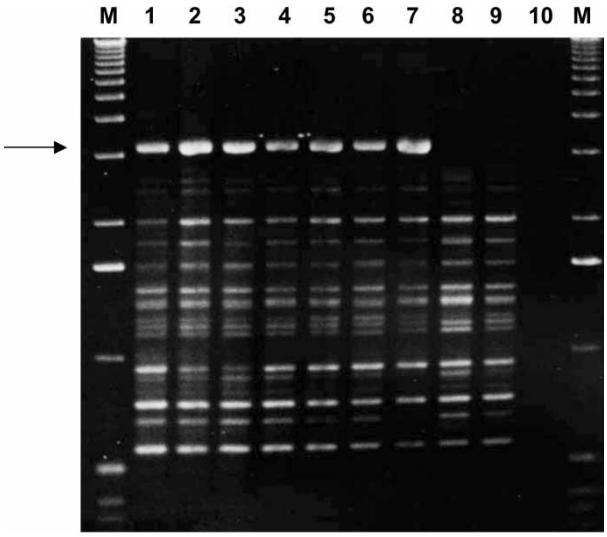

Figure 1. Sex-specific polymorphism revealed by the randomic primer OPA-06. The arrow shows the 3.216 bp male-specific band. Male DNA samples: Nelore (1); Gir (2); Guzera (3); Tabapuã (4); Holstein (5); Jersey (6); and Simmental (7). Female DNA samples: Nelore (8) and Holstein (9). Lane 10: negative control. Lane M: 1 Kb ladder (Gibco BRL).

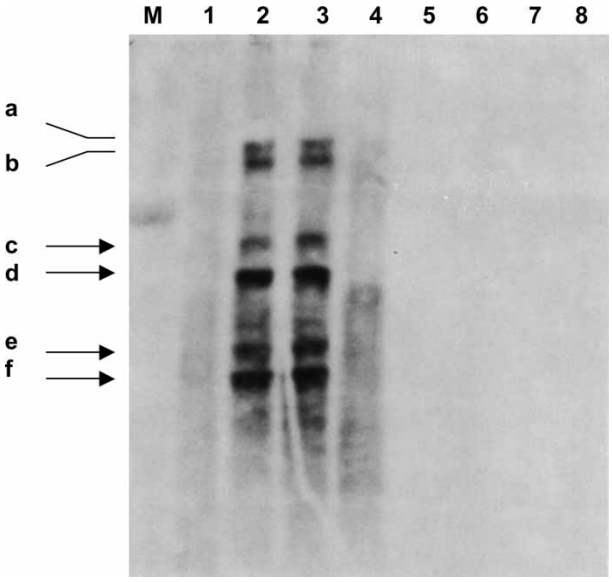

Figure 2. Southern blotting using OPA.06.3216 as a probe. The arrows show the 5700 bp (a); 4700 bp (b); 2900 bp (c); 2300 bp (d); 1200 bp (e); and 1100 bp (f) fragments present in zebu (lane 2) and taurine (lane 3) bulls. These fragments are absent in murine (lane 6) and in human (lane 7) males, as well as in zebu, taurine, murine and human females (lanes 1, 4, 5 and 8, respectively). M indicates the 1 Kb ladder (Gibco BRL).

hybridization to taurine and zebu *Pst* I digested male genomic DNA. The probing revealed the two products—1096 and 1202 bp—predicted by sequencing and restriction map of OPA.06.3216. In addition, four other fragments, of 2300, 2900, 4700 and 5700 bp (Figure 2) were revealed. No hybridization could be seen in bovine female genomic DNA, or in human and murine samples.

When used as a probe in FISH experiments, zebu OPA.06.3216 sequence generated a signal observed only on the long arm of the taurine Y chromosome (Figure 3).

A BLAST search of the OPA.06.3216 bovine sequence was performed using the non-redundant GenBank database. It was revealed that 60% of OPA.06.3216 (from bases 1050–3193 bp) have an extensive homology (86% sequence similarity) with the *Ovis aries* repeat region OY9 DNA sequence (accession no. U30306).

The OPA.06.3216 sequence showed no similarity with the *B. taurus* repeating sequence family BRY, which occurs throughout the Y chromosome (Matthews and Reed 1991; Schwerin et al. 1992), the bovine repeated sequence FBNY (Weikard et al. 2001) or even with the bovine repeated sequence S4, also localized throughout the long arm of the Y chromosome (Kageyama et al. 2004).

Discussion

In contrast to the results obtained by Antoniou and Skidmore (1995), we detected the presence of the OPA.06.3100 male-specific marker in both taurine and zebu breeds. Its extension—3216 bp-was precisely determined by DNA sequencing.

FISH experiments mapped the OPA.06.3216 sequence on the long arm of the Y chromosome of a taurine animal (Holstein breed). FISH analysis has also corroborated the Y-specific nature of this sequence, since no other marker was found either on the X chromosome or on any of the 29 autosomic

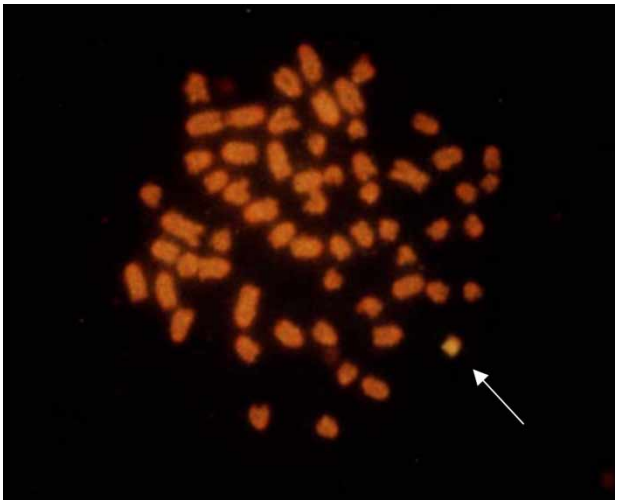

Figure 3. FlSH of the OPA.06.3216 male-specific fragment on taurine metaphasic chromosomes. The arrow indicates the hybridization on the Y chromosome long arm.

pairs. The male-specific nature of this marker was also confirmed by Southern blotting results, which showed hybridization only in males. It's interesting to see that Southern blotting results have also indicated that this sequence is bovine-specific, since no cross hybridization was observed with human or murine DNA.

The comparison of OPA.06.3216 sequence with others deposited in databanks has shown that approximately 60% of the bovine fragment has a high homology (similarity average of 85%) to a region of repetitive DNA in Y of *Ovis aries,* known as OY9. Species-specific repeats have been isolated from the Y chromosome of mouse (Nishioka and Lamothe 1986), human (Kunkel et al. 1976), pig (Mileham et al. 1988) and cattle (Popescu et al. 1998). Matthews and Reed (1991) describe a repetitive region BRY.1 associated exclusively to the Y chromosome of Bovinae and Caprinae. To the authors, these results suggest the presence of repetitive structures in the ancestor of the *Bovidae* Y chromosome. So far, BRY.1 was the only Y-associated repetitive sequence found to be shared exclusively by Bovinae and Caprinae.

The conservative evolution of these chromosome-specific repetitions shows that the Y chromosome of *Bovidae* was kept considerably stable. The conservation of a repetitive sequence could be an indication that such sequence is located in a region of the Y chromosome protected from rapid alterations, probably because this region harbors genetic information crucial for the Y chromosome biological functions. Subsequent speciation events would tend to conserve these ancestral families of repeated sequences.

Conserved sequences present in taurine and zebu Y chromosomes have evolutionary significance (Teale et al. 1995) and can be broadly used for bovine embryo sexing (Cotinot et al. 1991). Indeed, the authors successfully used the OPA.06.3216 marker for embryo sexing (Alves et al. 2003). Moreover, this marker is also highly similar to an ovine Y chromosome repetitive sequence, thus being the second *Bovidae* Y chromosome conserved sequence so far described (the first were the BRY sequences described by Matthews and Reed 1991).

In conclusion, our results show that the OPA.06.3216 male-specific marker is conserved in zebu and taurine breeds and that RAPD analysis is a reliable and powerful tool for identifying new sequences of interest for evolutionary and structural studies on the Y chromosome.

Acknowledgements

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References

Alves BCA, Hossepian de Lima VFM, Teixeira CM, Moreira-Filho CA. 2003. Use of primers derived from a new sequence of the bovine Y chromosome for sexing *Bos taurus* and *Bos indicus* embryos. Theriogenology 59:1415–1419.

Antoniou E, Skidmore CJ. 1995. A bovine Y-specific marker amplified by RAPD. Anim Genet 26:444–445.

Bello N, Sachez A. 1999. The identification of a sex-specific DNA marker in the ostrich using a random amplified polymorphic DNA (RAPD) assay. Mol Ecol 8:667–669.

Cotinot C, Kirszenbaum M, Leonard M, Gianquinto L, Vaiman M. 1991. Isolation of bovine Y-derived sequence: Potential use in embryo sexing. Genomics 10:646–653.

Kageyama S, Yoshida I, Kawakura K, Chikuni K. 2004. A novel repeated sequence located on the bovine Y chromosome: Its application to rapid and precise embryo sexing by PCR. J Vet Med Sci 66:509–514.

Kunkel LM, Smith KD, Boyer SH. 1976. Human Y-chromosome-specific reiterated DNA. Science 191:1189–1190.

Lahn BT, Page DC. 1997. Functional coherence of the human Y chromosome. Science 278:675–680.

Leite-Silva C, Santos N, Fagundes V, Yonenaga-Yassuda Y, de Souza MJ. 2003. Karyotypic characterization of the bat species *Molossus ates*, *M. molossus* and *Molossops planirostris* (Chiroptera, Molossidae) using FISH and banding techniques. Hereditas 138:94–100.

Matthews ME, Reed KC. 1991. A DNA sequence that is present in both sexes of Artiodactyla is repeated on the Y chromosome of cattle, sheep and goats. Cytogenet Cell Genet 56:40–44.

Mileham AJ, Siggens KW, Plastow GS. 1988. Isolation of a porcine male-specific DNA sequence. Nucleic Acids Res 16:11842.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Nishioka Y, Lamothe E. 1986. Isolation and characterization of a mouse Y chromosomal repetitive sequence. Genetics 113:417–432.

Popescu CP, Cotinot C, Boscher J, Kirszenbaum M. 1998. Chromosomal localization of a bovine male specific probe. Ann Génét 31:39–42.

Sambrook J, Maniatis T, Fritsch EF. 1989. Molecular cloning: A laboratory manual. 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schwerin M, Gallagher DS Jr, Miller JR, Thomsen PD. 1992. Mapping of repetitive bovine DNA sequences on cattle Y chromosomes. Cytogenet Cell Genet 61:189–194.

Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, et al. 2003. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 423:825–837.

Teale AJ, Wambugu J, Gwakisa PS, Stranzinger G, Bradley D, Kemp SJ. 1995. A polymorphism in randomly amplified DNA that differentiates the Y chromosome of *Bos indicus* and *Bos taurus*. Anim Genet 26:243–248.

Tiepolo L, Zuffardi O. 1976. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the Y chromosome long arm. Hum Genet 34:119–124.

Weikard R, Kuhn C, Brunner RM, Roschlau D, Pitra C, Laurent P, Schwerin M. 2001. Sex determination in cattle based on simultaneous amplification of a new male-specific DNA sequence and an autosomal locus using the same primers. Mol Reprod Dev 60:13–19.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbritary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535.

Xiong S, Park RL, Evans RP, Andersen RW, Fairbanks DJ. 1995. Bovine Y- and X- chromosome DNA markers detected by 10-mer primers and bulked samples. J Anim Sci 73:111.