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Molecular markers in the Portuguese Bísaro pig: screening for breed specific microsatellites

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SUMMARY - A large number of autochthonous breeds of domestic animals are endangered. The Portuguese indigenous pig breed Bísaro is reduced to a small number of animals mostly restricted to the Trás-os-Montes province. In order to prevent its extinction and preserve genetic variability fast measures are needed. Among molecular markers to assess the genetic variability microsatellites are the most widely used. No such markers have yet been described to be specific for this breed. This work aims to identify VNTRs (Variable Number of Tandem Repeats) among the populations of Bísaro and to sequence their conserved flanking regions to develop primers for PCR (Polymerase Chain Reaction) amplification of microsatellites in order to assess the genetic variability within and among populations. Such data can be used in future selection programs. A large number of recombinant clones is being screened by hybridization using $(TG)_{10}$ and $(TC)_{10}$ synthetic oligonucleotides as probes and $(TG)_n$ microsatellites were found to be more frequent in this breed.

Key words: Microsatellites, Bísaro, pig, biodiversity.

RESUME - *"Marqueurs moléculaires chez la race porcine portugaise Bísaro : criblage pour des microsatellites spécifiques de cette race". De nombreuses races d'animaux autochtones sont menacées. Le cochon indigène Bísaro est réduit à un petit nombre d'animaux localisés pour la plupart à Trás-os-Montes (province du nord-est portugais). Pour empêcher son extinction et préserver sa variabilité génétique des mesures urgentes sont nécessaires. Parmi les marqueurs moléculaires qui permettent d'estimer la variabilité génétique les micro-satellites sont les plus utilisés. Aucun micro-satellite spécifique pour cette race n'a encore été décrit. Le présent travail cherche à identifier des VNTRs parmi les populations de Bísaro et à séquencer leurs régions flanquantes conservées pour développer les amorces pour amplification par PCR de micro-satellites, dans le but d'estimer la variabilité génétique dans et entre populations. Ces résultats pourront être utilisés dans de futurs programmes de sélection. Un nombre significatif de clones recombinants a été criblé par hybridation en utilisant les oligonucléotides synthétiques* $(TG)_{10}$ *et* $(TC)_{10}$ *comme sonde, et les micro-satellites* $(TG)_n$ *se sont avérés les plus fréquents chez cette race.*

Mots-clés : *Micro-satellites, Bísaro, porcin, biodiversité.*

Introduction

For the pig breeds, Europe shares a large part both of the world population (30%) and of its genetic diversity (37%). This makes Europe an important region for all aspects of pig genetic diversity with respect with the Convention on Biological Diversity (CBD) and the need for such a study in Europe is urgent. Several minor autochthonous pig breeds are indeed presently at risk of extinction (FAO/UNEP, 1993). The Portuguese indigenous pig breed Bísaro is no exception and is reduced to an extremely low number of animals mostly restricted to the Northern Portugal (200 in 1994 860 in 1998 and registered in the Herd Book according to data from the breeders association- Associação Nacional de Criadores de Suínos de Raça Bísaro). In order to facilitate and rationalize the maintenance of genetic diversity it is essential to quantify the biodiversity within Bísaro. Specific genetic markers can be used as a tool for this purpose. Until recently, many studies of genetic structure have used allele frequency data at protein coding (primarily allozyme) *loci* (e.g., Ward *et al.*, 1992). Since the late 1970s, molecular methods have provided new markers for the study of genetic variation, even to the level of analysis at the DNA sequence itself (Hillis and Moritz, 1990; Avise, 1994).

Microsatellites are highly polymorfic DNA markers with discrete *loci* and codominant alleles. They are short tandemly repeated sequence motifs consisting of repeat units of 1-6 bp in length (Tautz, 1994 cited in Schlötterer, 1998). These molecular markers have been found to be

common in all eukaryotic genomes (Miesfeld *et al.*, 1981; Hamada *et al.,* 1982; Epplen, 1988; Litt and Luty, 1989; Weber and May, 1989; Stallings *et al.*, 1991; Beckmann and Weber, 1992; Robic *et al.*, 1995) including the pig genome (Winterø *et al.*, 1992).

They provide extremely useful markers for comparative studies of genetic variation, evolutionary studies (Takezakin and Neim, 1996; Crawford *et al.,* 1998; Paszek *et al.*, 1998), parentage assessment and studies of gene flow and hybridization (Bruford and Wayne, 1993; Roy *et al.*, 1994), and could well be the markers of choice for analyses of population structure in both wild (Scribner *et al.*, 1994) and domesticated species (MacHugh *et al.*, 1994; van Zeveren *et al.*, 1995).

Because microsatellites are highly polymorphic they can be easily and reproducibly analysed by PCR and gel electrophoresis (Ma *et al.*, 1996). Primers flanking the repeats are used for PCR amplification (Tautz, 1989, Rassamann *et al.*, 1991 cited in Schlötterer, 1998). Sizing of the PCR products on high resolution gels allows the determination of the number of repeats in the different alleles. Before a specific microsatellite *locus* can be amplified one needs sequence information for the flanking DNA to allow the design of specific primers (Schlötterer, 1998). No such markers have yet been described to be specific for this breed. It is our goal to search for conserved sequences flanking VNTRs regions and use them to design primers that can be used for PCR amplification and analysis of the biodiversity of Bísaro.

Materials and methods

Pig genomic DNA was isolated from blood samples collected from unrelated animals of Bísaro breed by a standard saline method Montgomery and Sise, 1990. The DNA from five animals was digested overnight (37°C) with *Hae* III (10 U/µl New England Biolabs). Fragments ranging from 150-600 bp were eluted (QIAquick Gel Extraction, Qiagen) from 1% agarose gels (NA Pharmacia), ligated into pUC 18 *Sma* I/BAP- 0.1 µg/µl (Pharmacia Biotech) and used to transform JM 109 *E. coli* competent cells. Recombinant clones were selected on Luria-Bertani LB (Duchefa) plates supplemented with 50 mg/ml ampicillin (Promega), 40 mg/ml X-gal (Sigma) and 100 mM IPTG (Sigma). Over 2500 clones were transferred on to Hybond – N^+ membranes (Amersham) and screened with the synthetic oligonucleotide $(TG)_{10}$ (0.05 µmol-operon technologie) $(TC)_{10}$ (0.05 µmol-operon technologie) end-labelled with (γ ^{32}P)-ATP (500 µCi Amersham). The membranes were exposed to X-ray films (Kodak X-OMA) during 48 h. Positive recombinant clones were selected, grown (18h) on LB liquid cultures (Duchefa) and plasmid DNA was extracted (QIAprep Spin Miniprep, Qiagen) and sequenced by using T7 Sequenase (Amersham Life Science version 2.0).

Results and discussion

DNA digested with *Hae* III produced a strong smear (Fig. 1). Fragments ranged from small sizes and up to above 23 kb. 5 µg de DNA were digested with 2 U of enzyme in a volume of 42 µl. Reactions were divided in 7 µl aliquots and loaded on different lanes of an 1% agarose gel.

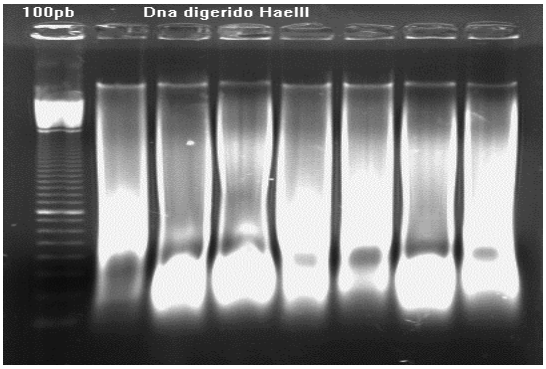

Fig. 1. DNA digestion with *Hae* III. Lane 1, 100 bp ladder; Lanes 2-8, DNA.

Fragments sized 150-600 bp were cut off and purified from a 1% agarose gel, (Fig. 2) and ligated into phosphorilated *Sma* I digested pUC18.

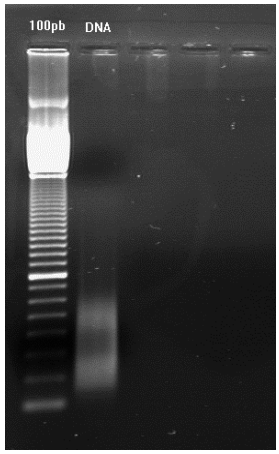

Fig. 2. Fragments purified. Lane 1, 100 bp ladder; Lane 2, fragments ranging from 150-600 bp.

From the fragments library, twenty two positive clones were selected with the $(TG)_n$ probe (Fig. 3) and eight clones were selected with the $(TC)_n$ (Fig. 4) out of over 250 clones analysed. A higher number of positive clones were found using $(TG)_n$ rather than $(TC)_n$ as a probe. Also the signal was stronger in the first case. Therefore, it can be assumed that $(TG)_n$ VNTRs are bound to be more frequent than $(TC)_n$ in this breed, which is the case for most mammalian (Ellegren *et al.*, 1992).

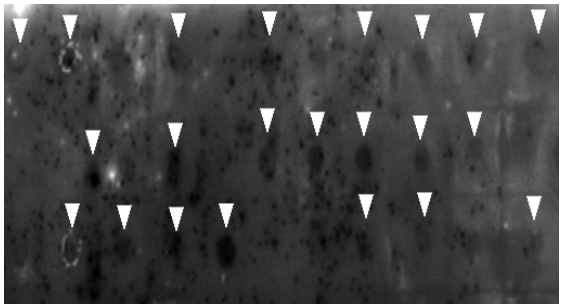

Fig. 3. Autoradiograph-recombinant positive clones using $(TG)_{10}$ probe. Stringency hybridizations (Tm - 15°C).

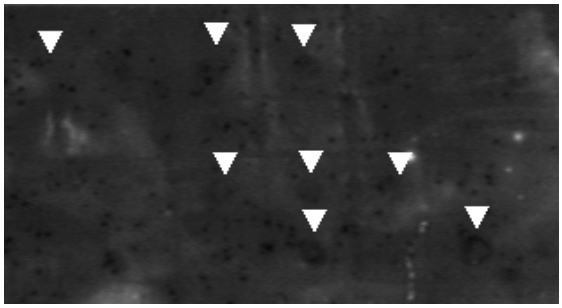

Fig. 4. Autoradiograph-recombinant positive clones-$(TC)_{10}$ probe. Stringency hybridizations (Tm - 15°C).

The thirty positives clones are presently being sequenced by the dideoxy chain termination method and automated sequencing. Consensus sequences flanking VNTRs regions are expected to be achieved in the near future. Such data can be used on the characterization of genetic variation in the breed Bísaro.

References

Avise, J.C.(1994). *Molecular Markers, Natural History and Evolution.* Chapman & Hall, London, pp. 511.

Beckmann, J.S. and Weber, J.L. (1992). Survey of human and rat microsatellites. *Genomics*, 12: 627-631.

Bruford, M.W. and Wayne, R.K. (1993). Microsatellites and their application to population genetic studies. *Curr. Opin. Genet. Develop.,* 3: 939-943.

Crawford, A.M., Kappes, S.M., Paterson, K.A. and deGotari, M.J. (1998). Microsatellite evolution: testing the ascertainment bias hypothesis. *J. Mol. Evol*., 46(2): 256-260.

Ellegren, H., Johansson, M., Sandberg, K. and Andersson, L. (1992). Cloning of highly polymorphic microsatellites in the horse. *Anim. Genet.*, 23: 133-142.

Epplen, J.T. (1988). On simple repeated sequences in animal genomes: a critical reappraisal. *J. Hered.,* 79: 409-417.

FAO/UNEP (1993). *World Watch List for Domestic Animal Diversity*, 1st Edition. Loftus, R. and Scherf, B. (eds). FAO, Rome, Italy, p. 376.

Hamada, H., Petrino, M. and Kakunaga, T. (1982). A novel repeated element with Z-DNA forming potential is widely found in evolutionary diverse eukaryotic genomes. In: *Proceedings of the National Academy of Sciences of the USA*, 79: 6456-6459.

Hillis, D.M. and Moritz, C. (eds) (1990). *Molecular Systematics*. Sinauer, Sunderland, MA.

Litt, M. and Luty, J.A. (1989). A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.*, 44: 397-401.

Ma, R.Z., Russ, I., Park, C., Heyen, D.W., Beever, J.E., Green, C.A. and Lewin, H.A. (1996). Isolation and characterization of 45 polymorphic microsatellites from the bovine genome. *Anim. Genet.*, 27: 43-47.

MacHugh, D.E., Loftus, R.T., Bradley, D.G., Sharp, P.M. and Cunningham, P. (1994). Microsateliltes DNA variation within and among European cattle breeds. In: *Proceedings of the Royal Society,* London B, 256: 25-31.

Miesfeld, R., Krystal, M. and Arnheim, N. (1981). A member of a new repeated sequence family which is conserved throughout eukaryotic evolution is found between the human delta and beta globulin genes. *Nucleic Acids Res.,* 9: 5931-5947.

Montgomery, G.W. and Sise, J.A. (1990). Extraction of DNA from sheep white blood cells. *N. Z. J. Agr. Res.,* 33(3): 437-441.

Paszek, A.A., Flickinger, G.H. and Fontanesi, L. *(*1998). Evaluating evolutionary divergence with microsatellites. *J. Mol. Evol*., 46(1): 121-126.

Robic, A., Parrou, J.L. and Yerle, M. (1995). Pig Microsatellites isolated from cosmids revealing polymorphism and localized on chromosomes. *Anim. Genet.,* 26(1): 1-6.

Roy, M.S., Geffen, E., Smith, D., Ostrander, E.A. and Wayne, R.K. (1994). Patterns of differentiation and hybridization in North American wollike canids, revealed by analysis of microsatellites loci. *Mol. Biol. Evol.,* 11: 553-570.

Schlötterer, C. (1998*). Microsatellites. Molecular Genetic Analysis of Populations. A Practical Approach.* Second Edition. Hoelzel, A.R. (ed.). pp. 238-261.

Scribner, K.T., Arntzen, J.W. and Burke, T. (1994). Comparative analysis of intra and interpopulation genetic diversity in *Bufo bufo*, using allozymes, single-locus microsatellite, minisatellite, and multilocus minisatellite data. *Mol. Biol. Evol.*, 11: 737-748.

Stallings, R., Ford, A., Nelson, D., Torney, D., Hilebrand, C. and Moyzis, R. (1991). Evolution and distribution of $(GT)_n$ repetitive sequences in mammalian genomes. *Genomics,* 10: 807-815.

Takezaki, N. and Neim, M. (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*, 144(1): 389-399.

van Zeveren, A., Peelman, L., van de Weghe, A. and Bouquet, Y. (1995). A genetic study of four Belgian pig populations by means of seven microsatellites loci. *J. Anim. Breed. Genet.*, 112: 191-204.

Ward, R.D., Skibinski, D.O.F. and Woodwark, M. (1992). Protein heterozygosity, protein structure, and taxonomic differentiation. *Evol. Biol.*, 26: 73-159.

Weber, J.L. and May, P.E. (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.*, 44: 388-396.

Winterø, A.K., Fredholm, M. and Thomsen, P.D. (1992). Variable $(dG\text{-}dT)_n$ - $(dC\text{-}dA)_n$ sequences in the porcine genome. *Genomics*, 12: 281-288.