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Single strand conformation polymorphism (SSCP) detection in six genes in Portuguese indigenous sheep breed "Churra da Terra Quente"

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Evaluation of the genetic diversity for six genes in forty animals of the Portuguese indigenous sheep breed (*Ovis aries*) "Churra da Terra Quente" was done. A non-radioactive method to allow single-strand conformation polymorphism (SSCP) detection was optimised, starting from genomic DNAand PCR amplification of seven fragments: exon 1 of the α-lactalbumin gene; exons 10 and 11 of the α_{s1}-casein gene; exon 7 of the β-casein gene; exon 4 of the κ-casein gene; exons 4 and 5 of the growth hormone gene and exon 6 of the growth hormone receptor gene. Polymorphisms were detected in five of the seven PCR products. Only κ-casein and growth hormone receptor were monomorphic. α-lactalbumin and α_{s1}-casein exons showed three conformational patterns, β-casein and growth hormone exon 4 showed two electrophoretic patterns and growth hormone exon 5 showed five conformational patterns. These data provide evidence that "Churra da Terra Quente" has a high genetic variability, which opens interesting prospects for future selection programs and also for preservation strategies. Also, our data show that PCR-SSCP is an appropriate tool for evaluating genetic variability.
Keywords. Single Strand Conformation Polymorphism – SSCP, genetic variability, growth hormone, lactoproteins, sheep, landraces, Portugal.

Detection de polymorphismes de conformation monocaténaires (SSCP – *Single Strand Conformational Polymorphism*) dans six gènes de la race indigène ovine portugaise "Churra da Terra Quente". L'objectif de ce travail a été l'étude de la variabilité génétique de six gènes dans 40 animaux de la race ovine (*Ovis aries*) indigène portugaise "Churra da Terra Quente". Une méthode non-radioactive a été optimisée pour la détection de polymorphismes de conformation monocaténaires (SSCP – *Single Strand Conformational Polymorphism*). À partir d'ADN génomique, il a été amplifié par PCR six fragments derivant de l'exon 1 du gène de l'α-lactoalbumine; des exons 10 et 11 du gène de l'α_{s1}-caseíne ; de l'exon 7 du gène de la β-caséine ; de l'exon 4 du gène de la κ-caséine ; des exons 4 et 5 du gène de l'hormone de croissance et l'exon 6 du gène du récepteur de l'hormone de croissance. On a observé des polymorphismes pour cinq des produits PCR. Les fragments de la κ-caséine et du récepteur de l'hormone de croissance se sont montrés monomorphiques. Pour l'α-lactoalbumine et l'α_{s1}-caséine on a observé trois profils de conformation, pour la β-caséine et l'exon 4 de l'hormone de croissance deux profils et pour l'exon 5 de l'hormone de croissance cinq profils. Nos résultats montrent que la race ovine "Churra da Terra Quente" a une variabilité génétique élevée, ce qui permet d'envisager des applications intéressantes dans une programme de sélection et aussi la définition d'une stratégie pour la préservation de la diversité génétique. Ces résultats montrent que PCR-SSCP est un outil intéressant pour l'évaluation de la variabilité génétique.
Mots-clés. Polymorphismes de Conformation Monocaténaires-SSCP, variabilité génétique, hormone de croissance, lactoprotéine, ovin, race indigène, Portugal.

1. INTRODUCTION

Conservation of animal genetic resources is a topic of discussion since the 1950s (Simon, 1984). Indeed, biological, economical, cultural and emotional reasons are some of the arguments that support this initiative. It is essential to avoid the loss of genetic variability since these resources may be valuable for future breeding requirements (Hodges, 1984).

Genetic variability in indigenous breeds is a major concern considering the necessity of preserving what may be a precious and irreplaceable richness, regarding new productive demands. Conservation should be based on a deep knowledge of the genetic resources of the specific breed. It is therefore important to make efforts in order to characterize genetically indigenous breeds.

"Churra da Terra Quente" is an interesting Portuguese indigenous sheep breed (*Ovis aries*) not only because of its economic importance to the Northeastern region of Portugal, but also because of its variability. This breed is the result of crosses between two Portuguese breeds ("Badana" and "Mondegueira") that occurred in the 19th century. Presently, there are more than 200,000 animals, used for milk, meat and wool production. There is a remarkable variability in the daily milk production that ranges from 0.25 to 1.5 litres. This variation is not only associated with management differences but also to possible genetic variability (Azevedo *et al.*, 1994). The study of genetic polymorphisms of milk proteins and hormones associated with its production is a major goal that will allow not only the genetic characterization of this breed but also the identification of correlations between genotypic variants and productive parameters.

It is well established that growth hormone (GH) plays an essential role in the lactation process (Peel, Bauman, 1987). There are many studies correlating milk traits and polymorphisms at GH gene (Lucy *et al.*, 1993; Hoj *et al.*, 1993). There is also extensive literature on the possible relationship between genetic polymorphisms of milk proteins and milk production and composition (Ng-Kwai-Hang, 1997), showing the great interest of using GH and milk protein genes as molecular markers for molecular assisted selection to increase milk production and improve milk composition.

Progress in molecular biology brought a lot of information about DNA sequences. Using different techniques of DNA analysis in combination with published existent data, it is possible to identify variability within and between populations. Some of the methodologies more frequently used for the identification of point mutations are DGGE (Denaturing Gradient Gel Electrophoresis) (Fischer, Lerman, 1980), TGGE (Temperature Gradient Gel Electrophoresis) (Riesner *et al.*, 1989), Ribonuclease (Myers *et al.*, 1985) and Chemical (Cotton *et al.*, 1988) Cleavage and SSCP (Single Strand Conformation Polymorphism) (Orita *et al.*, 1989).

SSCP is a simple and reliable technique, based on the assumption that changes in the nucleotide sequence of a PCR product affect its single strand conformation. Molecules differing by as little as a single base substitution should have different conformers under non-denaturing conditions and migrate differently. Therefore, those differences can be detected as a shift in the electrophoretic mobility (Hayashi, 1991). Early reports used radioactivity, what limited its widespread use. To simplify and make SSCPanalysis more efficient, alternative staining methods with ethidium bromide (Yap, McGee, 1992) and with silver (Ainsworth *et al.*, 1991) were described.

The aim of this study is the evaluation of genetic variability of milk protein, growth hormone and growth hormone receptor genes using a non-radioactive SSCP protocol. This intends to be a first step for a deeper study of "Churra da Terra Quente" breed in order to establish a breeding program based on marker-assisted selection.

2. MATERIALAND METHODS

2.1. DNA extraction

Forty "Churra da Terra Quente" animals (7 males and 33 females) from a flock of the "Direcção Regional da Agricultura de Trás-os-Montes" were analysed. Blood samples (10 ml) were obtained by jugular venipuncture, using vacuum tubes treated with 0.25% ethylene-diaminetetracetic acid (EDTA). DNA extraction was performed within 24 h according to Sneyers *et al.* (1994) with minor modifications. After measuring DNA concentration and its purity by spectrophotometry, DNA was diluted to a final concentration of 50 ng/µl in water and stored at 4 ºC.

2.2. DNA amplification by PCR

Twenty five µl of polymerase chain reaction (PCR) mixture were carried out in 0.5 ml PCR tubes, using a PCR kit (Pharmacia 27-9555-01) with the lyophilized components. Each tube contained 1.5 units of Taq DNA polymerase, 10 mM of Tris-HCl (pH 9), 50 mM of KCl, 1.5 mM of $MgCl_2$ and 200 mM of each dNTP. To this mixture, 1 µl of each primer (50 ng/µl), 22 µl of water, 1 µl of DNA(50 ng/µl) and two drops of mineral oil were added. The primers for the specific amplification of the seven fragments (**Table 1**) were the ones described by Barracosa (1996). For exon 1 of α-La, 150 ng of each primer and 3.5 mM of $MgCl_2$ were used and for exon 6 of growth hormone receptor gene, 100 ng of each primer and 3 mM of $MgCl_2$ were used.

Table 1. PCR targets with sequences of primers required for the amplification and sizes of the resulting products — *Cibles PCR avec les séquences des amorces requises pour l'amplification et les tailles des produits obtenus*.

Gene	Size of PCR prod. (bp)	Primersequence	N°
α-La (E*1)	166	5'-CTC TTC CTG GAT GTA AGG CTT-3'	1
		5'-AGC CTG GGTGGC ATG GAA TA-3'	2
α_{s1}-Cn (E 10 and 11)	314	5'-TGATGT GTC TGG TTAATTAGC-3'	3
		5'-CAC AAC ATT CTT GCTCAT TCC-3'	4
β-Cn (E 7)	510	5'-CTT CTTTCC AGG ATG AAC TCC-3'	5
		5'-GAC TTA CAAGAA TAG GGAAGG-3'	6
κ-Cn (E 4)	416	5'-GAG AAAGAT GAAAGATTC TTC G-3'	7
		5'-GCTTCT GGATTA TCTACAGTG-3'	8
GH (E 4)	214	5'-CCACCAACC ACC CAT CTG CC-3'	9
		5'-GAAGGG ACC CAAGAACGC C-3'	10
GH (E 5)	365	5'-GAAACC TCC TTC CTC GCC C-3'	11
		5'-CCAGGG TCTAGG AAG GCACA-3'	12
GHR (E 6)	155	5'-TTG GCC TCAACTGGACTC TAC T-3'	13
		5'-CCACTG GGT CTC ATT TAG TT-3'	14

* E = Exon

Amplification was carried out in a Perkin Elmer DNA thermocycler. Following a hot start (95 ºC for 5 min), 30 cycles were carried out (95 ºC for 30 seconds, 62 ºC for 30 seconds, 72 ºC for 30 seconds), ending with a 5 min final extension at 72 ºC. For growth hormone receptor gene a different annealing temperature (56 ºC) was used. Amplification was verified by electrophoresis on 2% (w/v) agarose gel in 1x TBE buffer (2 mM of EDTA, 90 mM of Tris-Borate, pH 8.3), using a 100 bp ladder (Pharmacia 27.4001-01) as a molecular weight marker for confirmation of the length of the PCR products. Gels were stained with ethidium bromide (1 μg/ml).

2.3. Single-strand conformation polymorphism analysis

PCR products were resolved by SSCP analysis. Several factors were tested for each fragment in order to optimize the methodology: amount of PCR product (4 μl to 10 μl), dilution in denaturing solution (30% to 66%), denaturing solution (A: 95% of formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue; B: same as A, plus 20 mM of EDTA), acrylamide concentration (5% to 20%), percentage of crosslinking (1.5% C and 2.5% C), presence (10%) or absence of glycerol, voltage (60 to 400 V), running time (4 to 17 h) and running temperature (4, 10, 15 and 20 ºC). Each PCR reaction was diluted in denaturing solution, denatured at 95 ºC for 5 min, chilled on ice and resolved on polyacrylamide gel. The electrophoresis was carried in a vertical unit (Hoefer Scientific SE600, 160×140×0.75 mm), in 1x TBE buffer. The gels were stained with ethidium bromide (1 μg/ml) or silver (Pharmacia, 17-6000-30).

3. RESULTS

3.1. DNA amplification by PCR

Figure 1 shows a typical result of a PCR amplification of the seven analysed fragments.

Primers 1 and 2 (**Table 1**), used for α-La (**Figure 1-A**) were designed to amplify the region between nucleotides 689 and 854 in goat (Genebank M63868) (Vilotte *et al.*, 1991) and nucleotides 657 and 822 in cattle (Genebank X06366), corresponding to the begining of exon 1. The sequence of this gene has not been published for sheep, only its mRNA sequence is known (Gaye *et al.*, 1987). Nevertheless, due to the high homology between sheep, goat and cattle, it was expected that the primers would amplify the same region. Indeed, we obtained a fragment with the expected length (around 166 bp).

Primers 3 and 4 (**Table 1**) designed for the amplification of a fragment of 314 bp (from nucleotide 11244 to 11557) that contains exons 10 and 11 and the respective intron of the α_{s1}-Cn gene in cattle (Genebank X59856) (Koczan *et al.*, 1991), allowed the amplification of a fragment of similar length in sheep (**Figure 1-B**).

Based on the sequence of the β-Cn gene in sheep (Provot *et al.*, 1995), primers 5 and 6 (**Table 1**)

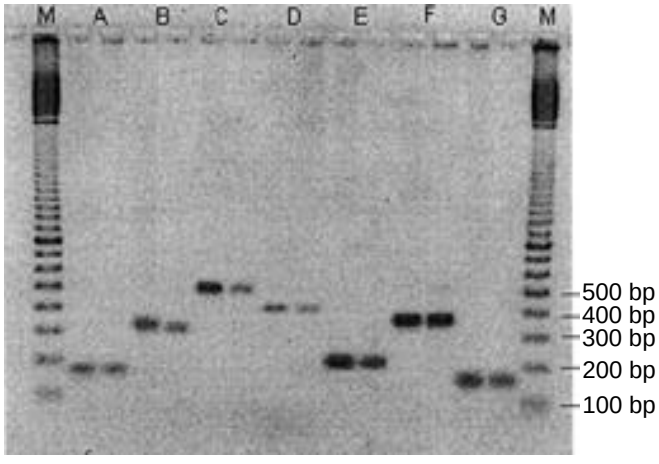

Figure 1. PCR products analysed by electrophoresis in a 2% agarose gel with ethidium bromide staining — *Produits de PCR analysés par électrophorèse sur un gel d'agarose 2 %, révélé avec du bromure d'ethidium*. A: α-La (exon 1); B: α_{s1}-Cn (exons 10 and 11); C: β-Cn (exon7); D: κ-Cn (exon 4); E: GH (exon 4); F: GH (exon 5); G: GH receptor; M: Molecular weight marker — *Marqueur de poids moléculaire* (100 to 100 bp) (Pharmacia, 27-4001-01).

allowed the amplification of a fragment of 510 bp (**Figure 1-C**) from nucleotide 11598 to 12107 (Genebank X79703) that corresponds to exon 7 of this gene.

Primers 7 and 8 (**Table 1**) were designed for the amplification of a fragment of 416 bp, from nucleotide 7 to 422 in goat (exon 4 of the κ-Cn gene, Genebank D14373) and 410 bp, for the same exon, from nucleotide 4936 to 5343 in cattle (Genebank X14908, Alexander *et al.*, 1988). We obtained a 416 bp PCR product that should correspond to the same exon in this species (**Figure 1-D**).

Based on the sequence of the GH gene in sheep (Orian *et al.*, 1988), the two sets of primers allowed the amplification of a fragment of 214 bp, from nucleotide 1288 to nucleotide 1501, corresponding to exon 4 of the GH gene (**Figure 1-E**, primers 9 and 10), and a fragment of 365 bp, from nucleotide 1634 to nucleotide 1998, corresponding to exon 5 of the same gene (**Figure 1-F**, primers 11 and 12) (Genebank X12546).

The GH receptor gene has not been entirely sequenced. Based on the work of Adams *et al.* (1990), we used primers 13 and 14 (**Table 1**) for the amplification of a 155 bp fragment (**Figure 1-G**) corresponding to exon 6.

3.2. Single strand conformation polymorphism analysis

After optimization of the parameters that affect the detection of SSCPs, we analysed the PCR products from 40 animals, with the conditions described in **table 2**. Electrophoresis were carried out at 400 V and 15 ºC in gels without glycerol, with the exception of exon 4 of the GH gene. In this case, electrophoresis were carried out at 300 V and 4 ºC and the gel contained 5% of glycerol.

Figure 2 shows the result of SSCPanalysis of exon 1 of α-La gene. We obtained three different conformational patterns. The frequencies were 57.5% for pattern 1, 22.5% for pattern 2 and 20% for pattern 3.

SSCP analysis of the 314 bp fragment corresponding to exons 10 and 11 of α_{s1}-Cn gene did not allow a good separation of the single strand bands. Although the separation of the bands was not perfect, three different patterns were detected (data not shown). The frequencies were 37.5% for pattern 1, 50% for pattern 2 and 12.5% for pattern 3.

Figure 3 shows the SSCP analysis of the 510 bp fragment corresponding to exon 7 of the β-Cn gene. Two conformational patterns were detected for this fragment. The frequencies were 37.5% for pattern 1 and 52.5% for pattern 2. In four animals (10%) the amplification was not possible.

SSCP analysis of the 416 bp fragment corresponding to exon 4 of the κ-Cn gene showed no polymorphisms.

Figure 4 shows the SSCP analysis for the 214 bp fragment of exon 4 of the GH gene. The addition of 10% of glycerol to the gel was favourable in this case, as it allowed a better separation of the single strands. Two conformational patterns were detected. The frequencies were 72.5% for pattern 1 and 22.5% for pattern 2. In 2 samples (5%) there was no amplification.

Figure 5 shows the SSCP analysis for the 365 bp fragment of exon 5 of the GH gene. This fragment showed a high level of polymorphism allowing the

Table 2. Conditions of electrophoresis in SSCP analysis — *Conditions de l'électrophorèse de l'analyse SSCP*.

Gene	Acrylamide	DNA	Denaturing solution	Duration
α-La (E 1*)	14%	10 µl	15 µl	7 h
α_{s1}-Cn (E 10 and 11)	12,5%	5 µl	15 µl	11 h
β-Cn (E 7)	15%	5 µl	15 µl	16 h
κ-Cn (E 4)	15%	5 µl	15 µl	17 h
GHR (E 6)	12,5%	6 µl	14 µl	4 h
GH (E 4)	15%	5 µl	15 µl	15 h
GH (E 5)	11%	5 µl	15 µl	11 h

* = Exon

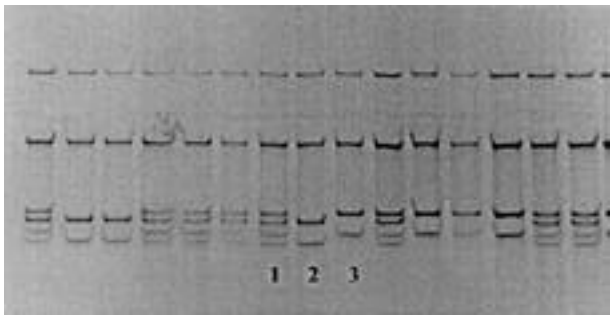

Figure 2. SSCP analysis of the 166bp fragment of α-La gene. Electrophoresis was performed in a 14% acrylamide gel, without glycerol, at 400 V and 15 ºC for 7 h, using 10 µl of DNA and 15 µl of denaturing solution. The frequencies of the three conformational patterns detected were 57.5% for pattern 1, 22.5% for pattern 2 and 20% for pattern 3— *Analyse SSCP du fragment de 166 pb du gene de α-La. L'électrophorèse a été réalisée sur un gel de polyacrylamide à 14 %, sans glycérol, à 400 V et à 15 ºC pendant 7 h, utilisant 10 µl de DNA et 15 µl de solution dénaturante. Les fréquences des trois profils conformationnels détectés on été 57,5 % pour le profil 1, 22,5 % pour le profil 2 et 20 % pour le profil 3*.

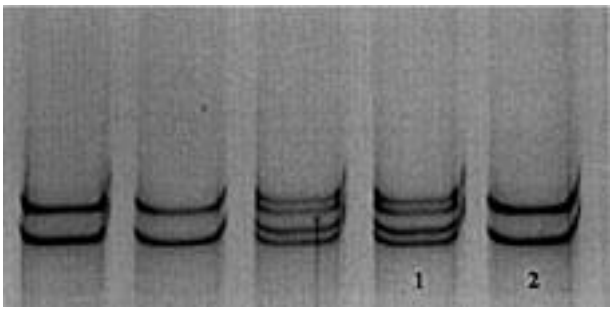

Figure 3. SSCP analysis of the 510bp fragment of β-Cn gene. Five μl of amplified DNA and 15 μl of denaturing solution were run in a 15% acrylamide gel without glycerol. Electrophoresis was performed at 400 V and 15 ºC for 16 h. The frequencies of the two conformational patterns detected were 37.5% for pattern 1 and 52.5% for pattern 2 — *Analyse SSCP du fragment de 510 pb du gene de β-Cn. Cinq μl d'ADN amplifié et 15 μl d'une solution dénaturante ont été placés sur un gel de polyacrylamide à 15 % sans glycérol. L'électrophorèse a été réalisée à 400 V, à 15 ºC pendant 16 h. Les fréquences des deux profils conformationnels détectés ont été de 37,5 % pour le profil 1 et de 52,5 % pour le profil 2.*

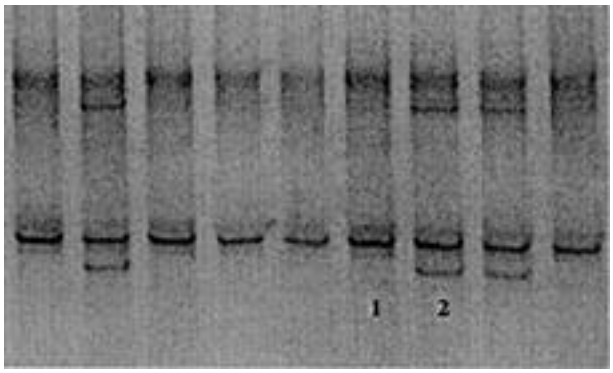

Figure 4. SSCP analysis of the 214 bp fragment of GH gene. Electrophoresis was performed in a 15% acrylamide gel with 10% of glycerol, at 300 Vand 4 ºC, for 15 h. Two conformational patterns were detected. The frequencies were 72.5% for pattern 1 and 22.5% for pattern 2 — *Analyse SSCP du fragment de 214 pb du gène de l'hormone de croissance. L'électrophorèse a été réalisée sur un gel de polyacrylamide à 15 %, avec 10% de glycérol, à 300 Vet à 4ºC, pendant 15 h. Deux profils conformationnels ont été détectés. Les fréquences ont été de 72,5 % pour le profil 1 et 22,5 % pour le profil 2.*

detection of five different conformational patterns. The frequencies were 47.5% for pattern 1, 5% for pattern 2, 22.5% for pattern 3, 12.5% for pattern 4 and 5% for pattern 5. In 7.5% of the samples there was no amplification.

For the 155 bp fragment of exon 6 of the GH receptor gene, the best results were obtained under the conditions described in **table 2**. Separation of single strands was clear, and in this case, animals were monomorphic (data not shown).

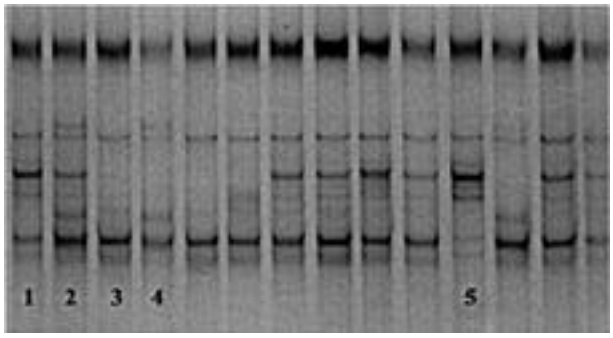

Figure 5. SSCP analysis of the 365 bp fragment of GH gene. The electrophoresis was performed in a 11% acrylamide gel without glycerol at 400 V and 15 ºC, for 11 h, using 5 μl of DNA and 15 μl of denaturing solution. The frequencies of the five conformational patterns detected were 47.5% for pattern 1, 5% for pattern 2, 22.5% for pattern 3, 12.5% for pattern 4 and 5% for pattern 5 — *Analyse SSCP du fragment de 365 pb du gene de GH. L'électrophorèse a été réalisée sur un gel de polyacrylamide à 11 %, sans glycérol, à 400 V et à 15 ºC, pendant 11 h, utilisant 5 μl d'ADN et 15 μl de solution dénaturante. Les fréquences des cinq profils conformationnels détectés on été de 47,5 % pour le profil 1, 5 % pour le profil 2, 22,5 % pour le profil 3, 12,5 % pour le profil 4 et 5 % pour le profil 5.*

4. DISCUSSION

It is currently accepted that genetic variability in sheep landraces is high. The maintenance of a great number of local sheep breeds with diversified production conditions offers resistance to the tendency towards the reduction of genetic variability which affects in larger scale other species (Flamant, 1991). Nevertheless, it is necessary to evaluate the variability within each breed.

Azevedo *et al.* (1994) proposed that the high variability in the daily milk production (from 0.25 to 1.5 l) in "Churra da Terra Quente" could be associated with genetic variability. Using PCR-SSCPanalysis, our data shows that there is a high level of polymorphism in "Churra da Terra Quente" breed, for specific genes.

For α-La exon 1, we detected three conformational patterns. Barracosa (1996) analysed the same exon with the same methodology and primers and found no variability in the Portuguese ovine breed "Serra da Estrela", while in the Portuguese caprine breed "Serrana" it was possible to detect two patterns.

In our study, the fragment including exons 10 and 11 and the respective intron of α_{s1}-Cn showed three conformational patterns. Barracosa (1996) pointed out that "Serra da Estrela" was monomorphic for this fragment, while two patterns were detected in "Serrana". The caprine α_{s1}-Cn *locus* is very polymorphic, showing 11 alleles, with some of them associated with unusual quantitative differences in casein synthesis (Grosclaude, Martin, 1997). Due to

this remarkable variability, similar studies have been done in the same gene, for ovine breeds. Ferranti *et al.* (1997) found variability at the protein level, Chianese *et al.* (1996) showed five electrophoretic variants (A, B, C, D and E) and Ferranti *et al.* (1995) determined the primary structure for A, C and D genetic variants. The D allele seems to be related with lower total casein content and is detrimental to ovine milk, even in association with other variants (Chianese *et al.*, 1997).

For β-Cn exon 7, we observed two electrophoretic patterns in "Churra da Terra Quente", while Barracosa (1996) could not find variability in "Serra da Estrela" breed. For "Serrana" breed, the same fragment showed three SSCP patterns. Barroso *et al.* (1999), using the same methodology, optimized the screen of the most frequent bovine variants found in the same exon.

In our study, κ-Cn exon 4 was monomorphic, while Barracosa (1996) found two patterns in "Serra da Estrela" breed. This locus is extensively studied in bovine breeds, reviewed by Ng-Kwai-Hang (1997). Barroso *et al.* (1998) optimized a SSCP protocol to screen the most frequent bovine variants found in the same exon of this gene.

For GH exon 4, we observed two electrophoretic patterns and for exon 5 it was possible to identify five conformational patterns. Barracosa (1996) detected a RFLP-*Eco*RI polymorphism in GH gene in "Serra da Estrela" breed. Gootwine *et al.* (1993) showed that the duplicated gene copy of the ovine growth hormone gene contains a *Pvu*II polymorphism in the second intron. In bovine breeds, GH polymorphisms are extensively studied and some associations between production traits and polymorphism have been made. Falaki *et al.* (1996, 1997) reported an association between the polymorphism GH-*Taq*I and milk traits for Simmental and Holstein-Friesian cattle. Lee *et al.* (1996) reported a positive association of a polymorphism in exon 5 with selection for milk yield in Holstein cows. Lagziel *et al.* (1996, 1999) found associations between milk protein percentage and SSCP haplotypes and *Msp*I polymorphism at bovine GH gene.

In our study, exon 6 of GH receptor showed no polymorphism. The absence of diversity does not imply that genes are not polymorphic. It only means that the primers used do not delimitate a polymorphic region. Nevertheless, in this study, the forty animals analysed were obtained from a single flock where it is possible to find some inbreeding, what may reduce the variability.

For β-Cn and GH genes, it was observed that some DNA samples could not be amplified. DNA sample quality could not be the reason for the absence of amplification, once the same samples showed amplification for all the other fragments. Therefore, it seems that specific DNA sequence differences could be the reason for this fact. This situation is probably due to a mutation in the primer region.

As previously reported by several authors (Barroso *et al.*, 1999; Sheffield *et al.*, 1993; Neibergs *et al.*, 1993) SSCPanalysis proved to be an effective technique for the detection of polymorphisms. Our results are consistent with these observations. Hayashi (1991) estimated PCR–SSCP analysis sensitivity (probability of detecting at least one strand shifted) as more than 99% for 100 to 300 bp fragments and 89% for 300 to 450 bp fragments. Comparing with other methods, for instance Denaturing Gradient Gel Electrophoresis, Temperature Gradient Gel Electrophoresis, Chemical and Ribonuclease Cleavage, SSCP has several advantages: it does not require specific equipment, it is technically simpler and faster, it can be used in most laboratories and is not very expensive. Thus, SSCP analysis is the technique of choice when screening for point mutations and minor deletions within a given fragment (Neibergs *et al.*, 1993) is concerned. Nevertheless, it is necessary to optimize, for each case, some parameters.

Temperature. The control of this parameter is essential for the reproducibility of SSCP analysis. In this work, we tested four different temperatures (4, 10, 15 and 20 ºC). The temperature of 4 ºC was beneficial only for exon 4 of growth hormone gene. For the others, 15 ºC was the most appropriate. A constant temperature is essential for band sharpness and reproducibility of strand separation (Hongyo *et al.*, 1993). This parameter was controlled by using a thermostatic bath adapted to the electrophoresis unit to control the buffer temperature.

Glycerol. For the analysed fragments, we only observed an improvement in the resolution for exon 4 of GH. In this case, the addition of 10% of glycerol to the gel allowed the differentiation of four single strand bands in comparison with the three bands observed in the absence of this reagent. For the other fragments, glycerol did not improve the resolution of the gel.

Acrylamide concentration and percentage of crosslinking. The acrylamide concentration of the gels ranged from 5 to 20% and two percentages of crosslinking (1.5 and 2.5%) were used. Low crosslinking did not improve resolution. On the other hand, we observed a clear increase in the detection of the conformation patterns when the acrylamide concentration was increased. Several authors reported similar results (Savov *et al.*, 1992; Glavac, Dean, 1993; Ravnik-Glavac *et al.*, 1994), specially for fragments with more than 400 bp (Savov *et al.*, 1992).

As mentioned in several reports (Bodenes *et al.*, 1996; Tokue *et al.*, 1995), it is sometimes possible to

observe multiple bands for some fragments under specific electrophoretic conditions. Theoretically, in a SSCP gel we have a maximum of four single strands for heterozygous samples. For some of the analysed fragments in this work, the presence of more than four bands was evident. Orita *et al.* (1989), Hayashi (1991) and Tokue *et al.* (1995) assumed that occasionally, a single strand can be separated in two or more bands, although the sequence is the same. This suggests that strands with the same sequence may have different molecular conformations, originating multiple bands under some electrophoretic conditions. Cai and Touitou (1993) and Nielsen *et al.* (1995) hypothezise that in some systems, excess of primers may interfere with the amplified sequence. The interaction is likely due to amplified DNA-primer re-pairing during electrophoresis or bi- or tri-molecular self-annealing interactions between single strands at complementary regions. As SSCP is believed to result from intramolecular base-pairing between complementary regions within a single strand, additional intermolecular duplex between complementary regions of two or more molecules is conceivable (Kasuga *et al.*, 1995).

5. CONCLUSION

Our results provide evidence that there is a high variability within the Portuguese indigenous sheep breed "Churra da Terra Quente". Comparing with the study of Barracosa (1996) with another Portuguese sheep breed "Serra da Estrela", we detected more polymorphisms in the fragments of the milk protein genes. This data opens interesting prospects for future selection programs and also for preservation strategies.

Also, our data show that PCR-SSCP is an appropriate tool for evaluating genetic variability. After optimization of the parameters that affect the detection of conformation polymorphisms, this technique is reliable and reproducible.

This first molecular approach in "Churra da Terra Quente" intends to be a first step for the genetic characterisation of this indigenous breed. The use of a powerful and reliable molecular technique (such as SSCP) to help breeders in the selection of the animals is the major goal of future research. A larger and more representative sample will allow us to search for possible correlations between productive parameters and genetic variants.

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