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

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Received 7 August 2000, accepted 19 January 2001.

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 DNA and PCR amplification of seven fragments: exon 1 of the α-lactalbumin gene; exons 10 and 11 of the αs1-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 PCR products. Only κ-casein and growth hormone receptor were monomorphic. α-lactalbumin and αs1-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, land races, 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 “Mondegueria”) 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 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.

2. MATERIAL AND 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-diaminetetraacetic 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/ml in water and stored at 4°C.

2.2. DNA amplification by PCR

Twenty five ml 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 MgCl₂ 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 MgCl₂ were used and for exon 6 of growth hormone receptor gene, 100 ng of each primer and 3 mM of MgCl₂ were used.
Amplification was carried out in a Perkin Elmer DNA thermocycler. Following a hot start (95°C 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 electrophoresison 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°C). Each PCR reaction was diluted in denaturing solution, denatured at 95°C for 5 min, chilled on ice and resolved on polyacrylamide gel. The electrophoresis was carried in a vertical unit (Hoefer Scientific SE600, 160x140x0.75 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 beginning 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 αs1-Cn gene in cattle (Genebank X59856) (Koczlan 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)
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).

### 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 °C 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 °C and the gel contained 5% of glycerol.

**Figure 2** shows the result of SSCP analysis 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 αs1-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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Acrylamide</th>
<th>DNA</th>
<th>Denaturing solution</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-La (E 1*)</td>
<td>14%</td>
<td>10 µl</td>
<td>15 µl</td>
<td>7 h</td>
</tr>
<tr>
<td>αs1-Cn (E 10 and 11)</td>
<td>12.5%</td>
<td>5 µl</td>
<td>15 µl</td>
<td>11 h</td>
</tr>
<tr>
<td>β-Cn (E 7)</td>
<td>15%</td>
<td>5 µl</td>
<td>15 µl</td>
<td>16 h</td>
</tr>
<tr>
<td>κ-Cn (E 4)</td>
<td>15%</td>
<td>5 µl</td>
<td>15 µl</td>
<td>17 h</td>
</tr>
<tr>
<td>GHR (E 6)</td>
<td>12.5%</td>
<td>6 µl</td>
<td>14 µl</td>
<td>4 h</td>
</tr>
<tr>
<td>GH (E 4)</td>
<td>15%</td>
<td>5 µl</td>
<td>15 µl</td>
<td>15 h</td>
</tr>
<tr>
<td>GH (E 5)</td>
<td>11%</td>
<td>5 µl</td>
<td>15 µl</td>
<td>11 h</td>
</tr>
</tbody>
</table>

* = 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 °C 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 glycerol, à 400 V et à 15 °C pendant 7 h, utilisant 10 µl de DNA et 15 µl de solution dénaturante. Les fréquences des trois profils conformationnels détectés ont été 57.5 % pour le profil 1, 22.5 % pour le profil 2 et 20 % pour le profil 3.
Genetic diversity in a Portuguese indigenous sheep breed

The frequencies of the two conformational patterns detected were 37.5% for pattern 1 and 52.5% for pattern 2. 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).

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-SSCP anaysis, 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 αs1-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 αs1-Cn locus is very polymorphic, showing 11 alleles, with some of them associated with unusual quantitative differences in casein synthesis (Grosclaude, Martin, 1997).
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 possible to find some inbreeding, what may reduce the GH gene.

Barroso this remarkable variability, similar studies have been analysed were obtained from a single flock where it is 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) hypothesise 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.

Remerciements

The first author was sponsored by the Science and Technology Foundation (STF) (PRAXIS XXI-BM7008). This work was partially supported by STF (PRAXIS XXI 3/3.2/CA/1991/1995).

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