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# Identification of the species of origin of milk in cheeses by multivariate statistical analysis of polymerase chain reaction electrophoretic patterns

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ABSTRACT

PDO Portuguese cheeses were used as a source of DNA for analysis by the polymerase chain reaction (PCR). Good quality DNA was extracted from all samples of milk and cheese and was suitable for PCR amplification at least of fragments up to 600 bp. The presence of caprine, ovine and bovine DNA was assessed using sets of species-specific primers. Electrophoretic patterns of PCR products were analysed with a computer-based method to obtain mobility values for each sample. These data were subjected to multivariate statistical analysis to evaluate the potential of this approach for authentication of dairy products. This enabled unequivocal discrimination between bovine, ovine and caprine milks and their derived cheeses.



## 1. Introduction

Producers of dairy products, including cheeses, are increasingly being obliged to label their products with respect to the milk type used for production. In different European countries, bovine, ovine, caprine and buffalo milk are used to make cheese or other dairy products. Cheese milk adulteration by mixing milk from different species occurs primarily because of economic reasons, as there is a larger quantity of bovine milk available and the price of this type of milk is usually lower. Similarly, for ovine and caprine milk products there is an economic incentive for adulteration, since caprine milk yield is higher than ovine milk yield and its price is lower. For these reasons, it is desirable to ascertain that dairy products made with ovine and caprine milk are free from the milk of other species. With the increasing diversification of markets for dairy products and in particular the economic imperatives to develop value added products, it will be necessary to improve analytical capabilities to detect deliberate or accidental adulterations.

Hitherto, several analytical approaches have been developed to detect the presence of admixtures in milk, the principal ones being electrophoretic-, immunological-, chromatographic- and DNA-based techniques (Mafra, Ferreira, & Oliveira, 2008).

Techniques using DNA analysis are increasingly being developed for species identification, since DNA can potentially provide more information than protein due to the degeneracy of the genetic code and the presence of many non-coding regions (Lockley & Bardsley, 2000). Molecular biology tools such as sequencing, hybridization to DNA probes and especially the polymerase chain reaction (PCR) have been used for species identification (Lockley & Bardsley, 2000; Mafra et al., 2008). Nucleic acids can also be found since DNA from somatic cells is known to persist in ripened cheese and may be amplified by PCR and analysed for species discrimination (Ulberth, 2003).

Using species-specific primers, several authors have used different types of PCR to differentiate bovine, caprine and ovine milks or cheeses (Bania, Ugorski, Polanowski, & Adamczyk, 2001; Bottero et al., 2003; Calvo, Osta, & Zaragoza, 2002; Dalmasso, Civera, La Neve, & Bottero, 2011; Diaz et al., 2007; Feligini et al., 2005; Lopez-Calleja et al., 2004, 2005a, 2005b, 2007a, 2007b; Mafra, Ferreira, Faria, & Oliveira, 2004; Mafra, Ferreira, Roxo, & Oliveira, 2007; Maudet & Taberlet, 2001; Mayer, 2005). In the present work, a combination of universal and species-specific PCR primers has been used to characterise Portuguese PDO caprine, ovine and bovine cheeses prior to application of statistical analysis. Preliminary work using DNA extracted from caprine, ovine, bovine and porcine blood and milk was used for optimisation, since the results are usually unequivocal and easily interpreted by eye, which helped us to derive the appropriate multivariate statistical analytical approach. The validation of the statistical methodology with the blood and milk DNA samples paved the way for the use of the same approach with the more complex results obtained for cheese.

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## 2. Material and methods

### 2.1. Samples

Blood was collected in heparin-coated tubes from three animals representative of each of the ovine, caprine, bovine and porcine species and stored at −20 °C. Separate milk samples from three individual ovines, caprines and bovines were collected in sterile Falcon tubes and stored at −80 °C. Thirteen kinds of PDO Portuguese cheeses with the EU symbol and the label of certification were obtained from a local market in northern Portugal. Cheese samples were classified in three groups: soft (Serra da Estrela, Serpa and Pico cheeses), semi hard (Terrincho, Azeitão, Nisa, Évora, Picante Beira Baixa, Amarelo Beira Baixa, Castelo Branco and S. Jorge cheeses) and extra hard (Terrincho Old and Cabra Transmontano cheeses). Samples were obtained from the interior of the cheese using sterilized instruments to avoid contamination, cut into small pieces (approximately 2 g), distributed and stored at −80 °C.

Protein and fat content of the cheeses were determined following standard procedures (James, 1995), namely protein (N×6.38) by the Kjeldahl method and fat by the Soxhlet method.

### 2.2. DNA extraction

DNA isolation from blood was performed using the Wizard Genomic DNA Isolation Kit (Promega, Madison, WI, USA) and from milk and cheese samples using the DNeasy tissue Kit (Qiagen, Germantown, MD, USA), following the handbook procedures for animal tissues. Before DNA isolation, milk samples were thawed and 500 μL of defatted milk was transferred to a 1.5 mL sterile microcentrifuge tube containing 400 μL of Buffer AL (cell lysis solution), supplied by the manufacturer and 40 μL of 20 mg $mL^{-1}$ Proteinase K (Sigma, St. Louis, MO, USA), with the mixture being incubated for 10 min at 70 °C, before proceeding to the next stage. The cheese samples (three from each cheese) were left at room temperature until thawed, before 200 mg aliquots were transferred to a 1.5 mL sterile microcentrifuge tube containing 400 μL of Buffer AL (cell lysis solution) and 40 μL of 20 mg $mL^{-1}$ Proteinase K (Sigma) and in this case the mixture was incubated for 5 h at 55 °C. After the incubation period the procedure was the same for both milk and cheese samples and was performed according to the DNeasy™ Tissue Kit Handbook (Qiagen).

### 2.3. Evaluation of DNA preparations

Spectrophotometry was used to assess the purity of the DNA (ratio of $A_{260}/A_{280}$) and the DNA concentration. The integrity of the DNA was assessed by gel electrophoresis using a 0.7% agarose gel with ethidium bromide staining (0.5 μg $mL^{-1}$) in 1 × Tris–Borate–EDTA (TBE) buffer (8.9 mM Tris–HCl, 8.9 mM boric acid, 0.25 mM EDTA, pH 8.3) run at 80 V for 1 h. Stained gels were visualized using UV light. An approximate mass value was estimated by visual comparison to known λ *Hin*d III DNA markers run alongside.

### 2.4. Primer design

Based on the sequence reported by Irwin, Kocher, and Wilson (1991) for mitochondrial cytochrome b for *Ovis aries* (EMBL accession number X56284) a ovine primer was designed: Ovine primer (5′-GAGGATGAGGATTAGTAGGATAGCACCTAG-3′)-amplification product with 660 bp.

The common forward primer (5′-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3′) and the following species-specific reverse primers, as designed by Matsunaga et al. (1999) were used: (i) caprine primer (5′-CTCGACAAATGTGAGTTACAGAGGGA-3′), amplification product with 157 bp; (ii) bovine primer (5′-CTAGAAAAGTGTAAGACCCGTAATATAAG-3′), amplification product with 274 bp; (iii) porcine primer (5′-TACGGTCATCACAAAT CTACTATCAGC-3′), amplification product with 398 bp.

Ovine primer showed no cross reactivity in the amplification of caprine, bovine and porcine *mt* DNA. All primers were synthesised and supplied by Sigma-Genosys Ltd (Haverhill, United Kingdom).

### 2.5. PCR amplification and analysis

A multiplex PCR amplification was performed in a 50 μL reaction containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP mix, 20 pmol of each primer, 250 ng template DNA and 1.25 unit recombinant *Taq* DNA polymerase (MBI Fermentas, Baltimore, MD, USA). The PCR was carried out in a Maxi-Gene thermocycler (Stuart Scientific, Red Hill UK) with Iso-temp cover, using the following conditions: a single initial denaturation at 94 °C for 2 min followed by 30–35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 420 s. Positive controls (blood DNA from the four species) and a blank were included for each amplification in order to verify the PCR efficiency and to detect contamination.

PCR products (5 μL) and 2 μL of 50 bp DNA ladder were mixed with 5 × loading buffer (50%, v/v, glycerol, 50 mM EDTA, pH 8.0, 0.125%, w/v, bromophenol blue, 0.125%, w/v, xylene cyanol) and electrophoresed on a 4% agarose gel with ethidium bromide (0.5 μg $mL^{-1}$) for 30 min at 90 V in 1 × TBE buffer (8.9 mM Tris–HCl, 8.9 mM boric acid, 0.25 mM EDTA, pH 8.3) and visualized under UV light after migration. Product size was determined by comparison to marker DNA run alongside.

### 2.6. Statistical analysis

Electrophoretic gel images were analysed with 1D Image Analysis Software (Kodak, Rochester, NY, USA) and the mobility values obtained for each sample were subjected to cluster analysis with Statistica 6.0 (StatSoft, Inc., Tulsa, OK, USA) utilising cluster procedures with the single linkage amalgamation rule and Euclidean distances.

## 3. Results and discussion

### 3.1. DNA yield and cheese proximate composition

When milk was a source of DNA, the extractions were successful and reproducible between three replicates of the same sample. Between different samples from different animals of the same species and from different species more variation in DNA yield was noted. DNA extraction from milk is based on the somatic cells present in milk. Lipkin, Shalom, Khatib, Soller, and Friedmann (1993) extracted DNA from milk and noted the technical convenience of milk as a source of DNA to increase the field of application of marker-based methods for genetic analysis and genetic improvement of economic traits in dairy bovine. Since then, other authors have used milk as a source of DNA (Amills, Francino, Jansa, & Sanchez, 1997; Bania et al., 2001; Dalmasso et al., 2011; Lopez-Calleja et al., 2004, 2005a, 2005b; Murphy, Shariflou, & Moran, 2002).

The DNA yield from cheese varied between 0.04 and 0.1 μg DNA $mg^{-1}$ cheese (Table 1), but despite the fact that some cheeses gave almost four times more DNA than others, the classification of the cheese (soft, semi hard and extra hard), protein and fat content did not influence the efficiency of the DNA extraction (Table 1). ANOVA analysis showed no significant difference between protein, fat and DNA content of these three groups (Table 1). Branciari, Nijman, Plas,

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**Table 1**
Composition and DNA yield of thirteen Portuguese PDO cheeses.

| Cheese | Code | Species origin (as on label) | Classification | Protein (%) | Average[a] protein (%) | Fat (%) | Average[a] fat (%) | DNA yield (μg DNA $mg^{-1}$ cheese) | Average[a] DNA yield (μg DNA $mg^{-1}$ cheese) |
|---|---|---|---|---|---|---|---|---|---|
| Serra da Estrela | 3 | Ovine | Soft | 22.59 ± 1.16 | 20.91 ± 1.45 | 24.23 ± 0.11 | 33.27 ± 12.25 | 0.05 ± 0.01 | 0.07 ± 0.03 |
| Serpa | 7 | Ovine | Soft | 20.05 ± 0.50 | | 28.43 ± 0.85 | | 0.05 ± 0.01 | |
| Pico | 11 | Bovine | Soft | 20.11 ± 0.44 | | 47.15 ± 1.32 | | 0.11 ± 0.03 | |
| Terrincho | 1 | Ovine | Semi hard | 20.93 ± 1.00 | 20.39 ± 3.34 | 30.67 ± 1.23 | 39.23 ± 6.74 | 0.05 ± 0.02 | 0.06 ± 0.02 |
| Azeitão | 4 | Ovine | Semi hard | 18.58 ± 0.37 | | 42.20 ± 2.67 | | 0.03 ± 0.01 | |
| Nisa | 5 | Ovine | Semi hard | 27.23 ± 1.16 | | 40.72 ± 0.75 | | 0.04 ± 0.01 | |
| Évora | 6 | Ovine | Semi hard | 22.59 ± 0.31 | | 37.62 ± 0.25 | | 0.05 ± 0.02 | |
| Picante Beira Baixa | 8 | Ovine and Caprine | Semi hard | 18.47 ± 0.81 | | 32.77 ± 0.45 | | 0.08 ± 0.01 | |
| Amarelo Beira Baixa | 9 | Ovine and Caprine | Semi hard | 17.46 ± 0.97 | | 41.15 ± 1.69 | | 0.06 ± 0.01 | |
| Castelo Branco | 10 | Ovine | Semi hard | 17.09 ± 0.06 | | 36.21 ± 0.36 | | 0.09 ± 0.03 | |
| S. Jorge | 12 | Bovine | Semi hard | 20.77 ± 0.37 | | 52.53 ± 2.23 | | 0.07 ± 0.02 | |
| Terrincho Old | 2 | Ovine | Extra hard | 20.59 ± 1.11 | 20.75 ± 0.22 | 34.04 ± 1.88 | 39.45 ± 7.71 | 0.08 ± 0.01 | 0.07 ± 0.01 |
| Cabra Transmontano | 13 | Caprine | Extra hard | 20.94 ± 0.12 | | 44.93 ± 1.97 | | 0.06 ± 0.02 | |

[a] Averages are of the class of cheese (soft, smi hard, extra-hard) and are means ± standard deviation; ANOVA indicated no significant differences between cheese classifications for any parameter.

Di Antonio, and Lenstra (2000) also found that the DNA yield from cheese did not appear to be affected by the other cheese components, such as protein and fat.

### 3.2. Species-specific DNA amplification

Template DNA extracted from blood from three samples of each species was subjected to a multiplex PCR reaction, generating single band amplicons only of the predicted sizes, namely 157 bp for caprine; 274 bp for bovine, 398 bp for porcine and 660 bp for ovine, with no apparent cross reactivity. The PCR reactions optimised for blood DNA were repeated with exactly the same primer combinations and conditions, but using DNA prepared from milk samples, and confirmed that the species-specific tests exhibited no cross reactivity. PCR with a porcine-specific primer was used as a negative control since it should not react with any of the other DNA preparations. Fig. 1 shows the results obtained with a multiplex reaction with 20 pmol of each primer (common forward primer: caprine: bovine: porcine: ovine) using template DNA extracted from milk.

The gel shown in Fig. 1 was analysed by 1D Kodak 2.0 Image Analysis Software and the mobility values of the PCR products of each sample (matrix 9 × 3), considered as variables for multivariate statistical analysis, were subjected to cluster analysis with Statistica software. A similar approach was also used by Colombo, Mangiagalli, and Renon (2005) to analyse SSCP electrophoretic patterns to tuna speciation. Fig. 2 shows the dendrogram resulting from cluster analysis which reveals three clusters in accordance with the source of the DNA of the milk samples.

DNA templates extracted from cheeses were amplified in uniplex PCR reactions using the primers specific to bovine, caprine and ovine DNA. The electrophoretic mobility values obtained by this gel analysis were subjected to a similar statistical analysis as reported before for milk. For cluster analysis, the matrix used was 13 × 3 and the resulting dendrogram is shown in Fig. 3. Three clusters were seen, the first group comprising bovine cheese DNA samples 11 and 12, but also including sample 1, an ovine cheese sample, as claimed on the label, in which the presence of bovine DNA was also observed. The second cluster joins together the DNA from cheese samples 3–7 and 10. All these samples were manufactured with

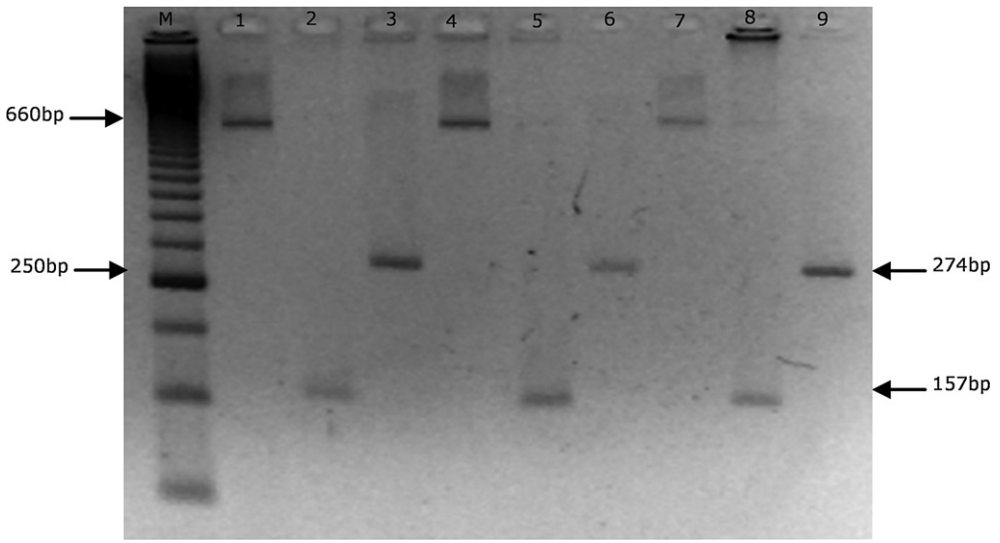


**Fig. 1.** Multiplex PCR amplicons from milk-derived DNA amplified with species-specific primers: lane M, 50 bp ladder; lanes 1, 4, and 7, sheep DNA; lanes 2, 5, and 8, goat DNA; lanes 3, 6, and 9, cattle DNA.

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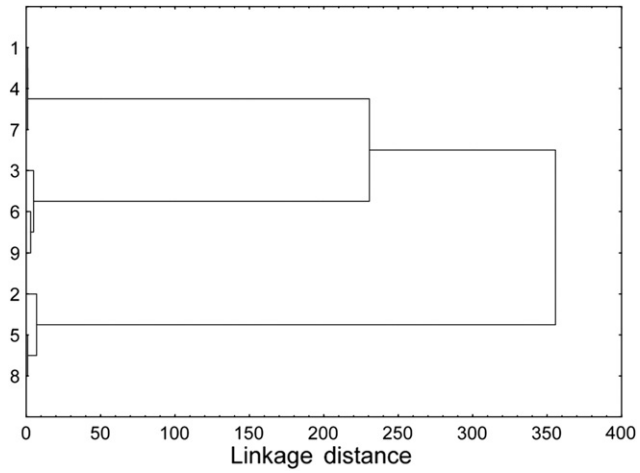


**Fig. 2.** Cluster analysis of amplicons generated by multiplex PCR using species-specific primers and milk-derived DNA. The numbers at the left hand side of the dendrogram branches correspond to sheep (1,4, and 7) goat (2,5, and 8) and cattle (3,6, and 9), species analysed in Fig. 1.

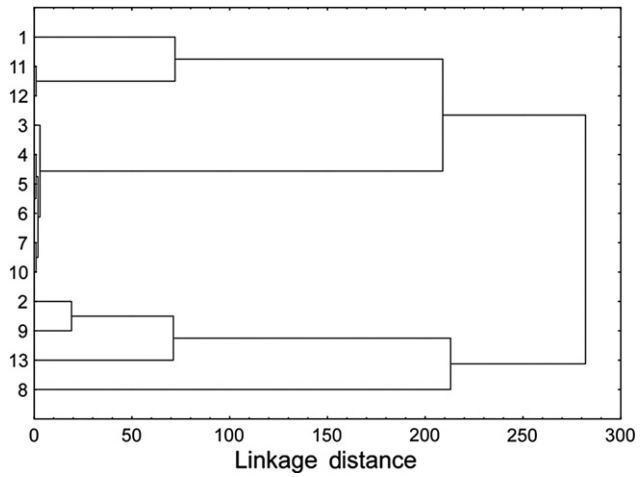


**Fig. 3.** Cluster analysis of amplicons derived from multiplex PCR using cheese-derived DNA. The numbers at the left hand side correspond to those of the thirteen PDO cheeses.

ovine milk and DNA analysis revealed that only DNA was detected. A third cluster group was comprised of samples 2 and 9 where both ovine and caprine DNA was found to be present. Sample 13, a caprine cheese milk, was also related to this group. Sample 8, appears separated from the others since all the three types of DNA were detected by DNA analysis.

Although the presence of bovine DNA or caprine DNA was not mentioned on the label, relevant can also be the fact that coagulation of milk using calf rennet can be a factor misleading to the interpretation of results since it can also be a source of additional DNA.

## 4. Conclusion

The use of multivariate statistics in DNA analysis is not a common practice and our goal was not to build a model for predicting the origin, but to evaluate this approach. One of the main advantages of multivariate techniques, such as cluster analysis, is to present data in a very informative and clear way allowing to reveal perhaps unexpected relationships between a large number of variables and samples. The results obtained with cluster analysis for samples that did not need complex approaches, such as blood and milk samples, encouraged us to use the same approach for cheese samples where the origin could be more complex. The results obtained revealed that these statistical tools could probably be useful in future to help explain and validate DNA analysis in food authentication.

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