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12S rRNA mitochondrial gene as marker to trace Sicilian mono-species dairy products

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ABSTRACT

For a rapid, specific and sensitive identification of cows', ewes' and goats' milk in mono-species Sicilian dairy products, species-specific duplex-PCR protocol was applied. DNA samples from blood and experimental cheeses of Sicilian autochthonous breeds were extracted to amplify the *12S rRNA* (and part of *16S rRNA* in case of *Ovis aries*) mitochondrial species-specific gene fragment. The use of species-specific primers for *Bos taurus*, *Capra hircus* and *Ovis aries* species, after electrophoresis on agarose gel, yielded fragments of 256 bp, 326 bp and 172 bp, respectively. Amplification by duplex-PCR of DNA pools from two species showed detection thresholds of 0.1% of "contaminant" DNA in each mixture. Finally, duplex-PCR assay was applied to experimental cheeses in order to detect the minimum threshold of DNA belonging to one species in cheese made with milk of two species. The results showed a sensitive threshold of 0.1% of ewes' milk in cows' and goats' cheeses, 0.1% of cows' milk in ewes' and goats' cheeses, and finally 0.1% of goats' milk in cows' and ewes' cheeses. The proposed assay represents a rapid and straightforward method of species traceability for the detections of adulteration in Sicilian mono-species dairy products.

1. Introduction

In general, traceability can be defined as the ability to follow food through all stages of production, processing and distribution (McKean, 2001). Traceability in animal food production is increasingly being demanded by consumers as an essential tool for food safety and quality monitoring. In fact, the ability to discriminate between livestock species or breeds is an element of quality control of animal products (Fernández et al., 2004).

Species identification in dairy products has received great attention in recent years (Mafra et al., 2004) since that authenticity assessment is an important issue regarding the consumers' interests not only for an economic point of view but also for food allergies (De La Fuente and Juárez, 2005). Moreover, species identification in dairy sector is important also to verify compliance with the Production Regulations of many typical dairy products (PDO/PGI) (Bánáti and Herman, 2011; Bottero et al., 2003).

The most common fraud of dairy products is due to the substitution of part of the raw material with another of different origin or with lower cost. Several analytical methods have been applied for species identification in milk and dairy products including immunological (Hurley et al., 2004; López-Calleja et al., 2007a; Xue et al., 2010; Zeleňáková et al., 2008), electrophoretic (Chianese et al., 1990; Mayer, 2005; Molina et al., 1999), chromatographic (Branciari et al., 2000; De Noni et al., 1996; Enne et al., 2005), and spectrometric (Nicolaou et al., 2011). For example, the European Commission Regulation (EC) No 273/2008 laid down detailed rules for the application of EC No 1255/1999 as regards methods for the analysis and quality evaluation of milk and milk products. Article 6 of EC No 273/2008 described isoelectrofocusing of γ-casein as reference method for the detection of cows' milk in cheeses from other dairy species (i.e. ewes', goats', buffalos' milk or mixtures of them), but chemical methods may fail in species identification after excessive proteolysis or heat-induced denaturation of proteins indicator (López-Calleja et al., 2005a).

Nowadays, species-specific PCR has shown to be a reliable method to control the authenticity of dairy products[20] because a specific target sequence (e.g. *12S rRNA*, *16S rRNA*, *cytochrome b*, and *cox1* gene) can be detected in matrices containing a pool of heterogeneous genomic DNA, such as milk (Galimberti et al., 2013; Mafra et al., 2008). Molecular methods may be the solution as the DNA from somatic cells persists even in the ripened cheese (Plath et al., 1997) and it is also possible to extract amplifiable DNA from pasteurized, filtered, and ultrafiltered milk (Bottero et al., 2002). Among the genetic markers used for species traceability of dairy products, the mitochondrial DNA

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(mtDNA) was mainly studied due to its unique characteristics among species (Bottero et al., 2003; European Commission, 2008; Plath et al., 1997). In fact, using species-specific primers, several authors have used different types of PCR to differentiate cows', goats', and ewes' milk or cheeses (Bottero et al., 2003; Dalmasso et al., 2011; Feligini et al., 2005; Mafra et al., 2004; Mafra et al., 2007; Maudet and Caberlet,2001; Mayer, 2005; López-Calleja et al., 2004; López-Calleja et al., 2005b; López-Calleja et al., 2007b; López-Calleja et al., 2007c).

The Sicilian dairy sector is characterized by several typical products resulting from the links between product-territory, territory-breed/species and breed/species-product. To avoid the possible substitution or the use of mixture of milk from different species, it is necessary to develop analytical procedures able to detect frauds and protect consumers from mislabeling (De La Fuente and Juárez, 2005; Mafra et al., 2007). In fact, the development of traceability systems can lead to the promotion of local and traditional cheeses (PDO and PGI), and thereby to the conservation and enhancement of the breeds of origin and/or local populations.

In the present work, a duplex-PCR method, to amplify *12S* and *16S rRNA* gene fragments of mtDNA, was applied for molecular traceability of Sicilian mono-species dairy products.

2. Materials and methods

2.1. Blood sampling and DNA extraction

Sample collection, animal management and care followed the recommendation of EU Directive 2010/63/EU. A total of 300 individuals were sampled and 10 ml of blood were collected from jugular vein through vacutainer tubes with EDTA as anticoagulant. We sampled animals from *B. taurus*, *O. aries*, and *C. hircus* species belonging to the most important Sicilian autochthonous breeds (*Modicana* and *Cinisara* cattle breeds, *Comisana*, *Pinzirita* and *Valle del Belice* sheep breeds, and *Girgentana*, *Maltese* and *Derivata di Siria* goat breeds). The individuals were collected from different farms located in Sicilian provinces. Genomic DNA was extracted from blood using a *salting out* method (Miller et al., 1988) and checked for quantity and quality by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples were diluted and stored at 4 °C until analysis.

2.2. Simplex-PCR and mono-species DNA pools preparation

In the first step, in order to check the specificity of primer pairs on each breed as tested by Bottero et al. (2003), a simplex-PCR protocol was applied on a total of 80 individual samples, 10 for each sampled breed in order to amplify *12S* and *16S rRNA* mitochondrial gene fragments using primers proposed by Bottero et al. (2003) (Table 1). In particular, both *12S* and *16S rRNA* gene fragments were amplified in *O. aries* species while only *12S rRNA* gene fragment was amplified in *B. taurus* and *C. hircus* species.

Table 1
Oligonucleotides used as PCR primer pairs for amplification of mitochondrial *12S* and *16S* gene fragments in *Ovis aries*, *Bos taurus*, and *Capra hircus* species.

Species and genes	Oligonucleotide primers	Amplicons
Ovis aries (12S and 16 S) (GenBank Acc. No. NC_001941)	FW: 5′-ATATCAACCACACGAGAGGAGAC –3′ RV: 5′-TAAACTGGAGAGTGGGAGAT–3′	172 bp
Bos taurus (12S) (GenBank Acc. No. NC_006853)	FW: 5′-GTACTACTAGCAACAGCTTA–3′ RV: 5′-GCTTGATTCTCTTGGTGTAGAG –3′	256 bp
Capra hircus (12S) (GenBank Acc. No. M55541)	FW: 5′-CGCCCTCCAAATCAATAAG–3′ RV: 5′-AGTGTATCAGCTGCAGTAGGGTT –3′	326 bp

PCR amplifications were performed in a final volume of 25 µl containing 1 µM of each primer, 0.8 mM of dNTP Mix, 1 U of Taq DNA polymerase (Fermentas, Hanover, MD, USA), 1X PCR buffer with KCl, 3 mM $MgCl_2$, and approximately 100 ng of genomic DNA. Thermal cycling conditions were an initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C, 53 °C and 72 °C for 2 min and 30 s each, and a final extension at 72 °C for 5 min. Amplified fragments were checked by electrophoresis on 2% agarose gel stained with ethidium bromide (Fig. 1A)..

For mono-species DNA pools preparation, samples were chosen after their positive amplification in simplex-PCR. In particular, 5 samples for each breed for each species were used. A total of 10 µl of diluted DNA (50 ng/µl) of each sample were used to constitute the three different pools. To evaluate data repeatability and reproducibility, individual samples and mono-species DNA pools were amplified in triplicate and analyses were carried out independently by two experienced operators. The same simplex-PCR protocol was applied to mono-species DNA pools to confirm the length of expected fragments and amplification products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide (Fig. 1B).

2.3. Duplex-PCR and multi-species DNA pools preparation

The second step involved the application of duplex-PCR protocol to detect, at the same time, each species present both in multi-species DNA pools and in reference experimental cheese samples. For duplex-PCR protocol, some tests with DNA of mono-species pools were carried out in order to verify the real specificity of each species-specific primer pairs. Thermal cycling conditions were the same as simplex-PCR protocol.

After these preliminary analyses, multi-species DNA pools containing known mixtures of DNA from two species at the same time were prepared as reported in Table 2. In order to evaluate repeatability and reproducibility of data from multi-species DNA pools, these samples were amplified in triplicate and analyzed as reported above. Moreover, some tests were carried out in order to choose the better amplification condition and the optimal primers concentrations. Duplex-PCR amplifications were performed in a final volume of 30 µl containing different concentration of each primers pair (from 0.5 µM for mono-species DNA pools to 0.8 µM for multi-species DNA pools), 0.8 mM of dNTP Mix, 1 U of Taq DNA polymerase (Fermentas, Hanover, MD, USA), 1X PCR buffer with KCl, 3.5 mM $MgCl_2$, and approximately 200 ng of genomic DNA. The amplification products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide (Fig. 2). Optimized duplex-PCR protocol was applied to evaluate the visual detection limit of each mixture on 2% agarose gel..

2.4. Reference cheeses samples and DNA extraction

Reference experimental cheeses were prepared in a dairy facility in Belmonte Mezzagno (Palermo province, Sicily, Italy), using mixtures of cows', ewes' and goats' raw milk from Sicilian local dairy farms. Experimental cheeses were prepared according to the classical Sicilian cheese-making procedure from raw milk starting from mixture with known concentration of two different milks.

In particular, for cows' cheeses, three mixtures were prepared adding ewes' milk and other three mixtures adding goats' milk in the following percentages: 0.1%, 0.5%, and 1% in cows' milk. The same six mixtures were prepared for ewes' cheeses adding separately cows' and goats' milk in ewes' milk, and for goats' cheeses adding cows' and ewes' milk in goats' milk.

For DNA extraction from cheeses the CTAB method of ISO 21571:2005(E) (International Organization for Standardization (ISO), 2005) was used, making some changes to the protocol for sample preparation (i.e. 5 g of cheese sample, use of proteinase K, and incubation overnight at 50 °C). Five samples from each cheese were

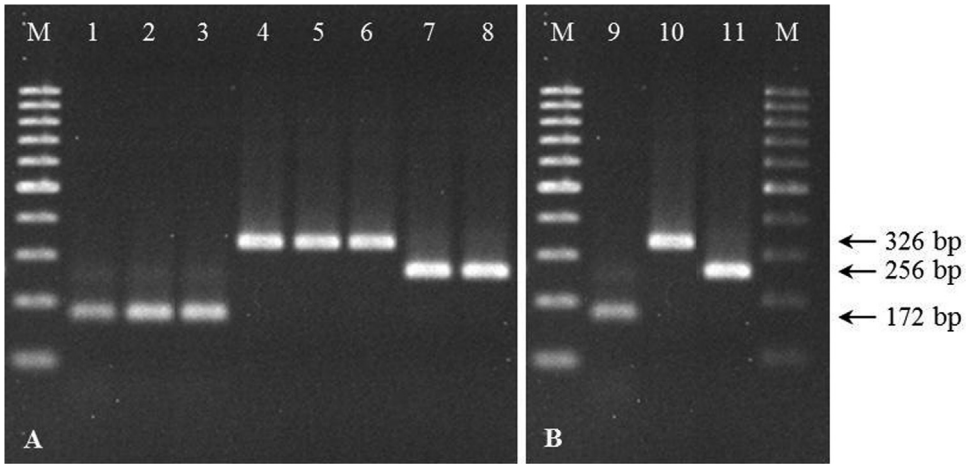

Fig. 1. (A) Agarose gel electrophoresis of simplex-PCR products of different ovine, caprine and bovine breeds. M, 100 bp ladder; lanes 1–3, *Comisana*, *Pinzirita* and *Valle del Belice* sheep breeds; lanes 4–6, *Girgentana*, *Maltese* and *Derivata di Siria* goat breeds; lanes 7–8, *Modicana* and *Cinisara* cattle breeds. (B) Agarose gel electrophoresis of PCR fragments of mono-species DNA pools from *Ovis aries* (lane 9), *Bos taurus* (lane 10), and *Capra hircus* (lane 11) species.

Table 2
Multi-species DNA pools containing different percentages (%) of *Bos taurus* (B), *Ovis aries* (O), and *Capra hircus* (C) mixtures of DNA. Indications within brackets are referred to Fig. 2 (Ref. Fig. 2).

Ref.Fig. 2	B+O (%) [2 A]	O+B (%) [2B]	B+C (%) [2 C]	C+B (%) [2D]	O+C (%) [2E]	C+O (%) [2 F]
Lane **1**	50.0 +50.0	50.0 +50.0	50.0 +50.0	50.0 +50.0	50.0 +50.0	50.0 +50.0
Lane **2**	75.0 +25.0	75.0 +25.0	75.0 +25.0	75.0 +25.0	75.0 +25.0	75.0 +25.0
Lane **3**	90.0 +10.0	90.0 +10.0	90.0 +10.0	90.0 +10.0	90.0 +10.0	90.0 +10.0
Lane **4**	99.5+0.5	99.5+0.5	99.5+0.5	99.5+0.5	99.5+0.5	99.5+0.5
Lane **5**	99.9+0.1	99.9+0.1	99.9+0.1	99.9+0.1	99.9+0.1	99.9+0.1

collected for DNA extraction and used as technical and biological replicates. The concentration of extracted DNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at 4 °C until use. Finally, duplex-PCR protocol was applied to experimentally cheese samples to evaluate the minimum (visual) threshold of each DNA mixture. Amplification products were checked on 2% agarose gel stained with ethidium bromide.

3. Results and discussion

Extracted DNA was used as a template to amplify species-specific fragments of mtDNA, *12S* and *16S rRNA*, through primers sets proposed by Bottero et al. (2003) in order to assess their applicability to Sicilian autochthonous breeds and species. Previous works showed the application of different PCR protocols on different mitochondrial genes to differentiate cows', goats' and ewes' milk or cheeses by means of species-specific primers as reported by Guerriero et al. (2012).

Since Lipkin et al. (1993) showed the technical convenience of milk as a source of DNA, several PCR-based methods have been developed for authenticity assessment of dairy products, as reported by Mafra et al. (2008).

3.1. Specificity of PCR protocol

In the first part of our work, DNA extracted from cows', ewes', and goats' blood samples (n=80) was used to verify the specificity of primer pairs proposed by Bottero et al. (2003). The optimized simplex-PCR protocol was applied on individual samples of each considered breed and subsequently on mono-species DNA pools in order to confirm the specific fragment lengths and 172 bp, 256 bp and 326 bp for *O. aries*, *B. taurus*, and *C. hircus* species. Electrophoresis on agarose gels of PCR products showed good species-specific amplifications and perfect separation of the three expected fragments both in individual samples (Fig. 1A) and in mono-species DNA pools (Fig. 1B). These results were in agreement with those reported by other authors on different cows', ewes' and goats' breeds (Bottero et al., 2003; Mafra et al., 2004; Mafra et al., 2007) using the same primer pairs proposed by Bottero et al. (2003).

The next step involved the application of duplex-PCR protocols on mono-species DNA pools in order to verify the real specificity of each species-specific primer pairs and then the optimization of primers concentrations and amplification conditions on multi-species DNA pools. Good results of amplification were obtained for duplex-PCR protocols on multi-species DNA pools as showed in Fig. 2. PCR amplifications for individual samples, mono-species and multi-species DNA pools gave satisfactory results in terms of reproducibility and repeatability and this simplified the laboratory work and reduced the analyses costs.

3.2. Sensitivity of PCR protocol

Electrophoretic patterns of all duplex-PCR protocols applied on multi-species DNA pools from known mixtures reported in Table 2, are showed in Fig. 2. In particular, for all the multi-species DNA pools the visual detection threshold was 0.1%. This means that 0.1% of ewes' DNA was found both in ewes'/cows' and ewes'/goats' DNA pools (Figs. 2A and F, lanes 5); 0.1% of goats' DNA was found both in goats'/cows' and goats'/ewes' DNA pools (Figs. 2C and E, lanes 5), and, finally, 0.1% of cows' DNA was found both in cows'/goats' and cows'/ewes' DNA pools (Figs. 2D and B, lanes 5). Moreover, duplex-PCR protocols were tested on experimental cheeses to confirm the identified visual detection threshold of 0.1% in multi-species DNA pools. Fig. 3 showed good PCR amplifications for all reference experimental cheeses and confirmed the visual detection threshold of 0.1% of “contaminant” DNA from another species..

In particular, lanes 1 of Figs. 3A and B showed ewes' and goats' DNA fragments in cows' cheeses; lanes 1 of Figs. 3C and D showed cows' and goats' DNA fragments in ewes' cheeses; and, finally, lanes 1 of Figs. 3E and F showed cows' and ewes' DNA fragments in goats' experimental cheeses.

Our results are in agreement with previous studies in which detection threshold of 0.1% of cows' milk in ewes' and goats' cheeses (Mafra et al., 2007) is reported. Moreover, other authors (López-Calleja et al., 2004; Maudet and Taberlet, 2001) reported the same detection

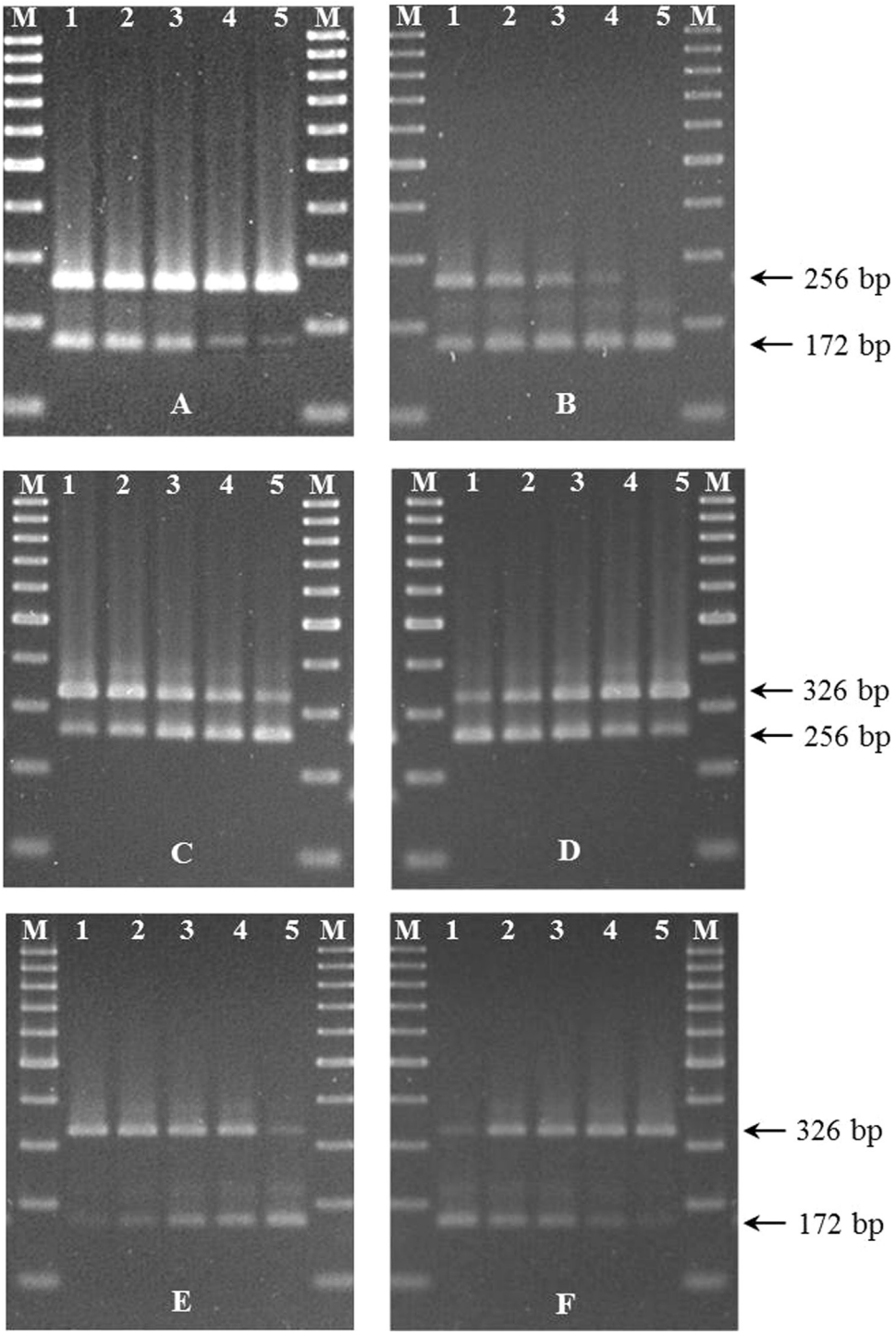

Fig. 2. Agarose gel electrophoresis of PCR products of multi-species DNA pools obtained using different percentages of *Ovis aries* (172 bp), *Bos taurus* (256 bp), and *Capra hircus* (356 bp) mixtures of DNA as reported in Table 2; M, 100 bp ladder.

threshold of 0.1% of cows' milk both in goats' cheeses (Maudet and Taberlet, 2001) and in ewes' and goats' milk (López-Calleja et al., 2004) using different primers targeting mitochondrial DNA control region and *12S rRNA* gene.

Our 0.1% detection thresholds were more precise than the ones reported by Bottero et al. (2003) which were only able to detect 0.5% of cow's milk in mixture of cows' and goats' milk using the same primers.

4. Conclusions

Nowadays, mitochondrial genome was successfully used by several authors as target to detect undeclared milk in dairy products thank to its several advantages over nuclear DNA as the abundance and the relatively high mutation rate to better define species differences.

The duplex-PCR protocols gave us satisfactory results in term of costs and time consuming. Therefore, it could be useful to use these protocols in species traceability system. Considering that typical Sicilian dairy products are important for economy and traditions, and for the conservation of livestock local populations, the ability to detect low levels of contaminating milk could be interesting to safeguard not only mono-species dairy products protected by European labels (PDO and PGI) but also allergic or intolerant subjects.

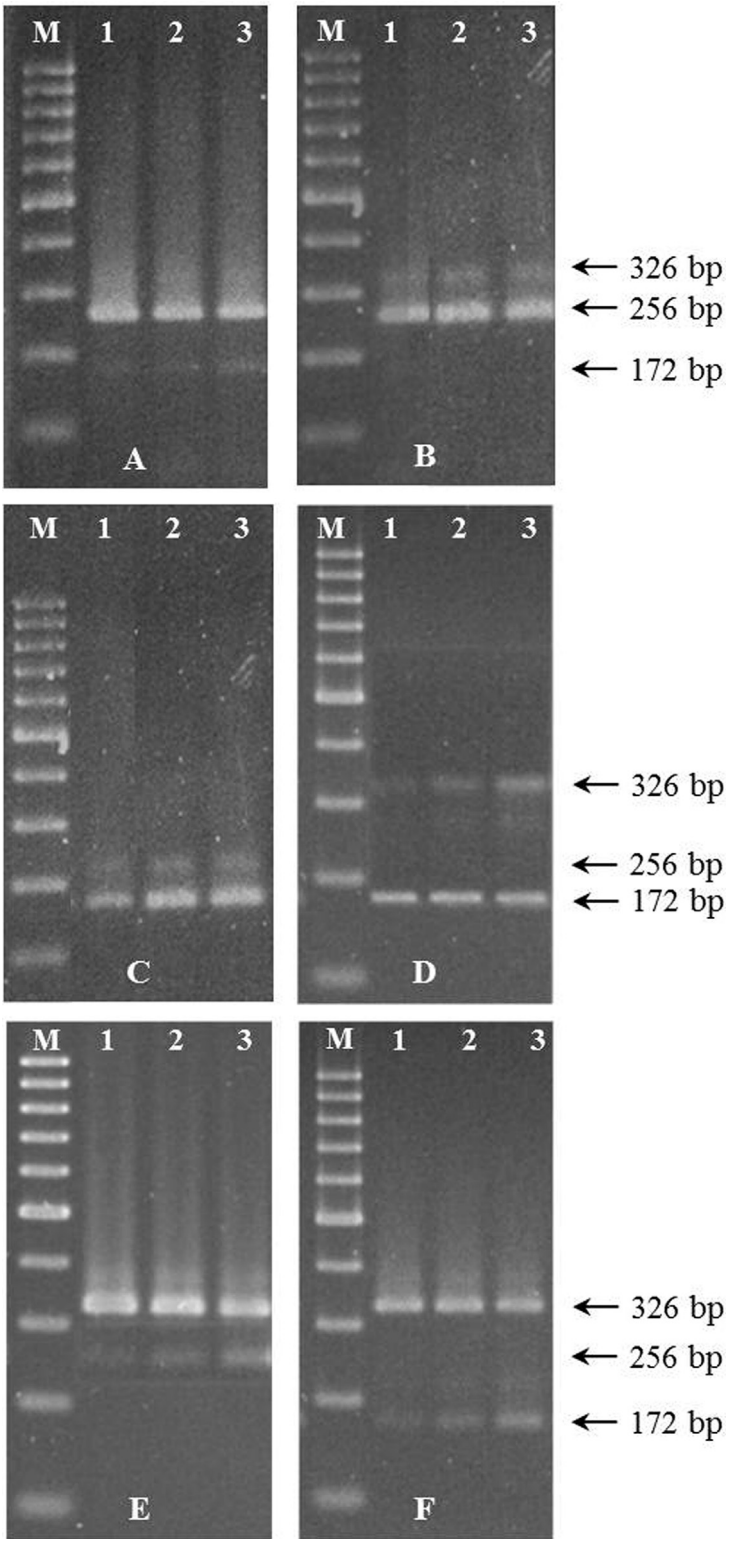

Fig. 3. Agarose gel electrophoresis of PCR products of DNA extracted from cows' cheeses with different percentages of ewes' (A) and goats' (B) milk; from ewes' cheeses with different percentages of cows' (3 C) and goats' (3D) milk; and from goats' cheeses with different percentages of cows' (3E) and ewes' (3 F) milk; lanes 1–3 of each section, from A to F, represent 0.1%, 0.5% and 1% of "contaminant" DNA in experimental cheeses; M, 100 bp ladder.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgement

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