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APPLICABILITY AND SENSITIVITY OF PCR SSCP METHOD FOR MILK SPECIES IDENTIFICATION IN CHEESE

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Corresponding Author:	Levente Czegledi, PhD University of Debrecen Debrecen, HUNGARY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Debrecen
Corresponding Author's Secondary Institution:	
First Author:	Adam Csikos
First Author Secondary Information:	
Order of Authors:	Adam Csikos
	Aida Hodzic
	Eva Pasic-Juhas
	Andras Javor
	Amina Hrkovic-Porobija
	Teufik Goletic
	Gabriella Gulyas
	Levente Czegledi, PhD
Order of Authors Secondary Information:	
Abstract:	Species identification in food has become a prominent issue in recent years as the importance of consumer protection has increased. DNA-based species identification methods were developed by researchers in last two decades, as these are reliable, accurate and low-cost techniques for species identification in raw and processed food products as well. In our study universal primers were designed to conserved regions of mitochondrial 12S rRNA. Amplicons were heat-denatured and a PCR single strand conformation polymorphism (SSCP) method was developed to identify cattle, buffalo, sheep and goat DNA. Sensitivity of this technique was tested on DNA mixtures of cattle-sheep, cattle-goat and cattle-buffalo and the threshold limit of cattle DNA was 5%, 5% and 3% respectively. 105 cheeses were purchased and collected from Bosnian and Hungarian farmers, retails and supermarkets to reveal fraud, 32 percent of them (34 cheeses) were found to be mislabelled by species.
Suggested Reviewers:	Attila Zsolnai, PhD University of Kaposvar zsolnai.attila@ke.hu A. Zsolnai has experience and a number of papers in molecular genetic studies on livestock species.
	Agnes Bali Papp University of West Hungary

	bali@mtk.nyme.hu Prof. Agnes Bali Papp works on polymorphism, genetic markers of farm animals. Her papers represent a wide range of molecular genetic studies.

APPLICABILITY AND SENSITIVITY OF PCR SSCP METHOD FOR MILK SPECIES IDENTIFICATION IN CHEESE

A. CSIKOS[a], A. HODZIC[b], E. PASIC-JUHAS[b], A. JAVOR[a], A. HRKOVIĆ-POROBIJA[b], T. GOLETIC[c], G. GULYAS[a], L. CZEGLEDI[a*]

[a]Institute of Animal Science, Biotechnology and Nature Conservation, University of Debrecen, 138 Boszormenyi Street, 4032 Debrecen. Hungary

[b]Department of Physiology, [c]Department of Avian Diseases and Management, Veterinary Faculty, University of Sarajevo, 90 Zmaja od Bosne, 71000 Sarajevo. Bosnia and Herzegovina

Short title: Milk species identification by PCR SSCP

Abstract

Species identification in food has become a prominent issue in recent years as the importance of consumer protection has increased. DNA-based species identification methods were developed by researchers in last two decades, as these are reliable, accurate and low-cost techniques for species identification in raw and processed food products as well.

In our study universal primers were designed to conserved regions of mitochondrial 12S rRNA. Amplicons were heat-denatured and a PCR single strand conformation polymorphism (SSCP) method was developed to identify cattle, buffalo, sheep and goat DNA. Sensitivity of this technique was tested on DNA mixtures of cattle-sheep, cattle-goat and

* To whom correspondence should be addressed. Phone: +36 52 508 444/88199; fax: +36 52 486 285; e-mail: czegledi@agr.unideb.hu

cattle-buffalo and the threshold limit of cattle DNA was 5%, 5% and 3% respectively. 105 cheeses were purchased and collected from Bosnian and Hungarian farmers, retails and supermarkets to reveal fraud, 32 percent of them (34 cheeses) were found to be mislabelled by species.

Keywords: milk species, fraud, PCR SSCP, 12S rRNA

Livestock species identification in food has received increasing attention in the last two decades. Deceptive and fraudulent behaviour of unfair producers impair the consumers and fair manufacturers. Species identification of dairy products such as commercial cheeses has outstanding importance because some proteins may cause allergic reactions in the human body. One of the most common foods responsible for allergic reactions is cow milk. It is a major source of anaphylactic reactions during infancy (MAHLMEDEN YMAN, 2004). However, allergic reaction is not the only problem, but the misdescription of dairy products and replacement of expensive and high quality milk with a cheap and low quality milk impair the consumers and religion questions raises on the issue of foodstuff adulteration as well (LOCCI et al., 2008; ABDEL-RAHMAN & AHMED, 2007).

In recent decades many analytical and molecular biological techniques were developed by researchers worldwide, simultaneously with spread of foodstuffs adulterations (MAYER, 2005). Beside protein methods and fatty acid composition analysis, a number of DNA based techniques were applied for species identification.

Most DNA methods are based on PCR (polymerase chain reaction) with the theoretical background that milk derives from healthy udder of ruminants contains a large number of somatic cells e.g. leukocytes predominantly. These cell populations are used as a

source of genomic and mitochondrial DNA which are suitable for PCR reactions (AMILLS et al., 1997).

Papers studied variable and conserved regions of mitochondrial genes, such as 12S rRNA (LÓPEZ-CALLEJA et al., 2005, 2007), 16S rRNA (GHOVVATI et al., 2009; NATONEK-WIŚNIEWSKA et al., 2013), cytochrome b (DOOSTI et al., 2014), cytochrome oxydase subunit I (HAIDER et al., 2012), D-loop (MANE et al., 2009) sequences with duplex-, multiplex-PCR, PCR-RFLP and DNA sequencing.

The aim of this study was to design universal primers for the same regions of 12S rRNA mitochondrial gene of cattle, buffalo, sheep and goat. Target DNA region of control (species known) milk samples and commercial cheese samples was amplified in single PCR reaction and species specific patterns were determined by SSCP method on polyacrylamide gel. The PCR assay was validated by amplifications of control samples mixed to a certain degree with known DNA concentrations.

1. Material and methods

1.1. Samples

Milk of cattle, buffalo, sheep and goat were obtained from commercial dairy farms in Hungary. Fresh samples were collected in a 50 ml centrifuge tube and transported to the laboratory, stored at -20 °C until further analysis. Cheeses were purchased from hypermarkets in Debrecen, Hungary and Travnicki sheep cheeses were collected in Bosnia and Herzegovina. Cheese samples were stored at -20 °C.

1.2. Extraction and quantification of DNA

Genomic DNA was extracted from 1000 µl milk of cattle, buffalo, sheep and goat according to ZSOLNAI and ORBÁN (1999) with slight modifications. In brief, milk fat was discarded after centrifugation at 13 000 rpm, 5 min. DNA of cheese samples was isolated from 70 mg of cheese according to DE and co-workers (2011). DNA concentration and quality were measured by NanoDrop 1000 (Thermo Fisher Scientific, USA) spectrophotometer.

1.3 Primer design

Nucleotide sequences of 12S rRNA of cattle (GQ926965.1), goat (GQ926969.1), sheep (JQ622016.1) and buffalo (GU119953.1) were used from NCBI GenBank database. Nucleotide sequences were aligned by CLUSTALW2 algorithm of European Bioinformatics Institute (Figure 1). The universal forward and reverse primers produce a 279 bp, 281 bp, 282 bp and 283 bp amplicons for cattle, goat, sheep and buffalo respectively. Primers were tested by Oligoanalyzer for self-dimer and hetero-dimer structures.

Figure 1

1.4. PCR assay with universal primers

The PCR amplification of DNA from milk and cheese was carried out in 20 µl volume containing 4 mM $MgCl_2$ (Fermentas), 200 µM dNTP mix (Fermentas), 10x Dream Taq buffer (Fermentas), 2 pmoles reverse primer R (5' TTTACTGCTAAATCCTCCTT 3') (Sigma), 2 pmoles forward primer F (5' ACTCTAAGGACTTGGCGGTG 3') (Sigma), 1U Dream Taq

polymerase (Fermentas) and 100 ng DNA template. The PCR amplification was running in an ABI Gene Amp 9700 PCR System. The PCR was made up of discrete steps, first step is denaturation at 95 °C for 1.5 min, followed by 35 cycles consisting of denaturation at 95 °C for 0.5 min, primer annealing at 60 °C for 0.5 min and extension at 72 °C for 0.5 min. The final extension step was 5 min.

Amplified PCR products were resolved on 2 m/v% agarose gel (Lonza) for 1h at 100V in TAE (Tris-acetate-EDTA, pH: 8) (Lonza) buffer and stained by GelRed (Biotium, USA).

1.6. PCR-single strand conformation polymorphism (PCR-SSCP)

Previously amplified PCR products were heat-denatured at 95 °C for 5 min in the presence of 90 v/v% formamide-dye (8 v/v% bromophenol blue, xylene cyanol stain (Sigma); 92 v/v% formamide) and chilled on ice. Single stranded fragments were loaded onto a 10% acrylamide:bis-acrylamide (39:1) non-denaturing polyacrylamide gel (20 cm × 16 cm × 0.75 mm). Polyacrylamide gel electrophoresis was performed at 10 °C for 10 h with 20 V/cm.

SSCP bands on polyacrylamide gel were visualized by silver staining method (MERILL et al., 1984) and documented by Uvipro Platinum (Uvitec) gel documentation system.

2. Results and discussion

2.1. PCR-single strand conformation polymorphism

Before SSCP analysis PCR amplicons were verified by agarose gelelectrophoresis. A sharp DNA band was detected at each DNA sample extracted from milk and cheese of cattle, goat, sheep and buffalo as well (Figure 2.). During the PCR-SSCP method, PCR products

were separated on polyacrylamide gel. Due to denaturation, each DNA-strand formed unique, species-specific conformations, thus each milk species can be differentiated from each other. Polyacrylamide gel electrophoresis of PCR product resulted no false positive patterns (Figure 3).

Figure 2

Figure 3

These results prove that the designed universal primers could unambiguously discriminate cattle, goat, sheep and buffalo species. To evaluate the sensibility of method an experimental design was tested with different DNA dilution series. Cattle DNA was mixed with sheep, goat and buffalo DNA, each DNA mixture contained cattle DNA in 0.1, 0.5, 1, 3, 5, 10, 20, 30, 40, 50%. Control samples were cattle, sheep, goat and buffalo DNA in 100%. The detection threshold of PCR-SSCP method in case of buffalo-cattle DNA mixture was 3% presence of cattle DNA and 5% of cattle DNA for goat-cattle and sheep-cattle DNA mixture as well (Figure 4; Figure 5). The DNA bands were detected by Uvipro Platinum (Uvitec) software. The documented gel was analyzed by Gel Analysis function of Uvipro Platinum (Uvitec) gel documentation software. Volume of each DNA band was calculated using pixel intensities data by Uvipro Platinum software.

Figure 4

Figure 5

Applicability of studied PCR-SSCP method was tested on 105 commercial cheese samples from hypermarkets and retails. 64 cheeses were labelled as sheep milk cheeses, 16 cheeses were made of cow milk, 11 cheeses were made of goat milk, 13 cheeses were labelled as sheep and cow cheeses and 1 cheese was labelled as it was made of goat and cow milk. We detected the presence of undeclared species in 34 cheeses, that is 32.4% of analysed samples. Regarding sheep cheeses, 59.4% of them (38 of 64 cheeses) contained only sheep DNA, 23.4% of cheeses were made of sheep and cattle milk, and in 17.2% of cheeses only cattle DNA was detected. In each cow cheese only the presence of cattle DNA was proved. 11 goat cheeses were involved in the study, 54.5% of them contained goat and cattle DNA, 45.5% contained only goat DNA. 13 cheeses were labelled as made of sheep and cow milk, 84.6% (11 products) of 13 cheeses contained sheep and cattle DNA, in one cheese only sheep DNA and in another only cattle DNA were detected. 1 cheese was labelled as goat and cow cheese and this cheese was confirmed as no fraud, it contained the indicated species.

Cow milk is cheaper than other milk, such as sheep, goat or buffalo. Consequently one can expect if there is a fraudulent, a low cost milk will replace an expensive one in a certain amount. 29 of 30 cheeses labelling cow milk, was proved to contain cow DNA, the only exception was a sheep and cow cheese in which no cattle DNA was found. This latter finding may due to the detection limit of this SSCP method. Results of commercial cheese products are listed in Table 1.

Table 1

Consumer’s demands have increased on quality of foodstuffs in recent years. In parallel with these increasing demands the European consumer protection authorities have created a number of regulations in order to suppress food adulterations. The European Union

has legislated Commision Regulations that contains rules concerning labelling of foodstuffs and public information for consumers (COMMISION REGULATION, No. 1898/2006).

Increasing demands of consumers and governmental regulations infer the development of different analytical techniques for detection of adulteration in food industry. A number of DNA-based methods exceed among these procedures because of these techniques have several advantages in contrast to fatty acid analysis and protein-based methods, such as PCR-based methods have high sensitivity, reproducibility, applicability and these are cost-effective techniques in species identification practice (DE et al., 2011). The simplest form of PCR-based methods is single-PCR in which using one primerpair to detect presence of specified species (SANTOS et al, 2012).

This paper reports a PCR-SSCP method for the detection of cow, sheep, goat milk in commercial cheeses. The target of this study was a highly conserved region of mitochondrial 12S rRNA gene. Designed primers allowed the amplification of DNA sequences of milk species in parallel. In cattle-buffalo DNA mix, the detection limit was 3 ng for cattle DNA and 5 ng in cattle-sheep and cattle-goat DNA mixture. In other studies researchers published different detection limits. ZHA and co-workers (2010) have described 1ng of porcine, 5ng of poultry and 0.5ng of bovine meat detection limit in a multiplex-PCR assay.

In a former PCR-SSCP study seven fish species from Sparidae family were included and five of them were successfully differentiated from each other (SCHIEVENHÖVEL & REHBEIN, 2013). SRIPHAIROJ and co-workers (2010) have been conducted a successfull species identification work on 5 Pangasiid species by PCR SSCP. The mobility of single-stranded DNA in polyacrylamide gel depends on its specific conformation(s) that is determined by nucleotide sequence. Beside this primary factor, duration of electrophoresis

(REHBEIN, 2005), acrylamide/bis-acrylamide ratio, gel concentration, buffer concentration and temperature also influence the mobility of DNA fragments (HAYASHI, 1991).

3. Conclusions

In this study, the applicability and detection limit of PCR-SSCP method for milk species differentiation was aimed. The application of this method was tested on 105 commercial cheeses and sensitivity threshold was tested on sheep-cattle, goat-cattle and buffalo-cattle 0.1, 0.5, 1, 3, 5, 10, 20, 30, 40, 50% DNA mixture. In each species mixture cattle DNA was detected at 5% ratio, however the sensitivity of method was better for buffalo-cattle DNA mix, as cattle DNA was detected at the concentration of 3%. Undeclared species were found in 34 of 105 commercial cheeses from Bosnian markets and Hungarian hypermarkets. Based on our results we found that PCR-SSCP protocol, described in this paper, is a reliable, low-cost and appropriate method to detect species in milk and milk products, such as cheese. In practice, this assay can be an appropriate, DNA based tool to detect fraudulent adulteration of milk species. For future work, we suggest to develop and apply a capillary electrophoresis SSCP method, which might be a more sensitive technique compared to polyacrylamide electrophoresis, to increase sensitivity for fraud detection (GARCÍA-CANAS et al., 2004; ICHIM, 2011).

*This work was supported by Federal Ministry of Education and Science, Federation of Bosnia and Herzegovina, B&H.

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Table 1 *Identification of species in commercial cheese samples with PCR-SSCP*

Label (species)	Number of cheeses	Detected species by PCR-SSCP
Sheep	64	38 sheep
		15 sheep + cow
		11 cow
Cow	16	16 cow
Goat	11	6 goat + cow
		5 goat
Sheep and cow	13	11 sheep + cow
		1 sheep
		1 cow
Goat and cow	1	1 goat + cow

Figure captions

Figure 1 Multiple alignment of 12S rRNA sequences

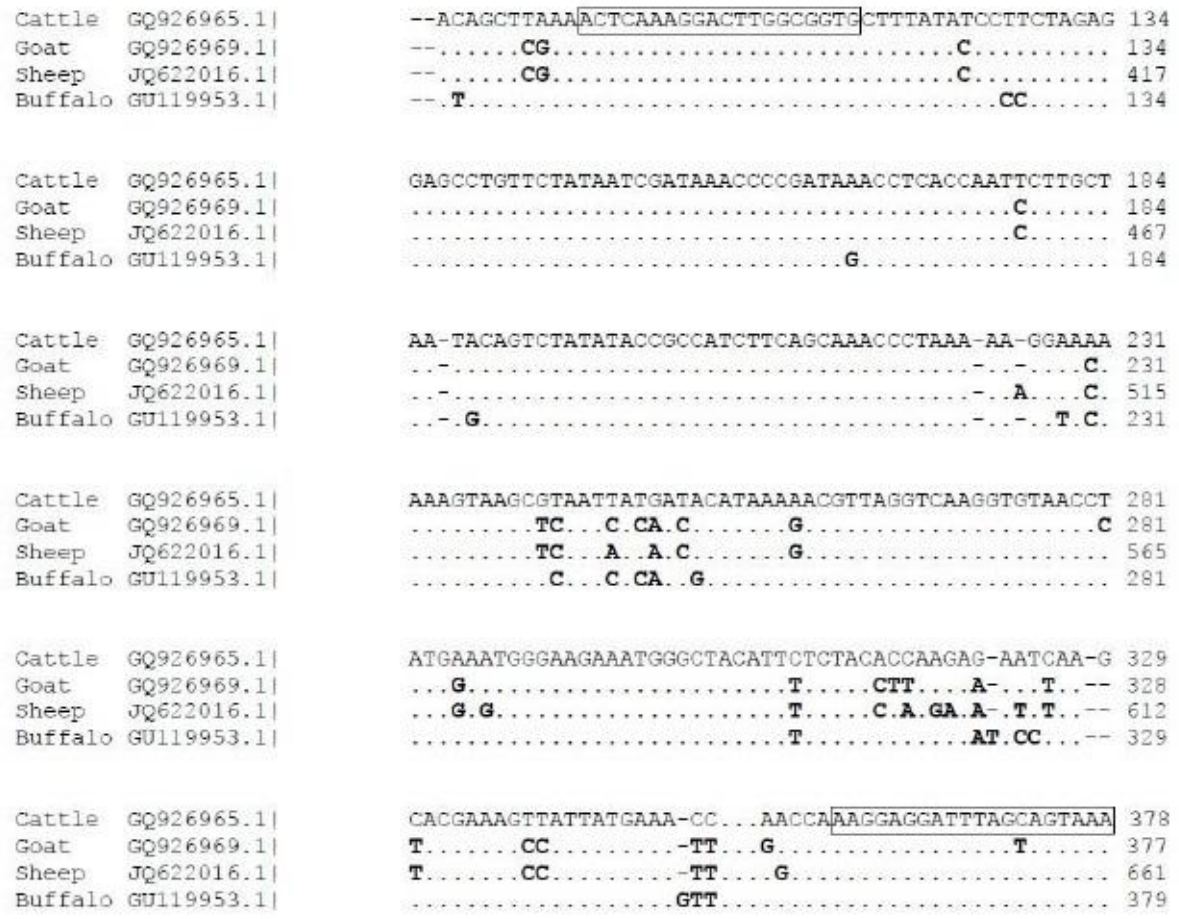

Figure 2 Electrophoretic analysis of the 12S rRNA PCR products on agarose gel. M: 50 bp DNA ladder; NC: negative control; lane 1: cow milk; lane 2: goat milk; lane 3: sheep milk; lane 4: buffalo milk; lane 5: sheep cheese; lane 6: sheep and cow cheese; lane 7: goat cheese; lane 8: goat and cow cheese; lane 9: cow cheese.

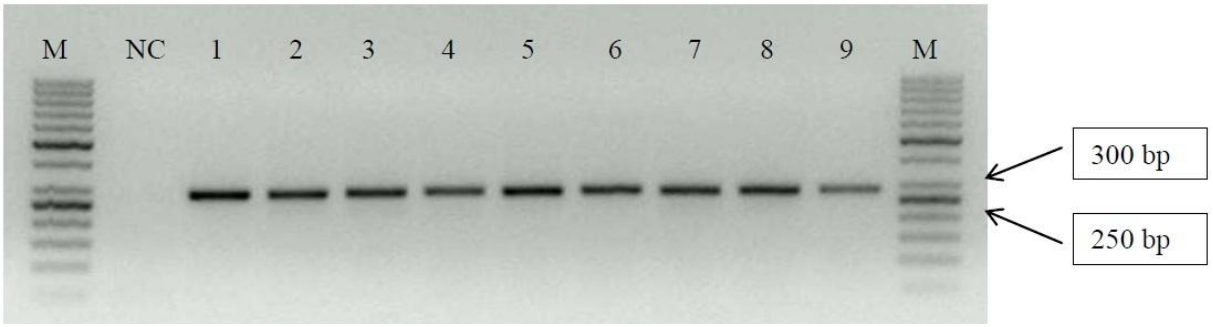

Figure 3 PCR-SSCP pattern of 12S rRNA of milk species. 1.: cattle; 2.: buffalo; 3.: sheep; 4.: goat; 5.: DNA mix of all species.

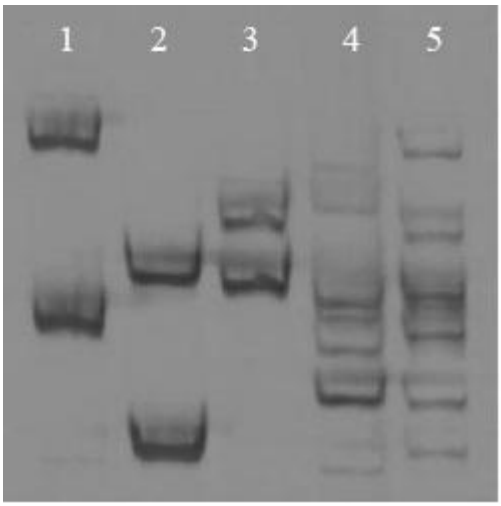

Figure 4 Electrophoretic analysis of PCR SSCP from mixtures of cow, buffalo, sheep, goat milk DNA. A: 0, 5, 10, 20, 30, 40, 50, 100% cow DNA in cow/buffalo DNA mixture; B: 0, 0.1, 0.5, 1, 3, 5, 100% cow DNA in cow/buffalo DNA mixture; C: 0, 5, 10, 20, 30, 40, 50, 100% cow DNA in cow/sheep DNA mixture; D: 0, 0.1, 0.5, 1, 3, 5, 100% cow DNA in cow/sheep DNA mixture E: 0, 5, 10, 20, 30, 40, 50, 100% cow DNA in cow/goat DNA mixture; F: 0, 0.1, 0.5, 1, 3, 5, 100% cow DNA in cow/goat DNA mixture.

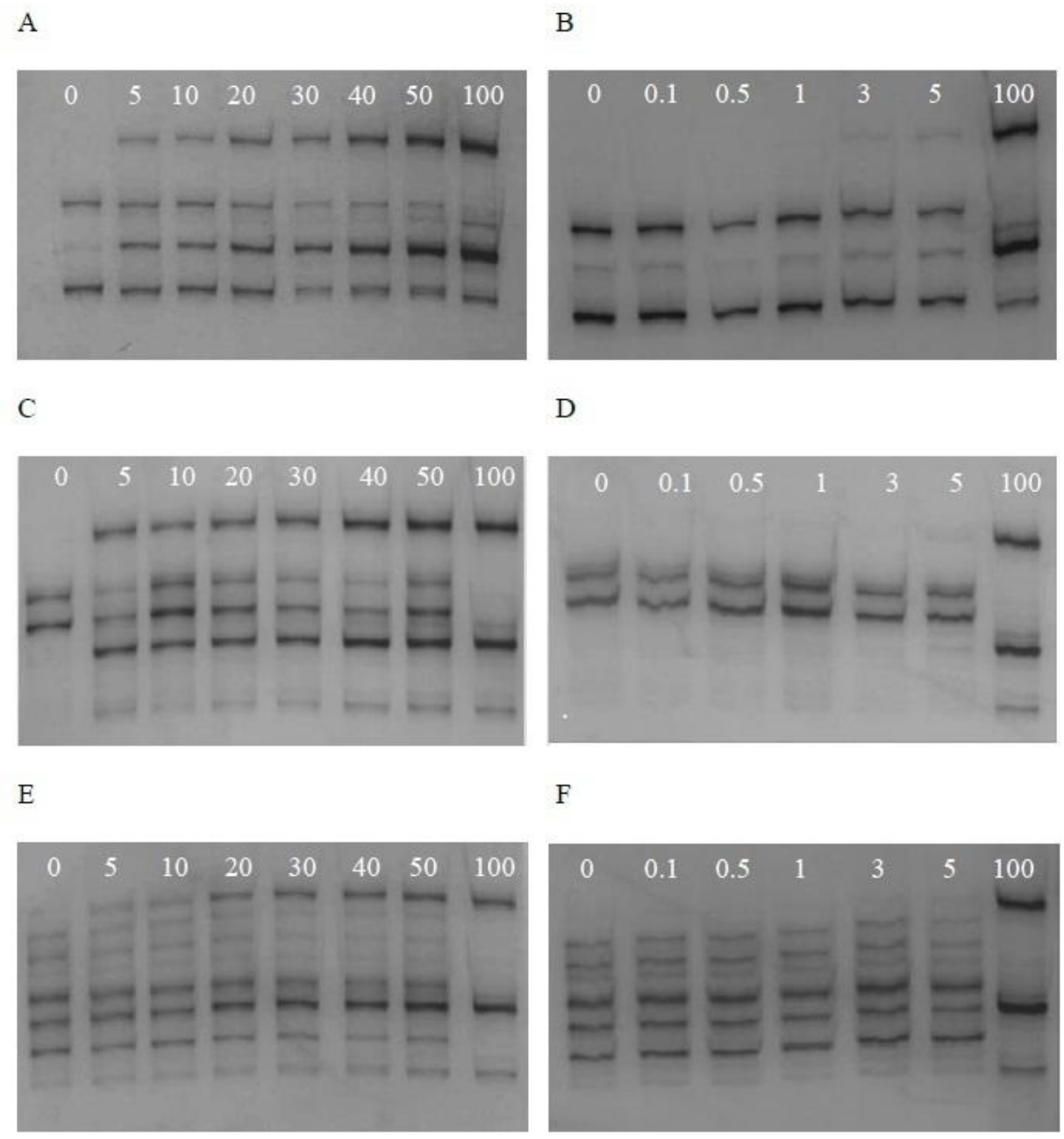

Figure 5 Volumes of PCR product bands of cattle (3 v/v%) and buffalo (97 v/v%) DNA mixture.

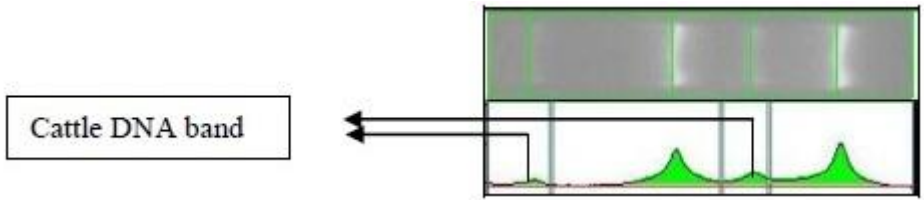

Figure1

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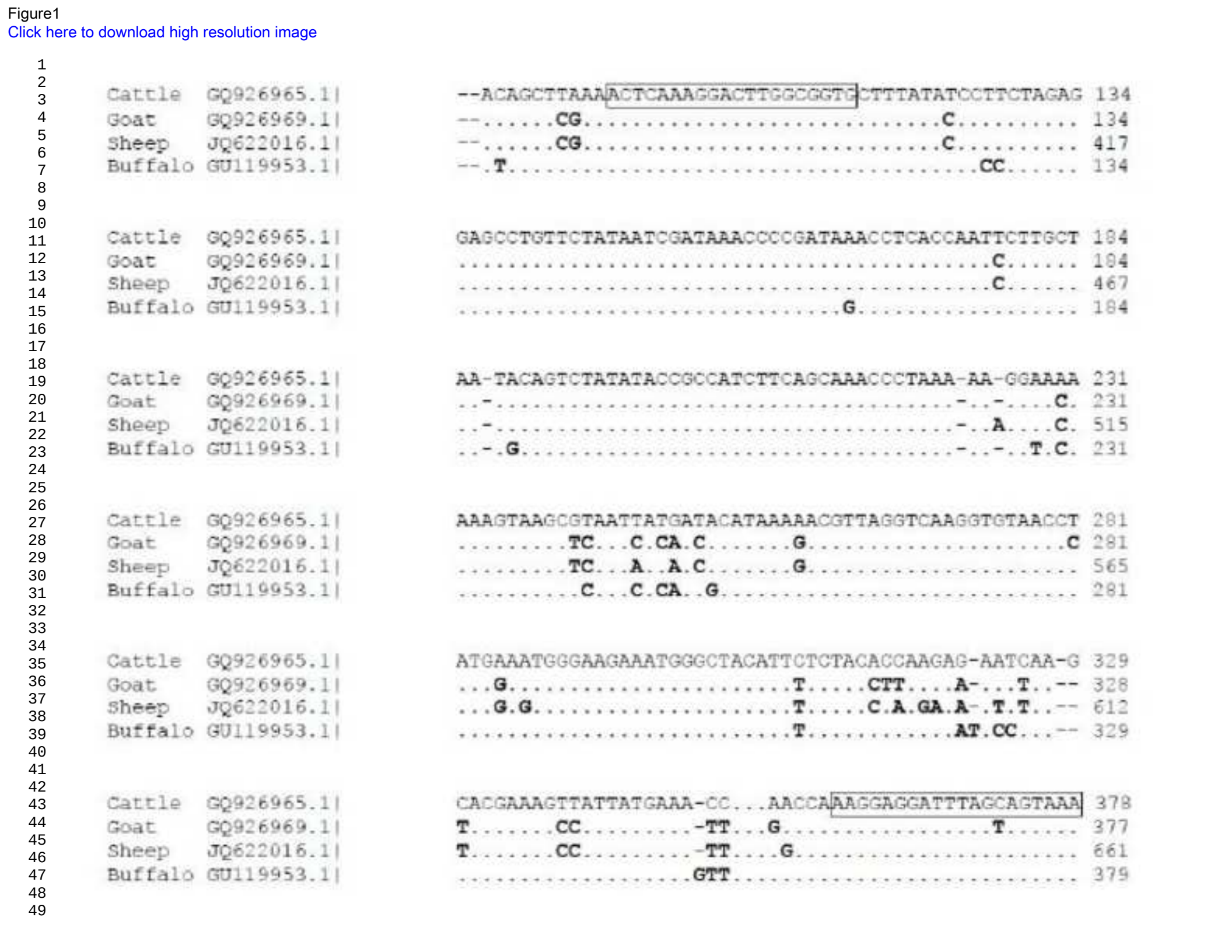

Figure2

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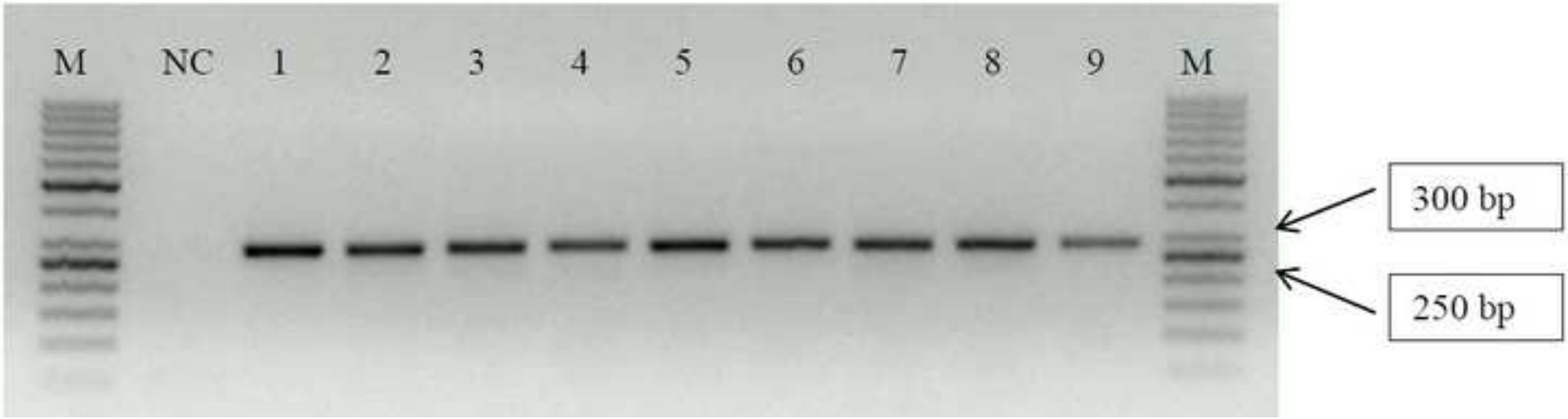

Figure3
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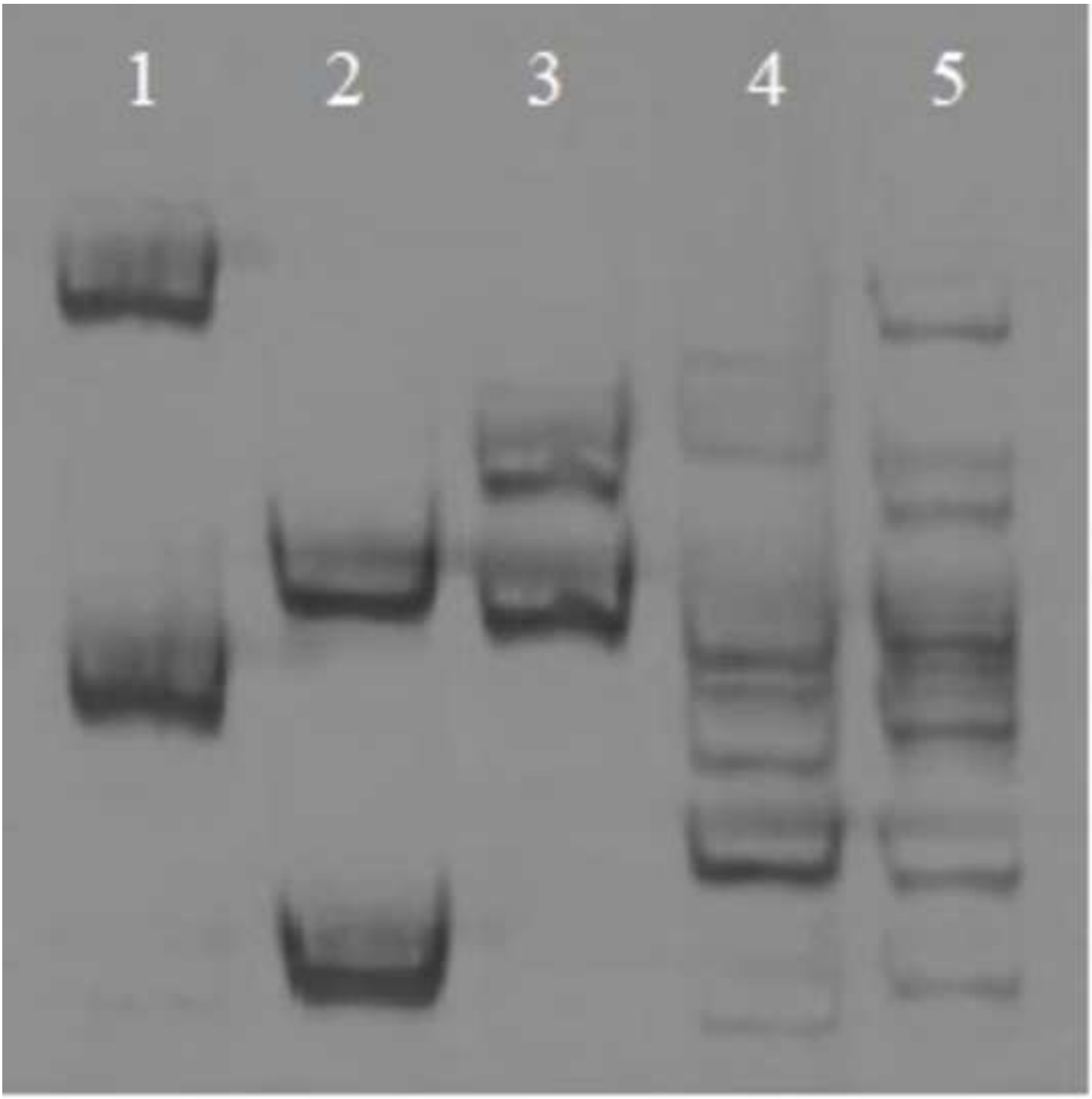

Figure4

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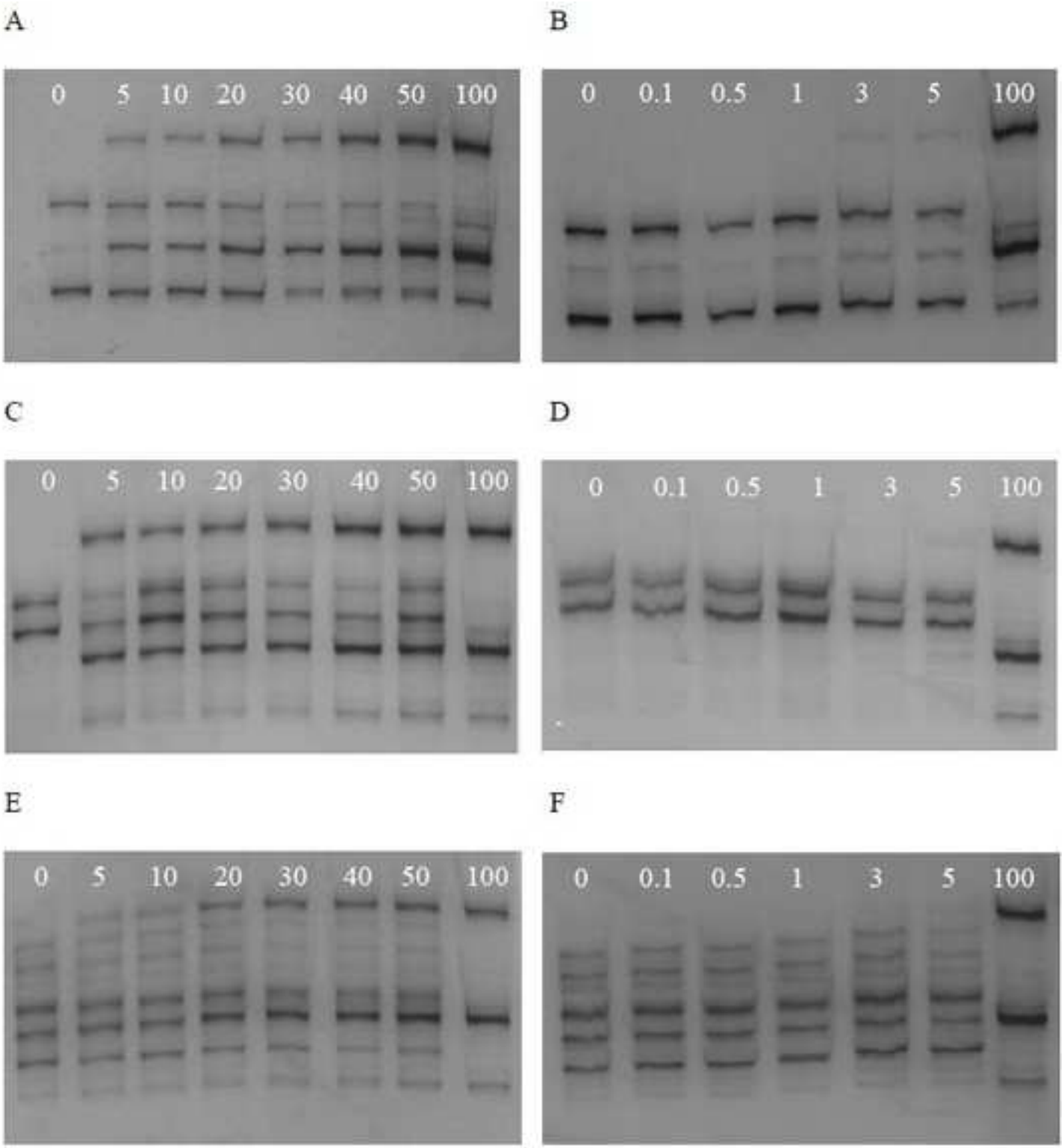

Figure5

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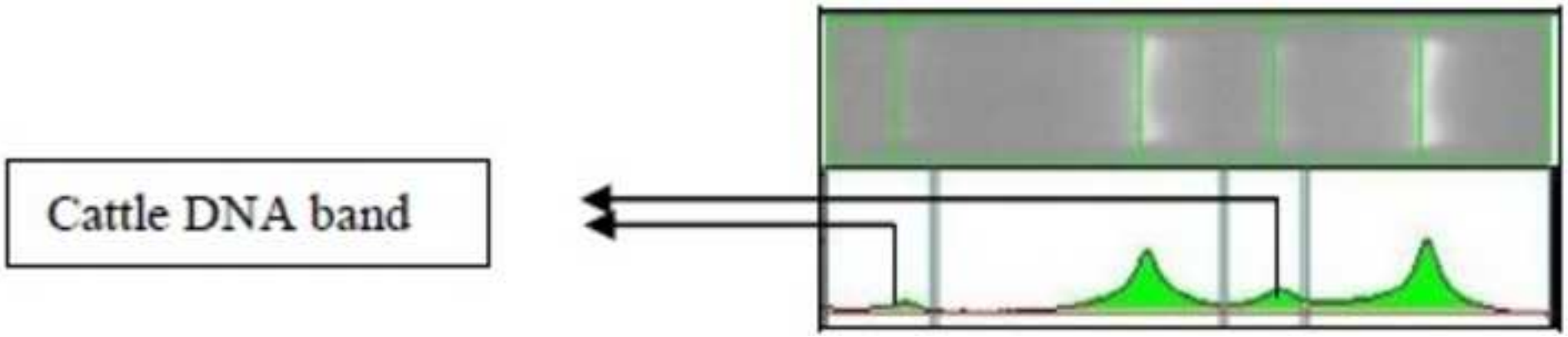


List of changes

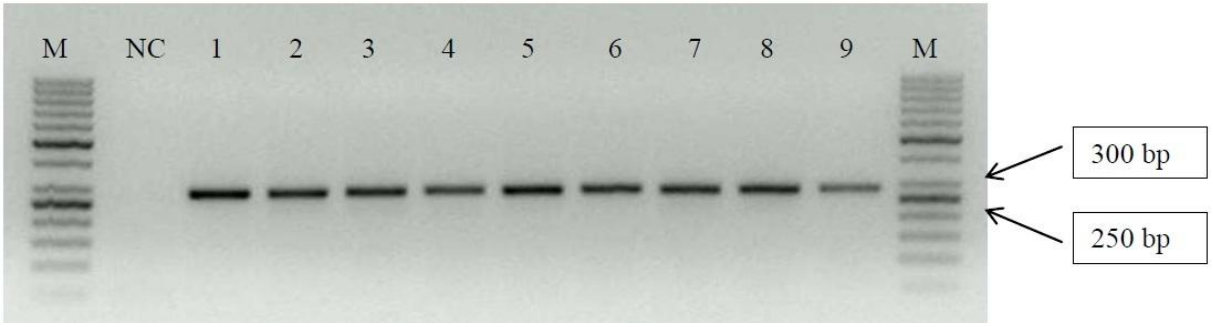
Applicability and sensitivity of PCR SSCP method for milk species identification in cheese by A. Csikos, A. Hodzic, E. Pasic-Juhas, A. Javor, A. Hrković-Porobija, T. Goletic, G. Gulyas, L. Czegledi

Reviewer 1:

Authors present figures of agarose gel results together from investigated sheep cheeses made from pure sheep milk or sheep and cow milk and from investigated goat cheeses made from pure goat milk or goat and cow milk.

Agarose gel result (Figure 2) was added to the manuscript (figure caption as well) and it has changed figure numbering in the text.

The recently added new figure, called Figure 2:



Its figure caption:

Figure 2 Electrophoretic analysis of the 12S rRNA PCR products on agarose gel. M: 50 bp DNA ladder; NC: negative control; lane 1: cow milk; lane 2: goat milk; lane 3: sheep milk; lane 4: buffalo milk; lane 5: sheep cheese; lane 6: sheep and cow cheese; lane 7: goat cheese; lane 8: goat and cow cheese; lane 9: cow cheese.

Changes, because of figure numbering:

Change a sentence in page 5:

Old sentence: A sharp DNA band was detected at each DNA sample extracted from milk and cheese of cattle, goat, sheep and buffalo as well.

New sentence: A sharp DNA band was detected at each DNA sample extracted from milk and cheese of cattle, goat, sheep and buffalo as well (Figure 2).

Change two sentences in page 6:

Old sentence 1: Polyacrylamide gel electrophoresis of PCR product resulted no false positive patterns (Figure 2).

New sentence 1: Polyacrylamide gel electrophoresis of PCR product resulted no false positive patterns (Figure 3).

Old sentence 2: The detection threshold of PCR-SSCP method in case of buffalo-cattle DNA mixture was 3% presence of cattle DNA and 5% of cattle DNA for goat-cattle and sheep-cattle DNA mixture as well (Figure 3; Figure 4).

New sentence 2: The detection threshold of PCR-SSCP method in case of buffalo-cattle DNA mixture was 3% presence of cattle DNA and 5% of cattle DNA for goat-cattle and sheep-cattle DNA mixture as well (Figure 4; Figure 5).

Reviewer 2:

How can the identification be made more sensitive? A possible answer is to use capillary electrophoresis if SSCP technique is maintained.

Added one sentence related to sensitivity of capillary electrophoresis-SSCP to Conclusions with two references.

New sentence: For future work, we suggest to develop and apply a capillary electrophoresis SSCP method, which might be a more sensitive technique compared to polyacrylamide electrophoresis, to increase sensitivity for fraud detection (GARCÍA-CANAS et al., 2004; ICHIM, 2011).

New references added in alphabetical order to References.

New references:

GARCÍA-CANAS, V., GONZÁLEZ, R. & CIFUENTES, A. (2004): The combined use of molecular techniques and capillary electrophoresis in food analysis. *Trends in Analytical Chemistry, 23, 9,* 637-643.

ICHIM, M.C. (2011): High-throughput screening for single nucleotide polymorphisms (SNPs) in specific DNA fragments by automated SSCP-based capillary electrophoresis. *Current Opinion in Biotechnology, 22S,* S103-S104.