Co-amplification and sequencing of a cytochrome *b* fragment affecting the identification of cattle in PCR-RFLP food authentication studies

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Abstract

Food authentication studies based on PCR-RFLP analysis are frequently targeted to well-conserved mitochondrial sequences, such as certain regions of the cytochrome *b* (*cytb*) gene. The use of mitochondrial L14841/H15149 universal *cytb*-targeted primers in PCR-RFLP assays revealed the existence of a complex restriction pattern in three genetically-unrelated Iberian (Northern Spain) cows, this being due to the simultaneous co-amplification of two 359 bp *cytb* fragments. Microsatellite analysis of 11 bovine-specific *loci* confirmed no familiar linkage among the animals investigated. Both co-amplification products were successfully separated by specific cleavage with endonucleases *Rsa*I and *Mva*I, which allowed the recovery of each amplification product, respectively. The new co-amplified *cytb* fragment described in this study was successfully sequenced, and exhibited a significantly high homology (95.1–99.3% range) with respect to mito- chondrial sequences previously described for a *Bos indicus* specimen and for another two Asian *Bos taurus*, this underlining that its presence in cattle may be more extended than initially thought. In contrast, the homology with the *cytb* sequence widely accepted for *B. taurus* was only 89.6%. The results presented in this work imply that food authentication studies by PCR-RFLP analysis may be complicated in the case of cattle by the co-amplification of two different *cytb* fragments.

Keywords: Food authentication; PCR-RFLP analysis; Species identification; Traceability; *Bos taurus* Cytochrome *b*; Mitochondrial DNA

1. Introduction

The use of mitochondrial universal primers for the amplification of well-conserved regions of the mitochondrial DNA (mtDNA) of land animals has been widely used for food authentication purposes (Bellagamba, Moretti, Comincini, & Valfre`, 2001; Meyer, Ho¨ felein, Lu¨ thy, & Candrian, 1995; Partis et al., 2000; Pascoal, Prado, Castro, Cepeda, & Barros-Vela´zquez, 2004; Prado et al., 2002; Wolf, Rentsch, & Hu¨ bner, 1999). This approach is based on the rationale that such primers normally allow the amplification of a single PCR product from each animal species that might be present in a food sample. However, genetic analyses based on mtDNA sequences may be sometimes complicated by mitochondrial heteroplasmy, this is the natural co-existence of two different mitochondrial genotypes in the same organism, an event that may lead to high variation in the mitochondrial genomic makeup within and between populations (Barr, Neiman, & Taylor, 2005). The natural co-existence of heteroplasmic genotypes has been reported in cattle (Hauswirth & Laipis, 1984; Hauswirth, Van de Walle, Laipis, & Olivo, 1984; Laipis, Van de Walle, & Hauswirth, 1988; Wu et al., 2000) or domestic pigs, among other animal species (Hsieh et al., 2001).

Another potential negative consequence linked to the use of universal primers targeted to mtDNA sequences is the potential co-amplification of mitochondrial pseudo- genes, relics of ancient mtDNA that are probably trans- ferred from the mitochondria to the nucleus (DeWoody, Chesser, & Baker, 1999; Perna & Ko¨ cher, 1996). Such non-functional pseudogenes can be co-amplified together with mtDNA functional genes and visualized as artefacts in routine PCR assays (Perna & Ko¨ cher, 1996; Zhang & Hewitt, 1996), thus complicating food authentication anal- yses based on PCR-RFLP studies. Although the amplifica- tion of pseudogenes has been described to be inefficient as compared with their mtDNA counterparts (DeWoody et al., 1999), especially when post-mitotic tissues exhibiting a high mtDNA/nuclear DNA ratio, such as skeletal mus- cle, are considered (Greenwood & Pa¨a¨bo, 1999; Sorenson & Quinn, 1998), their co-amplification is not fully pre- vented with universal primers.

Recently, we have reported the detection of a complex restriction pattern for beef in PCR-RFLP studies involving the analysis of the mitochondrial cytochrome *b* (*cytb*) gene of Iberian (Northern Spain) cattle (Prado, Calo, Cepeda, & Barros-Vela´zquez, 2005). Such complex restriction pattern is due to the co-amplification of an unexpected *cytb* frag- ment that may be widespread among Iberian cattle, with the subsequent negative implications on food authentica- tion studies. In this manuscript we provide molecular infor- mation of the co-amplified *cytb* fragment that may be useful for other authors with a view to prevent the misi- dentification of beef in food authentication studies based on the use of universal primers targeted to mitochondrial *cytb* sequences.

2. Materials and methods

2.1. DNA extraction and purification from the skeletal muscle of cattle

Muscle samples were obtained from the skeletal muscle of four different cows (*Bos taurus*), referred to by the codes *C*1, *C*2, *C*4 and *C*6. The animals belonged to local breeds from Northwestern Spain (*C*1 and *C*6), Northern Spain (*C*2), and Central Spain (*C*4). In all cases, samples were accurately labelled: the animal control number, place of origin, and name and location of the slaughterhouse were compiled. DNA was extracted from portions of 200 mg of the skeletal muscle of each animal using a commercial kit (DNeasy Tissue Isolation kit, QIAGEN, Darmstadt, Germany).

2.2. Amplification of a 359 bp cytb fragment with universal primers and restriction analyses

Amplification assays comprised in all cases template DNA (100 ng), a master mix (25 Ll) (BioMix, Bioline Ltd., London, UK) –this including reaction buffer, dNTPs, magnesium chloride and *Taq* DNA polymerase-, PCR water (Genaxis, Montigny le Bretonneaux, France) and each oligonucleotide primer (25 pmol), to achieve a final volume of 50 Ll. The universal CYTb primer pair, designed by Ko¨ cher et al. (1989), consisting of the set of primers: L14841 (5^0-CCATCCAACATCTCAGCATGATGAAA-3^0) and H15149 (5^0-GCCCCTCAGAATGATATTTGT CCTCA-3^0), was employed, these sequences being complementary to conserved areas from the vertebrate mitochondrial *cytb* gene, which yields a 359 bp PCR product. Amplification conditions were as follows: a previous denaturing step at 94 °C for 1 min 30 s was coupled to 35 cycles of denaturation (94 °C for 10 s), annealing (55 °C for 30 s), and extension (72 °C for 40 s), and to a final extension step at 72 °C for 15 min. When required, the annealing temperature was adjusted to either 52 °C or 56 °C. All PCR assays were carried out on a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA). Restriction enzymes *Pal*I(*Hae*III), *Mva*I, *Rsa*I, *Mbo*I and *Hin*fI were from Sigma (St. Louis, MO). Standard restriction assays were carried out for 2 h at 37 °C in a final volume of 20 Ll.

2.3. Microsatellite analysis in animals exhibiting a complex PCR-RFLP pattern

The amplification of bovine microsatellites in animals exhibiting a complex restriction pattern in the PCR-RFLP studies was carried out by means of the StockMarks For Cattle^{Ò} kit (Applied Biosystems), which contains a primer mix consisting of 11 primer sets, one for each of the micro- satellite markers recommended by the International Soci- ety for Animal Genetics (ISAG). Thus, the following 11 *loci* were analyzed: BM1824, BM2113, ETH3, ETH10, ETH225, INRA23, SPS115, TGLA53, TGLA122, TGLA- 126, and TGLA227. Once amplified the bovine DNA sequences by the eleven-plex PCR reaction, the polymor- phism of the microsatellites was analyzed by means of automated DNA sizing technology using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) provided with the GeneMapper^{TM} software (Applied Biosystems).[1]

2.4. DNA electrophoretic separation, elution and image analyses

PCR products were processed by horizontal 2.5% aga- rose electrophoresis. PCR-RFLP analyses were performed in both agarose gels and ExcelGel homogeneous SDS-poly- acrylamide (15%) gels (Amersham Biosciences, Uppsala, Sweden). Restriction fragments were visualized in poly- acrylamide gels by a standardized silver staining protocol (Amersham Biosciences). DNA elution from agarose gels

[1] The DNA sequences of each *cytb* fragment determined for animals *B. taurus* C1, C2 and C6 are available from GenBank (accession numbers: AY682374, AY682375, AY682376, AY682377, AY682379, and AY682380, respectively). The expected sequence is referred as genotype 1, while the co-amplified product is referred as genotype 2. The sequence corresponding to *B. taurus* C4 is also available at GenBank (accession number: AY682378).

was performed by means of the MinElute Gel Extraction kit (QIAGEN). Image analysis was carried out by means of the 1-D Manager software (TDI, Madrid, Spain).

2.5. DNA sequencing and computer analyses

Both strands of PCR products were sequenced to improve the reliability of the sequencing data by means of an automated DNA sequencing system (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems). The DNA sequences were carefully reviewed by eye using the Chromas software (Griffith University, Queensland, Australia). Prediction of restriction sites was carried out with the DNASIS software (Hitachi Software Engineering Co., Japan). Alignment of sequences was carried out using the CLUSTALW software (Thompson, Higgins, & Gibson, 1994).

3. Results

3.1. Electrophoretic detection of complex PCR-RFLP patterns in the skeletal muscle of cows

Restriction analyses of the 359 bp *cytb* amplification products with *Pal*I revealed different profiles for sample C4, on one hand, and for samples *C*1, *C*2 and *C*6, on the other (Fig. 1). Thus, C4 sample exhibited the pattern expected for cattle, consisting of restriction fragments of 285 bp and 74 bp, while samples *C*1, *C*2 and *C*6 showed a polymorphic restriction pattern for *Pal*I, DNA fragments of 285 bp and 74 bp co-existing with additional fragments of 159 bp and 126 bp (Fig. 1). Similar results – i.e., the presence of more complex restriction profiles– were also observed for samples *C*1, *C*2 and *C*6 when the 359 bp *cytb* fragment was treated with either *Mbo*I or *Hin*fI (data not shown). In contrast, in all cases sample *C*4 exhibited the restriction profiles expected for cattle according to the sci- entific literature (Anderson et al., 1982; Irwin, Ko¨ cher, & Wilson, 1991).

Microsatellite analyses confirmed that all three animals in which a complex PCR-RFLP pattern was observed, exhibited allele sizes that fell within the size ranges for all 11 bovine-specific *loci* analyzed (Table 1). As expected,

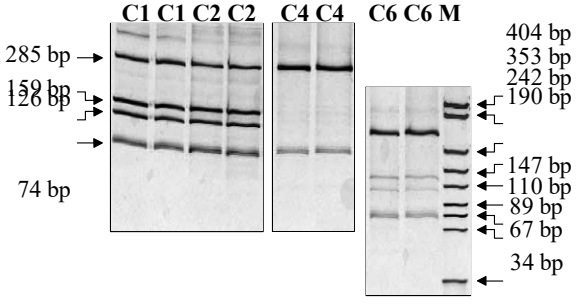

Fig. 1. PCR-RFLP analysis of the mixture of 359 bp *cytb* fragments in *B. taurus C*1, *C*2, *C*4 and *C*6 after cleavage with enzyme *Pal*I (*Hae*III). Unexpected restriction fragments of 159 bp and 126 bp are visible in

Table 1
Polymorphism of microsatellite sequences in animals exhibiting two *cytb* haplotypes

Locus	Chromosome	Expected size range (bp)	Allele size (bp)	Animals
BM1824	1	170–218	178/188	*C*1
			180/182	*C*2
			182/188	*C*6
BM2113	2	116–146	127/139	*C*1
			127/133	*C*2
			125/127	*C*6
ETH3	19	90–135	117/119	*C*1
			117/127	*C*2
			125/127	*C*6
ETH10	5	198–234	221/223	*C*1
			215/217	*C*2
			217/221	*C*6
ETH225	9	136–165	140/150	*C*1
			150	*C*2
			140/148	*C*6
INRA23	3	193–235	200/208	*C*1
			200/202	*C*2
			206/218	*C*6
SPS115	15	235–265	248	*C*1 and *C*2
			248/252	*C*6
TGLA53	16	147–197	172	*C*1
			160/172	*C*2
			170/186	*C*6
TGLA122	21	134–193	151/153	*C*1 and *C*2
			143/151	*C*6
TGLA126	20	104–131	115/121	*C*1
			115/117	*C*2
			115/123	*C*6
TGLA227	18	64–115	83/99	*C*1
			89/95	*C*2
			83/89	*C*6

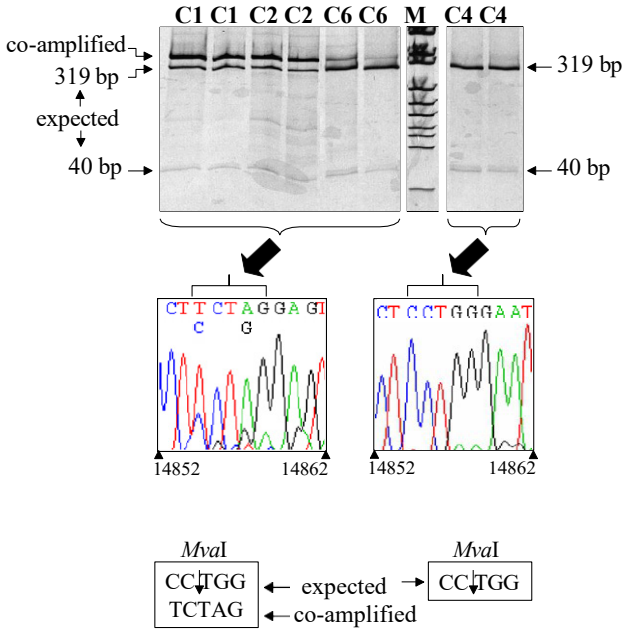

Fig. 2. PCR-RFLP and nucleotide sequence evidence of the specific animals *C*1, *C*2 and *C*6. *M*: molecular weight marker.

cleavage of the expected *cytb* fragment with the enzyme *Mva*I in animals *C*1, *C*2 and *C*6. The co-amplified 359 bp *cytb* fragment from animals *C*1, *C*2 and *C*6 remains intact. The presence of only the expected restriction pattern in animal *C*4 is also shown. *M*: molecular weight marker.

microsatellite analysis also confirmed that animals *C*1, *C*2 and *C*6 did not have any linkage among them. As it can be seen in Table 1, 40 bovine-specific alleles were identified in the 11 *loci* investigated. The greatest polymorphism was observed at *loci* INRA23 (5 alleles), while the lowest polymorphism was observed at *locus* SPS115 (2 alleles). Interestingly, none of the 11 *loci* proved to be monomorphic. The animal *C*6 resulted to be heterozygotic at all 11 *loci* investigated, while animals *C*1 and *C*2 exhibited homozygosis at 2 and 3 *loci*, respectively (Table 1).

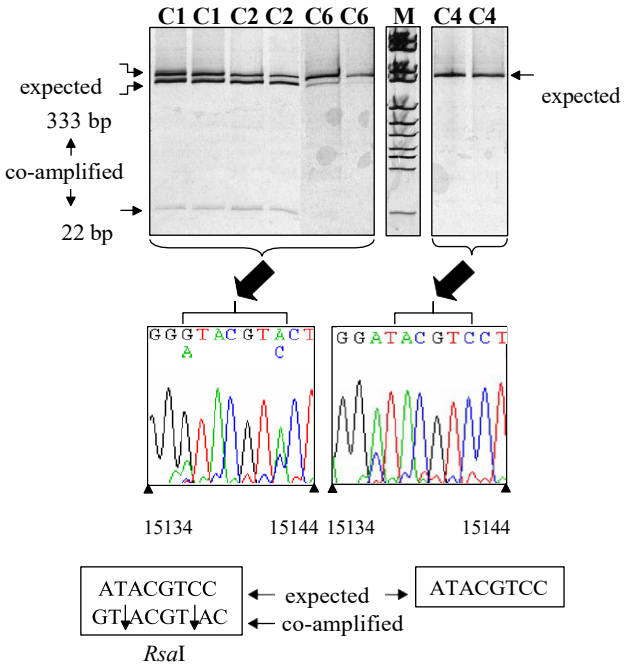

Fig. 3. PCR-RFLP and nucleotide sequence evidence of the specific cleavage of the co-amplified *cytb* fragment with the enzyme *Rsa*I in animals *C*1, *C*2 and *C*6. The expected 359 bp *cytb* fragment from animals *C*1, *C*2 and *C*6 remains intact. The presence of only the expected uncleaved 359 bp *cytb* fragment in animal *C*4 is also shown. *M*: molecular weight marker.

3.2. Isolation and sequencing of a new co-amplified cytb fragment from cattle

The variation of the annealing temperature of the amplification cycle in the 52–56 °C range allowed the preferential amplification of each individual *cytb* fragment over the other, although such strategy did not prevent the co-amplification of the non-predominant counterpart *cytb* frag- ment. Accordingly, the next goal of this study was to separate and purify the co-amplified *cytb* fragment prior to its sequencing. With this purpose in mind, having obtained preliminary information about the nucleotide sequence for each *cytb* fragment in C6 sample (Prado et al., 2005), we selected two restriction enzymes that would specifically cleave one of the *cytb* fragments but not the other, respectively. The results of such assumption are shown in Figs. 2 and 3. Thus, treatment with the endonuclease *Mva*I confirmed the presence of an uncut 359 bp amplification product (co-amplified product) and two smaller DNA fragments of 319 bp and 40 bp (expected PCR product) not only in sample *C*6, but also in samples *C*1 and *C*2 (Fig. 2). This result confirmed the co-existence in samples *C*1, *C*2 and *C*6 of a co-amplified 359 bp product not recognized by *Mva*I and the expected PCR product harbouring a restriction site for this endonuclease, while only the latter fragment was present in animal C4 (Fig. 2). Likewise, incubation with endonuclease *Rsa*I revealed the co-existence of an uncut 359 bp DNA band (expected PCR product) and two smaller DNA fragments of 333 bp and 22 bp (derived from the co-amplified product) in samples *C*6 and, interestingly, also in samples *C*1 and *C*2 (Fig. 3). The presence of a 4 bp fragment, predicted by sequence analysis and that would reconstitute the integrity of the co-amplified 359 bp fragment, could not be visualized owing to its very small size. These results also confirmed the co-existence of two *cytb* fragments in samples *C*1, *C*2 and *C*6: The expected PCR product was not cleaved by *Rsa*I while the co-amplified product harboured two

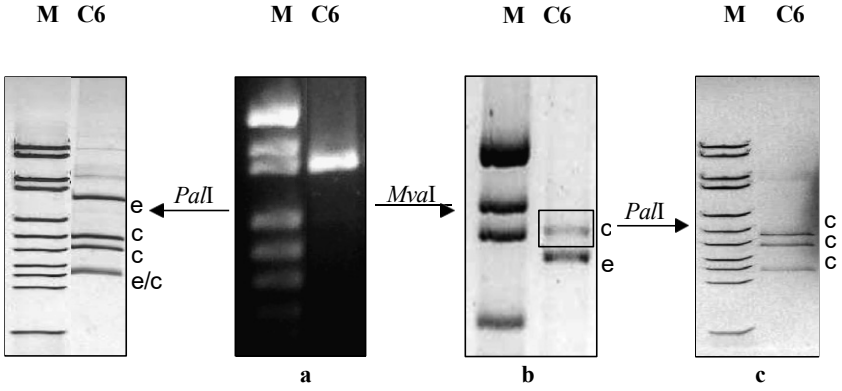

Fig. 4. Purification of the co-amplified *cytb* product from animal *C*1. Evidence of the presence of two *cytb* fragments was checked by treatment of the amplification products (a) with endonuclease *Pal*I: the mixture of restriction fragments corresponding to the expected PCR product – (e: 285 bp and 74 bp)– and the co-amplified product (c: 159 bp, 126 bp and 74 bp)– are indicated (left box). (b): cleavage with endonuclease *Mva*I allowed the separation of an intact 359 bp fragment, corresponding to the co-amplified *cytb* fragment (c), and a restriction fragment of 319 bp, corresponding to the expected *cytb* fragment (e). (c): the 359 bp fragment was eluted from the agarose gel and cleaved with the endonuclease *Pal*I: only restriction fragments corresponding to

the co-amplified *cytb* fragment (c: 159 bp, 126 bp and 74 bp) were observed. *M*: molecular weight marker.

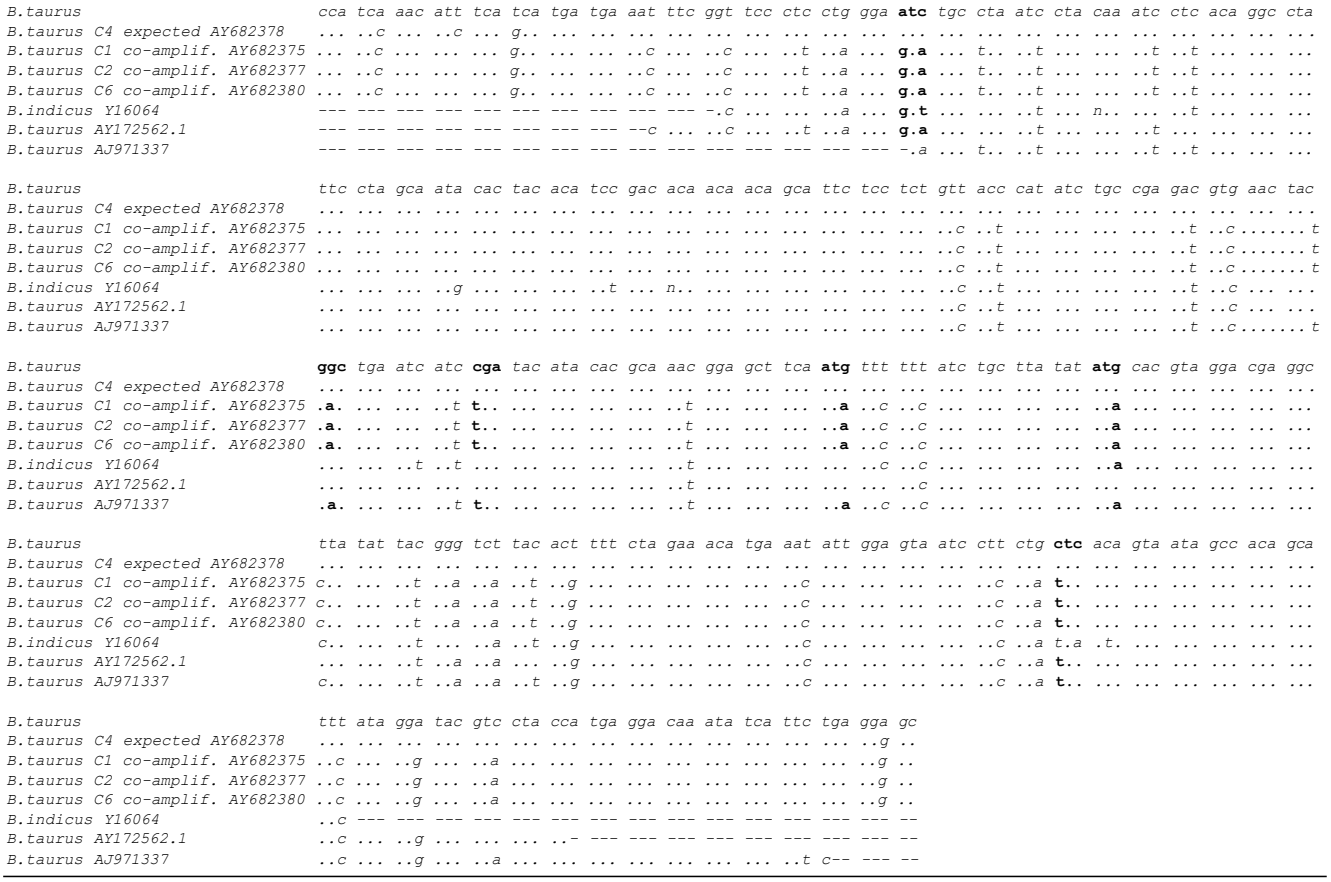

Fig. 5. Sequence variations observed in the 14816–15174 *cytb* region between the co-amplified fragments from animals *C*1, *C*2 and *C*6, with respect to the cow *C*4, *B. taurus* (Irwin et al., 1991), and homologous sequences from *B. taurus* AY172562, *B. indicus* Y16064 and *B. taurus* AJ971337. All sequences retrieved from the NCBI databases are identified with their accession numbers. Nucleotide changes implying amino acid substitutions are highlighted in bold characters.

restriction sites for this endonuclease. Only the former profile was present in the animal C4 (Fig. 3).

The application of this purification strategy to the *C*1 sample is shown in Fig. 4. Thus, after confirmation with *Pal*I that the 359 bp PCR products (Fig. 4a) from the sam- ple *C*1 included both *cytb* fragments (Fig. 4, left box), the mixture of amplification products was incubated in the presence of *Mva*I, specific for the expected amplification product, this allowing the separation of the intact 359 bp co-amplified product (Fig. 4b). The latter was eluted from the agarose gel, purified, and subjected again to cleavage with *Pal*I, only restriction fragments of 159, 126 and 74 bp, characteristic of the co-amplified product, being observed (Fig. 4c). This strategy proved to be simple and straightforward for achieving the separation and purifica- tion of co-amplified products in animals exhibiting com- plex PCR-RFLP patterns. Alternative purification of the intact expected PCR product could be achieved readily by digestion with enzyme *Rsa*I. Such enzyme specifically cleaved the 359 bp corresponding to the co-amplified prod- uct, yielding restriction fragments of 323 bp, 22 bp and 4 bp, this allowing the specific recovery of the uncut expected 359 bp *cytb* fragment by simple elution from the agarose gel.

Fig. 5 shows the DNA sequences obtained for the co-amplified *cytb* fragment in samples *C*1, *C*2 and *C*6. Such co-amplification product exhibited 89.6% homology with respect to the expected *B. taurus* sequence also found in such animals, and shared 95.1% homology with a mtDNA *cytb* sequence from a *Bos indicus* specimen (GenBank accession number: Y16064.1) (Schreiber, Seibold, Noetz- old, & Wink, 1998), 96.1% homology with a mtDNA *cytb* sequence from an Asian *B. taurus* specimen (GenBank accession number: AY172562.1) (Lei et al., 2002), and up to 99.3% homology with a *cytb* mitochondrial sequence recently reported for *B. taurus* in Central Asia (GenBank accession number: AJ971337) (Oshaghi, Chavshin, Vatandoost, & Yaaghoobi, 2005) (Fig. 5).

4. Discussion

Food authenticity is an issue of major concern for the administration, since incorrect labelling of animal foods may have remarkable negative consequences. On one hand, incorrect food labelling represents a commercial fraud to the consumer, especially when an animal species is replaced by other of lower commercial value. On the other hand, incorrect labelling may also have implications for health,

especially in the case of consumers who exhibit sensitivity to undeclared allergens. In addition, people following religious dietary restrictions are also defrauded with such replacements.

Among DNA-based methods for species identification, PCR-RFLP studies aimed at investigating mitochondrial sequences, such as the variable regions of the cytochrome *b* (*cytb*) gene, offer two main advantages: (i) mtDNA is present in thousands of copies per cell (as many as 2500 copies), especially in the case of post-mitotic tissues such as skeletal muscle, this increasing the probability of achiev- ing a positive result even in the case of samples suffering severe DNA fragmentation due to intense processing con- ditions; and (ii) the large variability of mtDNA targets as compared with nuclear sequences facilitates the discrimina- tion of closely-related animal species even in the case of mixtures of species.

Recently, an increasing number of scientific reports describing both the existence of mitochondrial heteroplasmy and the presence of nuclear pseudogenes of mitochondrial origin have complicated genetic analysis based on mtDNA sequences (for a review: Barr et al., 2005; Bensasson, Zhang, Hartl, & Hewitt, 2001). The discovery of mitochondrial genotypes by PCR-RFLP has proved to be a simple and useful method for checking the absence of *Bos gaurus* mtDNAs in a cloned animal, obtained by the fusion of somatic cells of *B. gaurus* with enucleated oocytes from *B. taurus* (Lanza et al., 2000). According to those authors, the PCR-RFLP approach would be able to detect as little as 1% of each individual mtDNA. Another previous study aimed at detecting mitochondrial genotypes proposed the use of an endonuclease specific for the mtDNA of the donor in order to determine the percentage of each mtDNA in different types of hetero- plasmic transgenic mice (Jenuth, Peterson, & Shoubridge, 1997). In that work, the mtDNA haplotype was also determined in PCR products by RFLP analysis (Jenuth et al., 1997).

In our study we picked up on these ideas, initially considered for animals derived from biotechnology, with a view to obtaining new evidence that the approach sug- gested by these authors may also be useful for the individ- ual purification of co-amplified PCR products from animals exhibiting complex restriction patterns in PCR- RFLP studies. Thus, RFLP analysis of mtDNA allowed us to identify endonucleases that would exhibit potential specificity for only one of the PCR products, this allowing the separation and individual characterization of the counterpart co-amplified PCR product.

Several authors have described that sequence variations between co-existing mtDNA molecules in heteroplasmic animals are frequently very low (Hauswirth et al., 1984; Hsieh et al., 2001; Laipis et al., 1988; Wu et al., 2000). In contrast, Boettcher et al. (1996) described a surprisingly high degree of sequence variation in the mtDNAs of Holstein cows with the same maternal lineage. On the other hand, nuclear pseudogenes of different sizes and exhibiting varying degrees of similarity to their mitochondrial counterparts have been described (Zhang & Hewitt, 1996).

In our work we observed a sequence variation as high as 10.4% between both 359 bp amplification products derived from the *cytb* gene of Iberian (Northern Spain) cattle. The fact that microsatellite analysis of 11 bovine-specific nuclear *loci* in the three animals exhibiting a complex PCR-RFLP pattern confirmed that all three animals did not have any linkage among them, and suggests that the natural co-existence of both *cytb* sequences could be more widespread than initially expected. Indeed, both *cytb* geno- types were found in three of the four animals investigated in this work, and an additional study on Iberian (Northern Spain) cattle involving 12 animals revealed the co-existence of both *cytb* genotypes in more than half of the animals investigated (data not shown). Moreover, the sequence of the *cytb* fragment co-amplified in this work shared high homology with respect to other mitochondrial sequences reported for cattle by other German (Schreiber et al., 1998) and Asian (Lei et al., 2002) scientists, and resulted to be almost identical to a *cytb* sequence recently described for *B. taurus* in Iran (Oshaghi et al., 2005), a result that underlines that the natural presence of the co-amplified *cytb* fragment characterized in this work may be more extended and frequent than expected in domestic cattle.

Although it is not the main objective of this work to elucidate the cellular location of the co-amplified *cytb* fragment characterized in this work, the following contradictory hypothesis might be especulated from the results obtained in this study: (i) the high ratio of divergence between the sequence of both co-amplification products (higher than 10%) would support the notion that the co-amplified product might represent a nuclear pseudogene; but (ii) the lack of frameshift mutations or stop codons confirmed by sequence analyses did not reveal non-gene-like features for such co-amplified PCR product, and would point towards a mitochondrial location. It should also be remarked that the fact that this study was focused on skeletal muscle, a post-mitotic tissue that exhibits a high mtDNA/nuclear DNA ratio, complicates but does not avoid completely the possibility of co-amplifying a nuclear pseudogene (Bensasson et al., 2001).

5. Conclusion

In summary, in the present work the use of universal primers aimed at amplifying mtDNA sequences in the skeletal muscle of cattle allowed the discovery of a complex PCR-RFLP pattern in cows from local breeds in Northern Spain. Such a complex pattern is derived from the co-amplification of a new *cytb* fragment that has been successfully isolated and sequenced in this study. The high homology between such co-amplified product and other *cytb* sequences found in other Asian bovines, underlines the fact that such sequence may be more extended than initially expected, with the subsequent negative implications concerning the potential misidentification of cattle in

PCR-RFLP food authentication studies involving the use of universal primers.

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