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Authentication of meat from game and domestic species by SNaPshot minisequencing analysis

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Abstract

The aim of the present study is to develop an assay for the specific identification of meat from *Capreolus capreolus*, *Cervus elaphus*, *Capra ibex*, *Rupicapra rupicapra*, targeting sequences of the cytochrome b (cyt b) gene of mitochondrial DNA. The assay is also intended to enable differentiation between meat from these wild species as well as *Ovis aries*, *Capra hircus*, *Bubalus bubalis*, *Bos taurus* and *Sus scrofa* domestic species.

The primers used in the preliminary PCR were designed in well conserved regions upstream and downstream of the diagnosis sites. They successfully amplified a conserved 232 bp region from the cyt b gene of all the species taken into consideration. The sites of diagnosis have been interrogated using a minisequencing reaction and capillary electrophoresis. All the results of the multiplex PER (primer extension reaction) test were confirmed by fragment sequencing. The assay offers the possibility of discriminating nine species at the same time.

Keywords: Species identification; Meat; Cyt b; SNaPshot

1. Introduction

In the last few years, the consumption of game meat has gained increasing favour among consumers, who appreciate its texture and flavour as well as the low fat and cholesterol content and its lack of anabolic steroids or other drugs. The high commercial value of game meat has sometimes induced fraud, such as mislabelling or selling less valuable meat as meat from more appreciated species (Fajardo et al., 2006). Therefore, it is important to establish the animal species in order to detect possible falsifications.

Nowadays a number of different assays have been developed in this field. Those based on protein analysis such as electrophoresis and immunoenzymatic tests (Hsieh, Sheu, & Brifgman, 1998) have been abandoned because of their low specificity (Berger, Mageau, Schwab, & Johnston, 1988). Game species are difficult to discriminate, as they are closely related to each other and are phylogenetically similar to some domestic species. Hence, the assays based on DNA analysis are preferable for differentiation and identification. In particular, due to the high mutation rate of mitochondrial DNA (mtDNA), 10 times greater than nuclear DNA, point mutations accumulate very quickly allowing the discrimination of closely related species (Jorde, Bamshad, & Rogers, 1998). Cytochrome b (cyt b) contains species-specific information and it has been widely used in a considerable number of studies on phylogenesis and in studies dealing with forensic science and food inspection. In these fields, the application of polymerase chain reaction (PCR) seems to give the most satisfactory results (Teletchea, Maudet, & Hänni, 2005). However, species-specific primers cannot be designed when species are very closely related. In these cases it is advisable to apply

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techniques such as PCR-RFLP (restriction fragment length polymorphism) which is based on the analysis of the single species-specific mutations (sites of diagnosis). This method allows the amplification of DNA by PCR and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes (Kelly, Carter, & Cole, 2003).

PCR-RFLP analysis of a conserved fragment from the mitochondrial 12S rRNA has been applied to identify several closely related domestic animal and game species (Fajardo et al., 2006; Pfeiffer, Burger, & Brenig, 2004; Wolf, Rentsch, & Hubner, 1999).

However, the possible individual mutations make the selection of endonucleases in conserved restriction sites difficult, and several enzymes are often needed to discriminate between phylogenetically related species and when several species have to be differentiated simultaneously (Wolf et al., 1999).

In addition the use of many restriction enzymes and analysis of more than one diagnosis site make PCR-RFLP difficult to automate.

Other method such as RAPD (random amplified polymorphic DNA) was successfully associated with PCR-RFLP for game species differentiation. The main advantage of RAPD is that the technique usually generates products which can be seen as DNA fingerprints on gel electrophoresis. However, the patterns are not always reproducible due to factors such as cycling conditions or intra-species polymorphism (Koh, Lim, Chua, Chew, & Phang, 1998). So, other techniques have been suggested. Among these, the sequencing is particularly suitable when a specific attributions needed and it is not sufficient to confirm or exclude the presence of a species (Bartlett & Davidson, 1992).

Recently Bottero, Dalmasso, Cappelletti, Secchi, and Civera (2007) successfully applied a method based on minisequencing reaction to differentiate closely related species of tuna fish in canned products. This technique consists in the analysis of the diagnosis sites present in a fragment previously amplified and is based on the dideoxy (ddNTP) single base extension of an unlabelled oligonucleotide (sequencing primer) at the 3′ end of the base immediately adjacent to the diagnosis site. Each ddNTP is labelled with different fluorescent dyes and a fifth color is used to label the internal size marker. In particular the fluorescent dyes are assigned to the individual ddNTPs as follows: A(dR6G, green), C(dTAMRA, black), G(dR110, blue) and T(dROX, red). The extended SNaPshot primers used to interrogate different diagnosis site differ in color and size (the length of a primer being modified by the addition of poly-T tails at the 5′-end) (Quintàns et al., 2004).

Meat from venison is becoming increasingly popular in European markets. The main species consumed in Europe are roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*).

Moreover the hunt of steinbock (*Capra ibex*) in some country is selective or prohibited.

In the present study we describe a rapid and reliable method for the specific identification of game meats from roe deer, red deer, steinbock and chamois based on minisequencing reaction and a capillary electrophoresis of a conserved fragment from the mtDNA cyt b gene. The assay is also intended to enable the differentiation between these wild ungulate meats and those from buffalo (*Bubalus bubalis*) sheep (*Ovis aries*), goat (*Capra hircus*), cattle (*Bos taurus*) and swine (*Sus scrofa*) domestic specie, which can be sold in place of more expensive venison meat.

2. Materials and methods

2.1. Samples and DNA extraction

Muscle samples of roe deer, red deer, steinbock and chamois, were obtained from the Department of Animal Production (Faculty of Veterinary Medicine, University of Torino, Italy) and from hunted killed animals in different Italian regions (Italian Wildlife Institute (INFS), Ozzano Emilia, Bologna, Italy). Goat, sheep, buffalo, cattle and swine meat samples were obtained from local abattoirs. Thirty specimens of each species were morphologically identified before sampling muscles for analysis.

The investigation was carried out on raw meat and on autoclave-treated meat (121 °C for 15 min.) of each species object of this study.

Genomic DNA was extracted from meat using Dneasy Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with slight modification (400 mg of sample and 100 μl the final elution volume).

The DNA was quantified by spectrophotometry (Biophotometer 6131, Eppendorf AG, Hamburg, Germany).

In order to evaluate the sensitivity of the preliminary PCR, dilutions of roe deer DNA (25, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025 ng) were prepared.

In addition, to evaluate the "dilution" effect on patterns reproducibility a series of dilutions of the purified PCR product has been prepared (0.4, 0.2, 0.1, 0.05, 0.01, 0.005, 0.001 pmol).

2.2. Primers design

The cyt b sequences downloaded from the GenBank database, corresponding to *Rupicapra rupicapra* (GenBank accession no. AF034725), *Capreolus capreolus* (GenBank accession no. AJ000024), *Cervus elaphus* (GenBank accession no. AJ000021), *Capra ibex* (GenBank accession no. AF034735), *Bubalus bubalis* (GenBanK accession no. AY488491), *Capra hircus* (GenBank accession no. X56289), *Ovis aries* (GenBank accession no. DQ097429), *Bos taurus* (GenBank accession no. AB074968) and *Sus scrofa* (GenBank accession no. NC_000845) were aligned with the Clustal X program (Higgins, Bleasby, & Fuchs, 1992) in order to detect polymorphic sites to be used as diagnosis position (Tables 1 and 2).

Table 1
Primer binding site for preliminary PCR primers and for PER primers

Species	GenBank Acc. n.	Aligned Sequences 5'----3'
Capreolus capreolus	AJ000024	CA**TGAGGACAAATATCATTCTG**AGGAAGCAACAGTTATTACCAATCTCCTC
Cervus elaphus	AJ000021	CATGAGGACAAATATCATTCTGAGGAAGCAACAGTCATTACCAACCTTCTC
Capra hircus	X56289	CATGAGGACAAATATCATTTTGAGGAGCAACAGTCATCACCAATCTCCTC
Capra ibex	AF034735	CATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACTAACCTTCTC
Ovis aries	DQ097429	CATGAGGACAAATATCATTCTGAGGAGCAACAGTTATTACCAACCTCCTT
Rupicapra rupicapra	AF034725	CATGAGGACAGATATCATTCTGGGGAGCAACAGTTATTACCAACCTCCTC
Bos taurus	AB074968	CATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCTTA
Bubalus bubalis	AY488491	CATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTTCTC
Sus scrofa	NC_000845	CCTGAGGACAAATATCATTCTGAGGAGCTACGGTCATCACAAATCTACTA
		* ******** ******** ** ** ** ** ** ** ** ** ** *
Capreolus capreolus	AJ000024	TCAGCAATTCCATATATCGGTACAAACCTAGTTGAATGAAATTTGAGGGGG
Cervus elaphus	AJ000021	TCAGCAATTCCATATATTGGTACAAACCTAGTCGAATGGATCTGAGGGGG
Capra hircus	X56289	TCAGCAATCCCATATATTGGCACAAACCTAGTCGAATGAATCTGAGGGGG
Capra ibex	AF034735	TCAGCAATCCCATATATTGGCACAAACCTAGTCGAATGAATCTGAGGGGG
Ovis aries	DQ097429	TCAGCAATTCCATATATTGGCACAAGCCTAGTCGAATGAATCTGAGGAGG
Rupicapra rupicapra	AF034725	TCAGCGATCCCGTATATTGGCACAGACTTAGTCGAATGAATCTGAGGAGG
Bos taurus	AB074968	TCAGCAATCCCATACATCGGCACAAATTTAGTCGAATGAATCTGAGGCGG
Bubalus bubalis	AY488491	TCAGCAATCCCATACATTGGTACAAGTCTGGTTGAATGAAATTTGAGGGGG
Sus scrofa	NC_000845	TCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAAATCTGAGGGGG
		***** ** ** ** ** ** *** * ** ***** ** ***** **
Capreolus capreolus	AJ000024	CTTTTCAGTAGACAAAGCAACCCTGACTCGATTTTTCGCTTTCCACTTTA
Cervus elaphus	AJ000021	CTTTTCAGTAGACAAAGCAACCCTAACCCGATTTTTCGCTTTCCACTTTA
Capra hircus	X56289	ATTCTCAGTAGACAAAGCCACTCTCACCCGATTCTTCGCCTTCCACTTTA
Capra ibex	AF034735	ATTCTCAGTAGACAAAGCCACTCTCACCCGATTCTTCGCCTTCCACTTCA
Ovis aries	DQ097429	ATTCTCAGTAGACAAAGCTACCCTCACCCGATTTTTCGCCTTTCACTTTA
Rupicapra rupicapra	AF034725	CTTCTCGGTAGACAAGGCTACCCTCACCCGATTCTTTGCCTTCCACTTCA
Bos taurus	AB074968	ATTCTCAGTAGACAAAGCAACCCTTACCCGATTCTTCGCTTTCCATTTTA
Bubalus bubalis	AY488491	ATTCTCAGTAGACAAAGCAACCCTCACCCGATTCTTCGCATTTCACTTCA
Sus scrofa	NC_000845	CTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTCCACTTTA
		** ** ** ***** ** ** ** ** ***** ** ** ** ** ** *
Capreolus capreolus	AJ000024	TCCTCCCATTTATCATTGCAGCACTTGCTATAGTCCATTTACTTTTCCTC
Cervus elaphus	AJ000021	TTCTCCCATTTATCATCGCAGCACTCGCTATAGTACACTTACTCTTCCTT
Capra hircus	X56289	TCCTCCCATTCATCATCACAGCCCTCGCCATAGTCCACCTACTCTTCCTC
Capra ibex	AF034735	TCCTCCCATTCATCATTACAGCCCTCGCCATAGTCCACCTGCTCTTCCTC
Ovis aries	DQ097429	TTTTCCCATTCATCATCGCAGCCCTCGCCATAGTTCACCTACTCTTCCTC
Rupicapra rupicapra	AF034725	TCCTCCCATTTATCATTGCAGCCTTAGCCCTAGTCCACCTACTCTTCCTC
Bos taurus	AB074968	TCCTTCCATTTATCATCATAGCAATTGCCATAGTCCACCTACTATTCCTC
Bubalus bubalis	AY488491	TCCTCCCATTCATTATCGCAGCACTTGCAATAGTCCACCTATTATTTCTC
Sus scrofa	NC_000845	TCCTGCCATTCATCATTACCGCCCTCGCAGCCGTACATCTCCTATTCCTG
		* * ***** ** ** ** ** * ** ** ** * * ** **
Capreolus capreolus	AJ000024	CACGAAACAGGATCA**AACAACCCGATAGGAATCC**CATCAAACGCGGACAA
Cervus elaphus	AJ000021	CACGAAACAGGATCTAATAACCCAACAGGAATTCCATCAGACGCAGACAA
Capra hircus	X56289	CACGAAACAGGATCCAACAACCCCACAGGAATTCCATCAGACACAGATAA
Capra ibex	AF034735	CACGAAACGGGATCCAACAACCCCACAGGAATTCCATCAGACACAGACAA
Ovis aries	DQ097429	CACGAAACAGGATCCAACAACCCCACAGGAATTCCATCGGACACAGATAA
Rupicapra rupicapra	AF034725	CACGAAACAGGATCTAACAACCCCACAGGAATCCCATCAGATGCGGACAA
Bos taurus	AB074968	CACGAAACAGGCTCCAACAACCCAACAGGAATTTCCTCAGACGTAGACAA
Bubalus bubalis	AY488491	CACGAAACAGGATCCAACAACCCAACAGGAATCTCATCAGACACAGACAA
Sus scrofa	NC_000845	CACGAAACCGGATCCAACAACCCTACCGGAATCTCATCAGACATAGACAA
		******** ** ** ** ***** * ***** * ** * ** **

Primers binding site for preliminary PCR primers (in bold) and for PER primers (in grey). The bases in the box correspond to the sites of diagnosis.

All the sequences available in GenBank for each species, were later compared so as to detect possible intraspecific variations of the chosen diagnosis sites. In addition, sequences of other species were aligned in order to evaluate theoretically some possible cross-reactions at the chosen sites (Table 2).

Primers for the preliminary PCR were designed in well conserved regions upstream and downstream of the diagnosis sites (Tables 1 and 3).

Primers were synthesized by Operon (Cologne, Germany). The obtained PCR products act as templates for the minisequencing reaction.

Extension primers Sp1, Sp2, Sp3, Sp4, Sp5 and Sp6 were designed upstream of the diagnosis sites and had varying lengths of poly(dT) non-homologous tails attached to the 5′end (Tables 1 and 3) and were synthesized by Sigma Genosys (St. Louis, MO, USA).

Table 2
Diagnosis site used

Species	Sp2 (489)	Sp3 (519)	Sp4 (549)	Sp1 (426)	Sp5 (576)	Sp6 (609)
Cervus elaphus	G	A	T	A	C	A
Capra ibex	A	C	C	G	C	G
Rupicapra rupicapra	A	T	C	A	A	A
Capra hircus	A	C	T	G	C	A
Capreolus capreolus	A	A	T	A	T	A
Ovis aries	A	T	T	A	C	A
Bos taurus	//	A	//	A	//	A
Sus scrofa	//	//	T	A	C	//
Bubalus bubalus	//	A	C	G	T	A
Rangifer tarandus	A	A	T	A	C	A
Alces alces	A	A	T	G	T	A
Dama dama	A	A	T	A	T	A
Cervus unicolor	A	C	T	A	C	A
Hydropotes inermis	A	T	C	A	T	A
Megaloceros giganteus	G	A	T	A	T	A
Syncerus caffer	A	A	C	G	T	A
Tragelaphus euryceros	A	A	T	A	A	A
Ovis ammon	A	C	T	C	C	A
Pseudois nayaur	G	C	C	G	C	A
Equus caballus	A	C	C	A	G	A
Oryctolagus cuniculus	A	C	C	A	A	T
Gallus gallus	A	A	C	G	T	A
Meleagris gallopavo	A	A	C	G	T	A
Homo sapiens	A	C	C	G	A	G

Missing peaks are indicated with // – The numbers in parenthesis indicates the position of the nucleotide within the cyt b gene.

Table 3
Primers for preliminary PCR and PER

	Primers	Position (*R. rupicapra* AF034725)	Oligonucleotides
Preliminary PCR	Cytbsn	403	5′–TGAGGACAAATATCATTCTG–3′
	Cytbasn	634	5′–GAATTCCGTGTGGGTTGTT–3′
PER	Sp1	406	5′–(22T)GGACAAATATCATTCTGRGG-3′
	Sp2	472	5′–ACARACYTAGTCGARTG-3′
	Sp3	502	5′–(8T)TTCTCRGTAGAYAARGC–3′
	Sp4	531	5′–(16T)ATTCTTCGCYTTYCACTT–3′
	Sp5	557	5′–(30T)CATTCATCATYRCAGCMYT–3′
	Sp6	592	5′–(38T)CTYTTYCTYCACGARAC–3′

2.3. Preliminary PCR

PCR amplification was performed in a final volume of 50 μl containing 75 mM Tris–HCl (pH 8.8), 1 unit of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Uppsala, Sweden), 2 mM $MgCl_2$, 25 pmol of primers Cytbs and Cytbasn (Table 3) and 50 ng of DNA template.

The PCR amplification was performed in an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) beginning with 10 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 7 min. Amplimers were resolved by electrophoresis on a 2.0% agarose gel (Invitrogen, Carlsbad, CA, USA).

2.4. SNaPshot reaction

To remove primers and un-incorporated dNTPs, amplicons were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were quantified by spectrophotometry. The minisequencing reaction was performed in a 2720 thermocycler (Applied Biosystems, Foster City, CA, USA) following the SNaPshot protocol (SNaPshot multiplex Kit Applied Biosystems, Foster City, CA, USA) with some modifications: 3.33 μl of SNaPshot Multiplex Ready Reaction Mix, 3 μl of a purified PCR products diluted to obtain range of 0.4–0.05 pmol, 0.02 μM, 0.033 μM, 0.05 μM, 0.04 μM 0.05 μM and 0.05 μM of extension primers Sp1, Sp2, Sp3, Sp4, Sp5, and Sp6, respectively, in a total volume 10 μl. The reaction mixture underwent 25 single base extension cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C during 30 s.

After minisequencing reactions, a post-extension treatment to remove the 5′-phosphoryl group of the ddNTPs helps to prevent un-incorporated ddNTPs co-migrating with the extended primers and producing a high background signal. For this purpose the final volume (10 μl) was treated with 1 unit of Calf Intestine Alkaline Phosphatase (CIAP) (Fermentas, Burlington, CA, USA). The tubes were incubated at 37 °C for 1 h and at 75 °C for 15 min.

The minisequencing products (1 μl) were mixed with 25 μl of Hi-Di formamide containing 0.4 μl of GeneScan-120 LIZ size standard (Applied Biosystems, Foster City, CA, USA) and electrophoresis was performed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Minisequencing products were injected electrokinetically for 15 s at 15 kV and electrophoresed for 16 min at 15 kV at 60 °C in a 47 cm length capillary column using the 310-POP-4 polymer (Applied Biosystems, Foster City, CA, USA). Resulting data were analysed with ABI Genescan E5 Run Module. Electropherograms analysis was performed using GeneMapper 4.0 software (Applied Biosystems, Foster City, CA, USA).

2.5. DNA sequencing

DNA sequencing was carried out as a confirmatory test. Amplification products of the preliminary PCR of samples were sequenced. Sequencing was carried out directly on purified fragments with ABI 310 Genetic analyser (Applied Biosystems, Foster City, CA, USA), using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA).

Sequences were aligned with Clustal X program (Higgins et al., 1992).

3. Results and discussion

The aim of the our study was the development of an assay for the specific identification of meat of some game and domestic animals targeting sequences of the cyt b gene of mtDNA. The mtDNA cyt b gene was selected as template for DNA amplification because it had been used in species identification and in taxonomic and phylogenetic studies (Cook, Wang, & Sensabaugh, 1999; Irwin, Kocher, & Wilson, 1991; Kocher et al., 1989; Kuwayama & Ozawa, 2000; Lau et al., 1998; Su, Wang, Lan, Wang, & Zhang, 1999).

Analysis of the alignment of the reference sequences obtained from GenBank showed that the bases in 426, 489, 519, 549, 576 and 609 position of cyt b could differentiate red deer, roe deer, steinbock, chamois, buffalo, sheep, goat, cattle and swine (Table 1). Alignments of all the sequences available in GenBank for each species object of this study, showed the absence of intraspecific variations of the chosen diagnosis sites.

The primers used in the preliminary PCR successfully amplified a conserved 232 bp region from the cyt b gene

of all the species taken into consideration. The PCR amplification from roe deer DNA template of 25, 5, 2.5, 1, 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025 ng showed a detection limit of 0.005; so the amplification could be possible also in case of low amounts of DNA.

When DNA extracted from roe deer, red deer, steinbock, chamois, sheep and goat was tested in singleplex PER with every single extension primer, it gave rise to a peak of the expected size and color which is specific to the diagnostic site. DNA extracted from cattle and swine originated only peaks in 426, 519, 609 and in 426, 549, 576 position, respectively. DNA extracted from buffalo originated peaks in 426, 519, 549, 576, 609. The absence of peaks in 489, 549 and 576 positions for cattle, of peaks 489, 519 and 609 positions for swine and in 489 position for buffalo was caused by a high mismatch of primers.

As expected, the detected size determined by the automated sequencer and the theoretical size of certain products were slightly different. The shift was constant according to [F]ddNTPs incorporated. This is due to

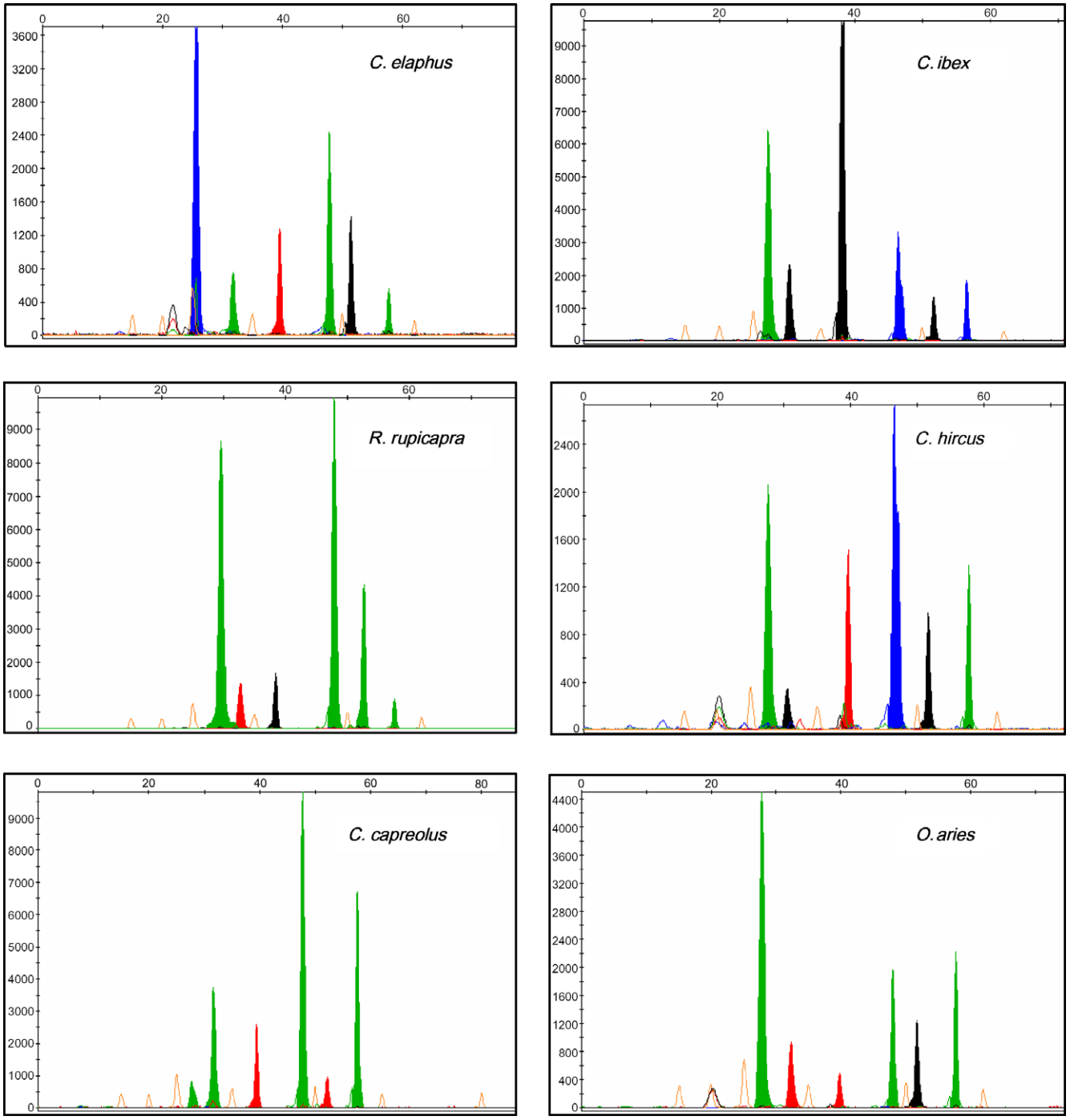

Fig. 1. Species-specific patterns observed in nine meat samples.

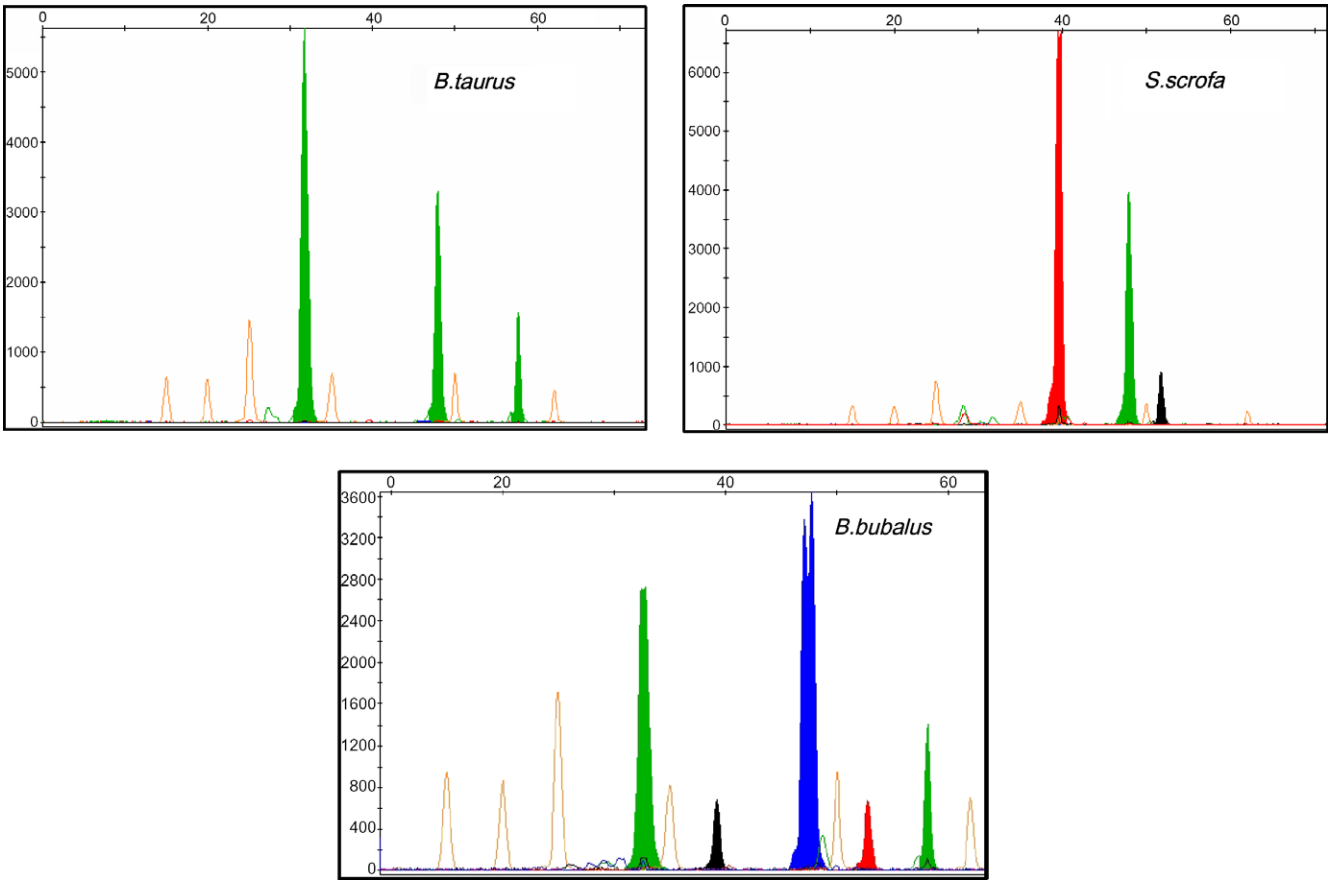

Fig. 1 *(continued)*

differences in electrophoretic mobility mainly determined by the length, sequence and dye used to label the extended primers. In particular, the greatest shift was observed in shortest fragments; this could be attributed to the algorithm of the software used for analysis (Quintàns et al., 2004). We estimated that to assure correct differentiation of extended primer, amplicons must differ by at least seven nucleotides in length.

As for the multiplex PER, the reaction was optimized and DNA extracted from the raw samples of the nine species gave species-specific patterns (Fig. 1).

Six peaks represented the patterns of red deer, roe deer, steinbock, chamois, goat and sheep, while five peaks were characteristic of buffalo pattern and only three peaks, still species-specific, were characteristic of swine and bovine patterns (Table 2 and Fig. 1). All samples gave specific patterns. With regard the species identified by the six peaks pattern, we evaluated the possibility of a misinterpretation of the results due to the low concentrations of DNA. We hypothesized the consequent disappearance of some peaks may result in a possible overlapping profile with swine or bovine patterns. So, a SNaPshot reaction from a serially diluted PCR product of roe deer (0.4, 0.2, 0.1, 0.05, 0.01, 0.005 and 0.001 pmol) was carried out. A clear six peaks pattern up to the dilution of 0.05 pmol was obtained, while only one peak was present in subsequent dilution. These results confirm that with a dilution in the range 0.4–0.05 pmol, the presence of three peaks is unambiguously associated to swine and bovine.

In addition, the small size of the fragment allowed amplification and species characterization of the samples which had undergone heat treatment (120 °C for 15 min) which resulted in DNA degradation. It is well known that in the case of severely degraded substrates the length of the fragment to be amplified should not exceed 200 bp (Unseld, Beyermann, Brandt, & Hiesel, 1995).

All the results of the multiplex PER test were confirmed by fragment sequencing.

Alignments of the species object of this study with the sequence of Homo sapiens excluded the possibility of cross-contamination carried by the operator (Table 2).

Unfortunately, the alignment of closely related species showed the impossibility of differentiating roe deer (*C. capreolus*) and fallow deer (*Dama dama*). However roe deer can be differentiated from all the other closely related species (e.g., *Alces alces*).

The SNaPshot minisequencing test is as precise and reliable as the sequencing test; moreover, it has the advantage of being quicker allowing immediate interpretation of results. In fact, the discrimination of the nine species is possible by means of a single reading of the generated pattern. Nonetheless the simultaneous presence of two or

more species could induce a misinterpretation. In addition although the application SNaPshot minisequencing to unadulterated animal tissues is simple and straightforward, the analysis of meat mixtures is problematic, as, besides, it is when PCR-RFLP is applied (Partis et al., 2000). This is the result of the differential template amplification, a phenomenon which also prevents a semi-quantitative analysis of meat mixture components.

Undoubtedly an advantage of the test described in this paper is that in case of a possible intraspecific variability of the single site of diagnosis, the species identification is made on the basis of an aplotype generated by six different sites. The mutation of a single site could cause a pattern modification, but it could not modify its specificity. In fact, the choice of the site did not aim only to the achievement of a specific pattern, but also a possible mutation (transitions and transversions) was hypothesized. So, even in this case the species attribution would be unambiguous. On the contrary, the variability of a single restriction site compromises the validity of PCR-RFLP.

For this reason some authors suggested taking into consideration at least two different diagnostic mutations in order to exclude intra-species polymorphism (Lenstra, Nikman, Van Cann, & Verkaar, 2003).

However, identification of species by analysis with endonuclease is not always easy. The possibility of amplifying not translated pseudogenes due to the transfer to nuclear genome of mitochondrial DNA gene cyt b can lead to artefacts as total DNA is analysed by PCR-RFLP (Bottero et al., 2003; Lenstra et al., 2003; Meyer, Hofelein, Luthy, & Candrian, 1995). The pseudogenes are characterised by an increased number of mutations, which can alter the distribution of restriction sites. These co-amplification phenomena have been described in deer (Partis et al., 2000). However, in the assay described in our study, the pattern is generated by the most significant sequence (mitochondrial DNA), while a double peak would mean an integrated copy of nuclear DNA. In this way the results are unambiguous.

The proposed assay can be considered an helpful tool for routine analysis aimed both at the authentication of food and at the detection of possible illegal trade of protected wildlife. Moreover could be useful for detection of possible illegal trade of wildlife. The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), which is the main international agreement to regulate the trade and captive reproduction of endangered plant and animal species has recently stressed the importance of applying reliable methods for the unmistakable identification of the species including food inspection, wildlife management and forensic evidences.

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