PCR−RFLP Analysis of Mitochondrial DNA: A Reliable Method for Species Identification

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A method for identification of game species has been developed on the basis of the amplification of a specific part of the mitochondrial genome (tRNA^{Glu}/cytochrome *b*) using the polymerase chain reaction (PCR). To distinguish between several game species, the obtained 464-bp-long PCR products were cut with different restriction endonucleases (RE) resulting in species-specific restriction fragment length polymorphism (RFLP). Even closely related deer species could be distinguished by application of one or two RE. Natural polymorphisms of the target sequence within one species were examined for red deer (*Cervus elaphus*), and base pair substitutions were identified affecting the RFLP pattern.

Keywords: *Species identification; game meat; PCR; RFLP; DNA sequence diversity; cyt b*

INTRODUCTION

Falsification of game meat and game-meat products is very common due to the tremendous profit that results from selling less costly meat as meat from much more demanded and higher priced species. These frauds are difficult to detect with protein-based methods of species identification such as isoelectric focusing (IEF) or immunological methods (Berger et al., 1988; Patterson and Jones, 1990). IEF, for example, is not suitable for processed meat products (heated or marinated) because the subject of analysis, the soluble muscle proteins, degrade very rapidly under such conditions (Jemmi and Schlosser, 1991; Sinclair and Slattery, 1982; Rehbein, 1990). In addition, for some species, such as chamois, at least two different IEF patterns are known (Jemmi, 1997). Immunological methods, relying on antibodies, can suffer from cross-reactions of proteins from closely related species.

For these reasons, nucleic acid based analytics, already widely used in many medical fields, became more and more popular for the differentiation and identification of food and food products (Allmann et al., 1993; Chikuni et al., 1994; Meyer et al., 1994; Meyer et al., 1995; Meyer, 1995; Meyer and Candrian, 1996). The advantages of DNA-based analysis are manifold. First, the ubiquity of DNA: all cell types of an individual contain identical genetic information rendering analysis independent of the origin of the sample (blood, muscle, bone etc.). Second, the information content of DNA is higher than that of proteins because of the degeneracy of the genetic code. Third, DNA is a rather stable molecule, allowing its extraction from many different types of specimens such as mummy tissue, insects enclosed in amber, or preserved museum samples (Cano et al., 1993; Pääbo, 1989; Pääbo et al., 1988; Shiozawa et al., 1992).

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Table 1: Investigated Species

English name	species (Latin name)	no. of animals
Alpine ibex	*Capra ibex ibex*	1
blesbok	*Damaliscus dorcas phillipsi*	2
buffalo	*Bubalus arnee*	1
fallow buck	*Dama dama*	2
moose	*Alces alces*	2
chamois	*Rupicapra rupicapra*	8
gnu	*Connochaetes*	1
hare	*Lepus europaeus*	2
kangaroo	*Marcropus giganteus*	1
Kudu	*Strepsiceros strepsiceros*	2
mouflon	*Ovis orientalis musimon*	1
nyala	*Tragelaphus angasi*	1
sable antelope	*Hippotragus niger*	1
roe deer	*Capreolus capreolus capreolus*	12
Chinese roe deer	*Capreolus capreolus pygargus*	1
Siberian roe deer	*Capreolus capreolus bedfordi*	1
reindeer	*Rangifer tarandus*	2
cattle	*Bos taurus domesticus*	4
red deer	*Cervus elaphus*	15
sheep	*Ovis ammon aries*	3
pig	*Sus scrofa domesticus*	4
sika deer	*Cervus nippon*	2
springbok	*Antidorcas marsupialis*	2
wild boar	*Sus scrofa mit*	4
goat	*Capra aegagrus hircus*	5

For this work, the mitochondrial (mt) DNA was chosen as the target of our investigations. Mt-DNA evolves much faster than nuclear (nc) DNA and thus contains more sequence diversity compared to nuclear DNA, facilitating the identification of closely related species (Brown et al., 1993; Brown et al., 1996; Vawter and Brown, 1986). In addition, maternal inheritance of the mt-DNA generally results in lack of heterocygosity (Hayashi and Walle, 1985; Lansman et al., 1983). Especially for analysis of processed samples, the high copy number of the mitochondrial genome which exceeds that of the nuclear genome by a factor up to 10 000 (1000 mitochondria per cell, each 10 copies of the genome (Alberts et al., 1990)) is advantegeous. Therefore, the required amount of tissue for mt-DNA-based analytics is very small.

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Table 2: Restriction Fragment Length Polymorphism of the cyt *b* Gene of Game Species

	cattle[a]	pig[a]	sheep[a]	goat[a]	chamois	roe deer	red deer	fallow buck	sika deer	mouflon	ibex
***Alu*I**	295	244	361	464	361	464	464[c]	409	464	361	229
	169	220	103		103			55		103	190
											45
***Ase*I**	464	464	464	400	464	464	464	464	400	464	400
				64					64		64
***Bam*HI**	464	349	464	328	464	464	464	464	464	464	328
		115		136							136
***Hae*III**	285	179	179	230	179	179	179	179	179	179	285
	179	153	159	179	159	159	159	159	159	159	179
		132	126	55	126	126	126	126	126	123	
***Hinc*II**	464	464	464	464	464	255	464	464	255	464	464
						209			209		
***Hin*fI**	198	464	296	266	266	315	315	266	464	296	464
	149		168	198	198	149	149	198		168	
	117										
***Mse*I**	464	430	307	400	307	254	464	307	400	307	400
		34	157	64	157	210		157	64	157	64
***Nla*III**	249	161	203	249	161	161	328	282	328	203	203
	125	125	125	125	125	125	112	66	66	125	125
	66	90	66	66	66	66	24	46	46	66	66
	24	88	46	24	46	46		46	24	46	46
			24		42	42		24		24	24
					24	24					
***Rsa*I**	464	464	464	464	464	464	343[d]	464	343	464	464
							121		121		
***Ssp*I**	378	464	188	302	302	188[b]	188	188	188	188	302
	86		162	162	162	162	162	162	162	162	86
			114			114	114	114	114	114	76
***Taq*I**	372	246	464	246	464	464	246	273	246	464	246
	48	218		218			191	191	218		218
	44						27				

	blesbok	springbok	nyala	Kudu	wild boar	reindeer	moose	hare	buffalo	gnu	kangaroo	sable antelope
AluI	464	464	464	464	244	409	464	250	295	464	220	409
					220	55		214	169		195	55
											49	
AseI	464	464	464	464	464	400	400	464	464	464	268	464
							64	64			196	
BamHI	464	464	328	328	349	464	349	464	464	464	464	464
			136	136	115		115					
HaeIII	338	179	274	464	179	338	464	332	285	338	285	179
	126	159	190		153	126		132	179	126	179	153
		126			132							132
HincII	209	255	464	464	464	464	464	386	464	209	386	255
	177	209						78		177	78	209
	78									78		
HinfI	212	464	315	198	464[e]	198	266	266	464	198	464	266
	198		149	149		149	198	126		159		198
	54			117		117		72		107		
MseI	387	292	464	464	430	307	307	387	464	210	200	307
	77	157			34	93	93	77		177	196	157
		15				64	64			77	68	
NlaIII	249	161	161	161	161	161	161	328	249	249	328	161
	125	125	125	125	125	125	125	112	125	125	66	125
	49	88	88	66	90	66	66	24	66	49	46	66
	24	66	66	46	88	46	46	24	24	24	46	
	17	24	24	44		42	42			17		38
				24		24	24					24
												4
RsaI	464	464	464	319	464	464	464	464	431	431	464	464
				145					33	33		
SspI	302	464	302	302	464	302	188	302	302	302	464	302
	162		162	162		86	162	162	162	162		162
						76	114					
TaqI	416	273	218	372	246	246	273	372	273	416	372	273
	48	191	154	48	218	218	191	92	191	48	92	191
			92	44								

[a] The species cattle, pig, sheep, and goat are given as references. [b] Three samples of 12 analyzed showed two fragments at 302 bp (188 + 114) and 162 bp instead of three at 188 bp, 162 bp and 114 bp. [c] Two samples of 13 analyzed (CW64/CW118) showed a digested PCR product with fragments of 409 bp and 55 bp instead of the undigested fragment at 464 bp (see Figure 4). [d] One sample of 13 analyzed (CW117) was not cut at this position (see Figure 4). [e] One sample of 2 analyzed was cut with *Hin*fI and gave two fragments at 266 bp and 198 bp instead of the uncut fragment at 464 bp.

Mitochondrial DNA of several vertebrates, including mammals, was mainly investigated for evolutionary studies (Anderson et al., 1981; Irwin et al., 1991). Comparisons of nucleotide sequences of the complete

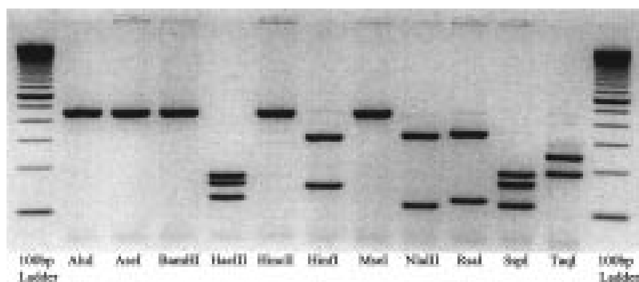

Figure 1. Restriction analysis of the of 464 bp cyt *b* amplicon of red deer (CW94) with 11 enzymes.

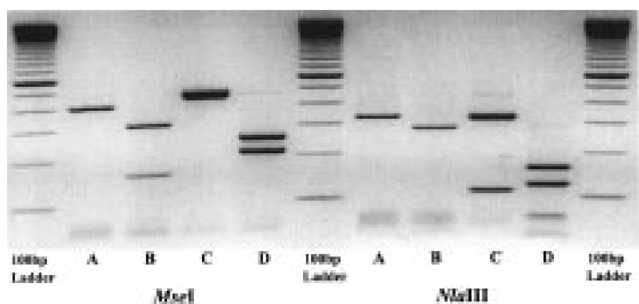

Figure 2. RFLP analysis (464 bp PCR amplikon) of sika deer (A), fallow buck (B), red deer (C), and roe deer (D) with *Mse*I and *Nla*III.

mt-DNA or of single genes (e.g., genes encoding the small ribosomal RNAs 12S and 16S and cytochrome *b*) (Esposti et al., 1993), were used to study inter- and intraspecies relationships of animals in order to establish the molecular phylogeny (Birstein and DeSalle, 1998; Birstein et al., 1998). Polymerase chain reaction (PCR) primers were described for amplification of the cyt *b* gene and for direct nucleotide sequencing (Kocher et al., 1989; DeSalle et al., 1993; Irwin et al., 1991; Saiki et al., 1988).

The analysis of restriction fragment length polymorphism (RFLP) of PCR fragments was already successfully applied for species differentiation (Meyer et al., 1994, 1995; Meyer and Candrian, 1996). PCR–RFLP of different genes was demonstrated to detect inter- and intraspecific variations in several animals such as Atlantic snapper (Chow, 1993), tuna (Chow and Inogue, 1993), lobsters (Silberman and Walsh, 1992), turtles (Karl et al., 1992), and pigs (Mayr et al., 1993). Recently, the coamplification of nuclear pseudo cyt *b* gene fragments was found to prevent the unambiguous identification of game species (Burgener and Hübner, 1998; Collura and Stewart, 1995; Perna and Kocher, 1996). However, these pseudo cyt *b* gene fragments were not amplified using a new PCR primer pair positioned on the $tRNA^{Glu}$ and the cytochrome *b* gene (Burgener and Hübner, 1998).

In this study we describe the application of this new PCR primer pair for the identification of game species. In addition, the cyt *b* sequence polymorphism within the species red deer (*Cervus elaphus*) was investigated in order to assess the applicability of PCR–RFLP for species differentiation in general.

MATERIALS AND METHODS

Meat Samples. Authentic samples of frozen meat or of lyophilized protein extracts, normally used for IEF, were used as game species references and were obtained either by the

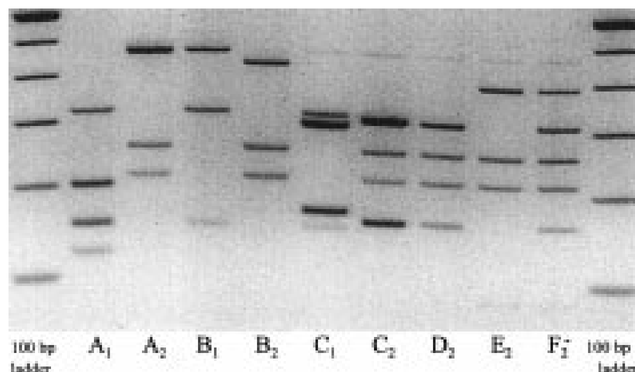

Figure 3. RFLP analysis (464 bp PCR amplikon) with *Hinf*I (1) and *Mse*I (2) of mixtures of two or three different species: roe deer + cattle (A); roe deer + pig (B); roe deer + sheep (C); roe deer + sable antelope (D), roe deer + blesbok (E), and roe deer + sable antelope + blesbok (F).

Swiss Federal Veterinary Office (BVET), the Federation of Migros Cooperatives Meat Laboratory (FMC) (Courtepin, Switzerland), Kantonales Laboratorium Basel, or a swiss hunter (Table 1).

DNA Extraction. A 300 mg meat sample was minced with a sterile surgical blade and transferred into a 2 mL Eppendorf tube. Extraction buffer (1 mL, 10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA, and 1% [w/v] sodium dodecyl sulfate [SDS]), 100 μL of 5 M guanidine hydrochloride and 50 μL of 20 mg/mL proteinase K (Merck, Darmstadt, Germany) were added. The mixture was incubated on a thermomixer at 57 °C for 2 h. After digestion, the samples were centrifuged at 20 000 $\times$ *g* for 15 min. A portion (500 μL) of the aqueous phase was added to 1 mL of Wizard DNA purification resin (Promega, Madison, WI) and mixed by gentle inversion. The mixture was transferred to a syringe plugged on a Wizard column, which was attached to a vacuum manifold. Vacuum was applied, and the column was washed with 2 mL 80% 2-propanol followed by centrifugation at 20 000 $\times$ *g* for 1 min. After incubation at 70 °C for 10 min the DNA was eluted with 50 μL of 70 °C elution buffer (10 mM Tris-HCl [pH 9.0]) and stored at −20 °C.

Lyophilized protein extracts were resuspended in 500 μL of extraction buffer, and 50 μL of 5 M guanidium hydrochloride and 25 μL of 20 μg/mL proteinase K were added. From here the samples were treated as described above.

PCR. DNA amplification was carried out in a final volume of 100 μL in 0.5 mL tubes containing PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 2 μg/mL BSA, 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each primer (L14735:aaa aac cac cgt tgt tat tca act a/H15149:gcc cct cag aat gat att tgt cct ca), 2 units of *Taq* DNA polymerase (Gibco) and 10 μL 1:10 diluted DNA solution (meat samples) or 10 μL undiluted DNA solution (lyophilized protein extracts). The cycling conditions on a Progene thermal cycler (Techne, Cambridge, U.K.) were as follows: 96 °C for 3 min for denaturation, 40 cycles of amplification (96 °C for 30 s, 65 °C for 1 min) and final extension at 72 °C for 3 h. PCR products were examined by electrophoresis through a 1.5% agarose (Agarose LE, Promega) gel in 0.5 TBE buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001 M EDTA, pH 8.0) and stained by ethidium bromide. As size reference, a 100 bp ladder (Gibco) was used.

RFLP. PCR mixture (20 μL) was digested using 10 units of RE at the recomended temperature for 2 h. Restriction fragments were separated on a 2.5% agarose gel, and the size of DNA fragments was determined in comparison with a 100 bp ladder (Gibco).

DNA Sequencing. PCR products were purified for direct sequencing by agarose gel electrophoresis if necessary and

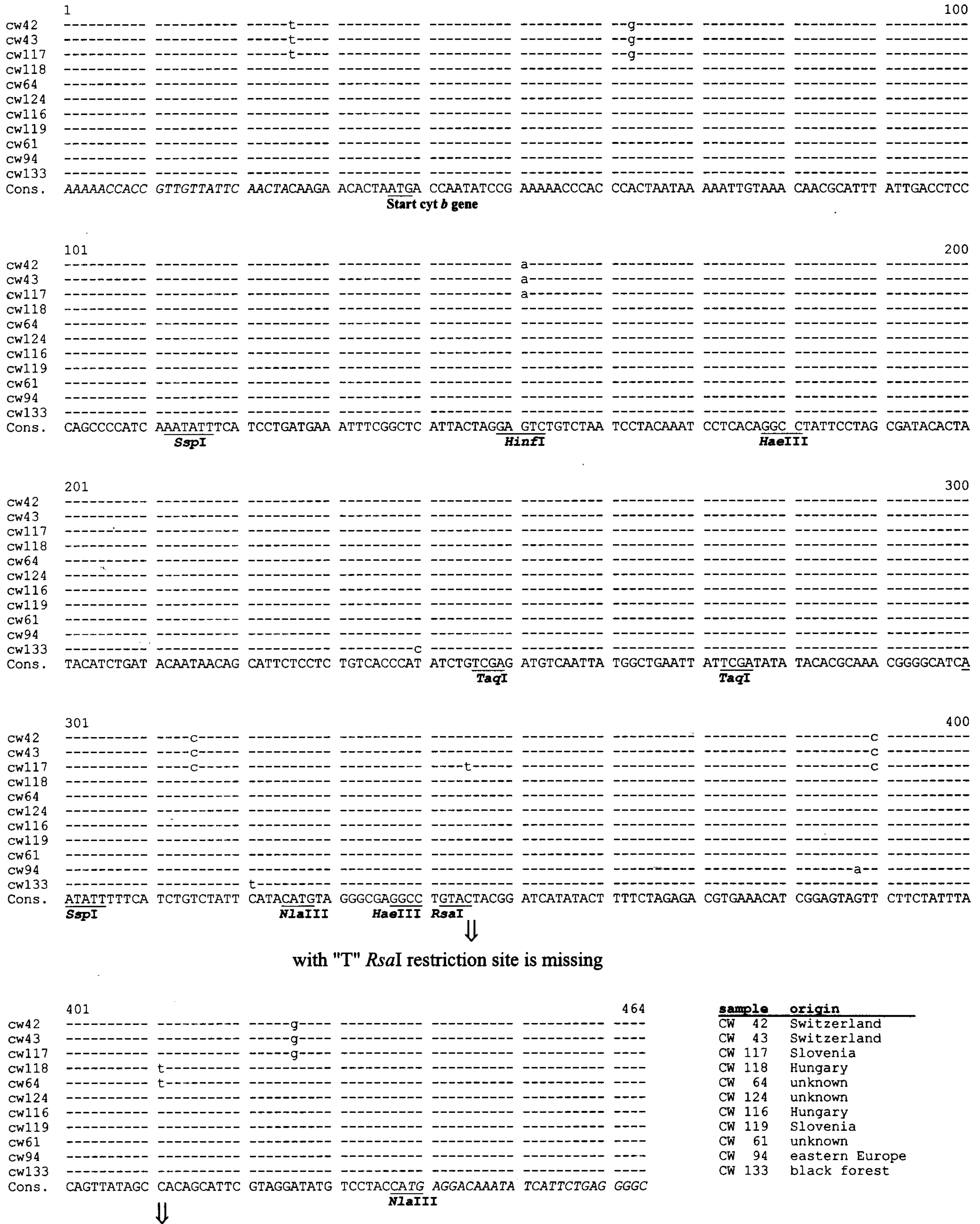

Figure 4. Intraspecies sequence polymorphism. The analyzed cyt *b* sequences of 11 individuals of the species C*ervus elaphus* (red deer) are listed together with the consensus sequence (cons). Individuals with the same base substitutions are written next to each other. PCR primer sequences are shown in italic, restriction sites are underlined, and the start of the cyt *b* gene is marked.

concentrated using Microcon 50 concentrators (Amicon). The purified 464 bp PCR products were sequenced on a DNA sequencer (Abi Prism 377, Perkin-Elmer) using fluorescence dye labeled dideoxynucleotides (Microsynth, CH-Balgach).

RESULTS AND DISCUSSION

DNA Sequence Analysis of Game Species. Total DNA was isolated from different game species (Table

1) and subjected to the previously described mitochondrial specific cyt *b* PCR (Burgener and Hübner, 1998). The 464-bp-long PCR product was amplified with all investigated samples and, with the exception of the species Kudu, no additional PCR fragments were detected. With Kudu, an additional, minor 700 bp DNA fragment was visible on agarose gels.

The amplified fragment length of 464 bp proved to be very suitable for DNA sequencing because the sequence of both strands could be completely determined. Complementary DNA sequences were aligned and compared with each other by biocomputing (GCG software), and obvious sequencing mistakes were removed. The corrected DNA sequences were the basis for calculations of RFLP patterns by biocomputing.

Differentiation and Identification of Game Species by Restriction Fragment Length Polymorphism (RFLP). Restriction enzymes for RFLP analysis were selected by the following criteria: they had to be easily available and low priced; they had to be reasonably active in the PCR mix in order to avoid tedious desalting and buffer exchanging steps; and they had to allow a maximal differentiation between the investigated game species. Nineteen REs fulfilled the first two criteria, and, after calculating all 19 corresponding RFLP patterns of 25 investigated species, 11 REs were found to allow all possible differentiations (Table 2). The results of an RFLP analysis of the red deer sample CW94 with these 11 REs are shown in Figure 1. It is important to note that in most cases the use of only two REs for the identification of an unknown sample or for labeling control will be sufficient. With the help of Table 2, such discriminating restriction enzymes can easily be identified. As an example, the differentiation of the four closely related species red deer, sika deer, fallow buck, and roe deer using the REs *Mse*I (*Tru*9I) and *Nla*III is shown in Figure 2. For the selection of appropriate REs for species differentiation, the analytical resolution capacity of the corresponding gels (e.g., agarose or polyacrylamide) has to be considered. In general, fragments smaller than 80 bp are difficult to identify on agarose gels.

A differentiation of the three closely related species roe deer, Chinese roe deer, and Siberian roe deer was not possible with the 11 selected restriction enzymes, although the two asian species differ significantly from roe deer at nine positions in their nucleotide sequence (data not shown). Wild boar and mouflon could not be distinguished from domestic pig and domestic sheep, respectively.

The use of appropiate restriction enzymes allows the identification of mixtures of different species occurring in falsificated game meat food products. Figure 3 shows the RFLP analysis of mixtures (PCR amplikon mixtures) of roe deer with one or two other species fraudulently used either because of their common availability (cattle, pig, and sheep) or their low price such as many African game species e.g., blesbok, antelope, etc. Using RE resulting in noticeable different restriction pattern for each single species (the single fragments should vary at least by 15−20 bp because of the resolution capacity of the agarose gel) allows easy differentiation between the species roe deer (two fragments of 149/315 bp) and cattle admixtures (three fragments of 117/149/198 bp). Because the two species both result in an RFLP fragment of 149 bp, just 4 fragments are expected on the agarose gel which are clearly visible in lane A_1 (Figure 3). Lane F_2 shows the restriction pattern of a mixture of the three species roe deer, blesbok, and sable antelope after *Mse*I digestion. The five fragments can be classified as follows: ∼160 bp, antelope; ∼210 bp, roe deer; ∼250 bp, roe deer; ∼310 bp, antelope; ∼390 bp, blesbok. The second fragment of the species blesbok (77 bp) is not visible on the agarose gel because of its low intensity.

Intraspecies DNA Sequence Polymorphism. The general applicability of RFLP for species differentiation might be hampered by intraspecies DNA sequence polymorphism. To get an estimate of this polymorphism, at least two individuals of 12 game species were investigated either by RFLP or by DNA sequence analysis. Polymorphic sequences were identified in chamois, red deer, roe deer, and wild boar. However, RFLP patterns were changed only in a few cases: roe deer (3 of 12 samples), wild boar (1 of 4 samples), and red deer (3 of 15 samples). The sequence polymorphism within the species red deer was examined in greater detail upon subjecting 11 individuals to DNA sequence analysis. Within the amplified 464-bp-long cyt *b* region, nucleotide substitutions at nine different positions were identified, three of them interfered with restriction sites (Figure 3). No correlation between the origin of the red deers and the nucleotide substitutions was found because individuals from Switzerland and Slovenia were found to have identical cyt *b* sequences, whereas another Slovenian and two Hungarian animals differed in their DNA sequences (see Figure 3). Although the RFLP pattern can be affected by intraspecific polymorphism, the use of two discriminating REs will nevertheless allow the unambigious identification of a particular sample.

A simple and reliable PCR−RFLP system for identification of falsification of game species is described. Two different restriction enzymes were found to be sufficient for the discrimination of all 25 investigated species. Intraspecies polymorphism occurs in some species at a frequency of roughly one base-pair substitution per 100 bp and can affect RFLP patterns as shown for the species red deer. However, no individual was identified as having two differing RFLP patterns compared to other individuals of the same species.

ABREVIATIONS USED

IEF, isoelectric focusing; bp, base pairs; PCR, polymerase chain reaction; dNTP, deoxynucleotide 5′-triphosphate; RFLP, restriction fragment length polymorphism; RE, restriction endonuclease; cyt *b*, cytochrome *b*; mt, mitochondrial.

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