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Brief communication

A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome *b* gene

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Abstract

We developed a simple, quick assay in order to discriminate forensic samples among human, and common domestic and livestock species of the Pampean region, Argentina. A mitochondrial cytochrome *b* fragment amplified with universal primers was separately digested with three restriction enzymes (*Alu*I, *Hae*III, and *Hinf*I) and the resulting fragments were resolved through electrophoresis in polyacrylamide gels. This PCR-RFLP method allowed us to identify the target species and worked on degraded samples. The assay was successfully applied in livestock robbery cases in Argentine, and may be useful when attempting a first assessment as to the specific status of a forensic evidence.
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1. Introduction

Several features of the mitochondrial DNA (mtDNA) explain its extensive use in phylogenetic and species identification studies. MtDNA genes exhibit a higher mutation rate than comparable nuclear sequences [1] and are transmitted through the maternal line as a single haploid block of completely linked genes. Besides, several hundreds to several thousand copies of the mtDNA genome are present in each cell [2], a relevant attribute when dealing with ancient, degraded and/or vestigial samples.

The early availability and good performance of conserved sets of primers for amplification of cytochrome *b* (cyt *b*) genes in vertebrates [3] allowed

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its wide application as marker for species DNA variability and ultimately for forensic, traceability and conservation studies [4–9]. Species identification studies based on cyt *b* have ranged from both short (<400 bp) to long (∼900 bp) PCR-RFLP, DNA sequencing, and variable size-species specific multiplex PCR [4–6,8,10].

Livestock robbery is a frequent transgression of law in Argentina. In a previous study, we described a cow stealing case where DNA profiling was used as evidence, supporting the prosecutor accusation in court [11]. In that case, the thieves left the stolen animal's remains on the owner's farm, who recognized them by the mark on the coat. Several meat and bone pieces were then taken from alleged animals in the suspected butchery in order to compare them with the remains found.

Our lab usually receives pieces of evidence from robbery cases as diverse as meat, leather, bones and blood stains on clothes, butchering devices and vehicles. Sometimes samples are inconclusive as to the species provenance (i.e. blood stains) and prosecutors ask for both the species determination and the individual identification.

In the current study, we report on the use of a partial cyt *b* sequence for species identification in forensic casework. In brief, a 358 bp fragment amplified with universal primers is separately digested with three restriction enzymes and the resulting fragments resolved through electrophoresis in polyacrylamide gels. This PCR-RFLP method can be used to identify common domestic and livestock species of the Pampean region (Buenos Aires Province, Argentina), and works on degraded samples.

2. Materials and methods

2.1. Reference sample collection and DNA extraction

Meat pieces or blood samples from the following animal species were provided by the CIGEBA tissue bank: cattle ($N=6$), horse ($N=6$), donkey ($N=1$), pig ($N=1$), sheep ($N=5$), dog ($N=4$), cat ($N=1$), rabbit ($N=1$), chicken ($N=2$) and human ($N=1$). Cattle and horse samples were selected in order to represent different mtDNA lineages as evidenced by control region sequencing [12,13]. For casework studies, 11 pieces of meat were supplied by the Justice Department of Buenos Aires Province (Argentina). These unknown casework samples were suspected to stem from cattle ($N=6$) and horse ($N=5$) by morphological identification.

Total DNA from blood lymphocytes was extracted by the DNAzol® method (Invitrogen, Carlsbad, CA) following the manufacturer's protocol, while DNA from muscle was purified using the protocol described by Giovambattista et al. [11].

2.2. PCR amplification

The universal oligonucleotide primers L14816 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and H15173 5′-CCCCTCAGAATGATATTTGTCCTCA-3′ [6] were used to amplify a 358 bp fragment of the cyt *b* gene by PCR. The amplification reactions were carried out on ∼100 ng of extracted total DNA in a 30 μl final volume containing PCR buffer (final concentration 2.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris, pH 8.4), 100 μM dNTP, 0.2 μM each primer and 0.5 U Taq polymerase (Invitrogen). After 5 min of initial denaturation (94 °C), 30 cycles were run on a thermal cycler (MJ Research, Boston, MA) each comprising 40 s denaturation (93 °C), 40 s annealing (52 °C), and 40 s extension (72 °C), followed by a final extension of 5 min (72 °C).

2.3. RFLP analysis of PCR products

Eight microliters of PCR product were singly digested with 2.5 units of *Hae*III, *Alu*I, and *Hinf*I (Invitrogen) in a final volume of 17 μl for at least 2 h at the temperature recommended by the suppliers. Restriction fragments were resolved by gel electrophoresis on 8% acrylamide–bis acrylamide (19:1) non-denaturing gels in 1× TBE running buffer for 40 minutes at 170 V and silver-stained following Bidler et al. [14].

2.4. Electronic RFLP analysis of cyt b DNA sequences

Fifty-eight cyt *b* sequences including the targeted fragment of interest reported for 12 species were gathered from the GenBank: cattle ($N=17$), horse

($N=3$), donkey ($N=1$), sheep ($N=4$), goat ($N=1$), pig ($N=8$), dog ($N=3$), cat ($N=3$), European hare ($N=7$), rabbit ($N=7$), chicken ($N=5$) and turkey ($N=2$). For humans, the appropriate fragment was obtained from the revised Cambridge Reference Sequence [15].

Initially, the theoretical restriction patterns for 14 enzymes (*Alu*I, *BamH*I, *Bfa*I, *BstY*I, *Dde*I, *Hae*III, *EcoR*I, *Hha*I, *Hind*III, *Hinf*I, *Msp*I, *Taq*I, *Pst*I, and *Rsa*I) were analyzed for the targeted fragment with Webcutter 2.0 [Max Heiman, Copyright 1997; http://searchlauncher.bcm.tmc.edu/]. Based on these preliminary results, three enzymes (*Alu*I, *Hae*III, and *Hinf*I) were selected for further experimental analysis. For each selected enzyme, the restriction band patterns were named sequentially with small letters.

3. Results and discussion

The aim of this study was to develop a simple PCR-RFLP assay in order to discriminate forensic samples among human, and common domestic and livestock species of the Pampean region, Argentina. We selected a 358 bp fragment of cyt *b* for which universal primers amplifying in several vertebrate species were available [3,6]. In addition, this fragment fulfilled the requirement of relative short size and enough sequence divergence to allow our objectives.

Cyt *b* fragments from the species of interest, homologous to the sequence between nucleotide position (np) 14816 and np 15173 in the human reference sequence [15] were gathered from GenBank and submitted to electronic RFLP analysis with 14 enzymes (data not shown). Selection of *Alu*I, *Hae*III, *and Hinf*I for further experiments was based on their putative ability to discriminate between the species of interest in polyacrylamide minigels. Shown in Fig. 1 are the expected restriction patterns for each enzyme, while Table 1 summarizes those patterns for each species. Two different RFLP patterns were obtained for turkey (for *Hae*III) and three rabbit (for *Hinf*I and *Hae*III) while the rest of the non-human species were monomorphic (Table 1). Further variability in humans was analyzed in the literature. High-resolution PCR-RFLP studies available for 2159 individuals from the major continental populations showed that >99% of them share the restriction pattern found in the reference sequence (populations and references are available from the authors upon request). Three minor variants with respect to the human reference sequence were the absence of the otherwise universal *Hinf*I (in 4 Siberians [16]) and *Hae*III (in 2 Africans, 17) sites, and the gain of an *Alu*I site at np 14899 in 1 European [18].

A single fragment of 358 bp resulted from PCR amplification in all the 13 species. PAGE resolution of digested amplicons confirmed the expectations about the number and size of bands (Fig. 2). For some species, however, additional fragments shown up in the gels. Repeated PCR-RFLP with different samples confirmed these results and allowed us to exclude partial digestion for cat (*Alu*I, *Hae*III and *Hinf*I), dog (*Hae*III), and horses and donkey (*Alu*I and *Hinf*I).

Possibly, these results could be attributable to a co-amplification of nuclear mitochondrial pseudogenes (Numts) [19–21], sequences inserted in the nuclear genome that diverge from their ancestral mitochondrial genes with a mutational spectra related to its loss of function. The presence of these mt pseudogenes integrated in nuclear genomes has been largely demonstrated for several taxa [6,22].

Our major goal was to design a simple and reliable PCR-RFLP method to discriminate the specific status of forensic samples. In order to test the performance of our method under 'real case conditions', i.e. overall bad quality of samples, we typed casework samples suspected to belong to cattle ($N=6$) and horse ($N=5$). Results obtained from these experiments showed a perfect match with the morphological identification (Fig. 1). Complete agreement was obtained with further molecular typing of microsatellites (data not shown).

In our experiments, a brief PCR-RFLP protocol with a set of selected enzymes allowed us to discriminate between common species of forensic interest in the area. This approach may be useful when attempting a first, quick assessment as to the specific status of a forensic evidence.

*Hinf*I
45 55 64 162 172 234 279
a b c d e f g h i j
358
294 294
234
196 196 186 234
162 162 162
124 124
117 117
79 79
72
64
55
45 45

*Hae*III
44 74 85 130 190 227 233 304
a b c d e f g h I j k
273
233 230 227
153 183
159 153
131 131 131 131
125 125 125 125
103
74 74 74 74 74 74 74
60
54 56 56
43 44 44
30

*Alu*I
85 109 115 190 304 310
a b c d e f g
358
304
249
243 243
190 190
168
148
120
115
109
85
54
30

Fig. 1. *Alu*I, *Hae*III, and *Hinf*I restriction patterns of cyt *b* based on the analysis of the GenBank DNA sequences. The full length of the PCR product is 358 bp. Sizes for restriction fragments are indicated above the bands. '|' indicates the location of the restriction sites.

Table 1
*Alu*I, *Hae*III and *Hinf*I restriction patterns predicted for cytochrome *b* (cyt *b*) DNA sequences analysis for human, cattle, horse, donkey, sheep, goat, pig, dog, cat, European hare, rabbit, chicken and turkey

Species	N_1	N_2	*Alu*I	*Hae*III	*Hinf*I
Human (*Homo sapiens*)[a]	1	2152	a	a	a
Cattle (*Bos taurus*)	6	17	b	b	b
Pig (*Sus scrofa*)	1	8	e	c	c
Sheep (*Ovis aries*)	5	4	a	d	d
Goat (*Capra hircus*)	0	1	a	e	a
Donkey (*Equus asinus*)	1	1	a	d	b
Horse (*Equus caballus*)	6	3	d	d	e
Dog (*Canis familiaris*)	4	3	g	a	f
Cat (*Felis catus*)	1	3	c	f	g
Rabbit (a) (*Oryctolagus cuniculus*)	1	5	a	h	j
Rabbit (b) (*O. cuniculus*)	0	1	a	k	j
Rabbit (c) (*O. cuniculus*)	0	1	a	k	i
European hare (*Lepus europaeus*)	0	7	f	i	j
Chicken (*Gallus gallus*)	2	5	a	d	h
Turkey (a) (*Meleagris gallopavo domestica*)	0	1	a	g	a
Turkey (b) (*Meleagris gallopavo domestica*)	0	1	a	J	a

N_1, the number examined; N_2, the number from the database. Restriction patterns were named sequentially with small letters (see Fig. 1).

[a] A worldwide sample of 2159 individuals resulted almost monomorphic for the pattern indicated, with three minor variants present in seven individuals: the absence of the otherwise universal *Hinf*I in four Siberians [16] and *Hae*III in two Africans [17] sites, and the gain of an *Alu*I site at 14899 in one European [18].

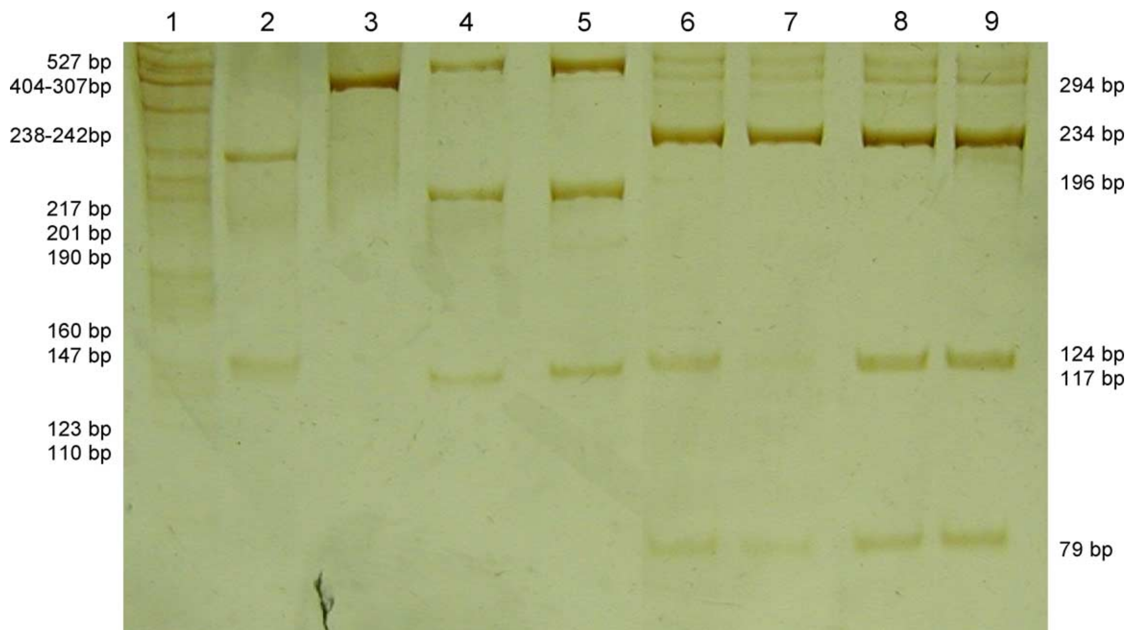

Fig. 2. PCR-RFLP patterns for cytochrome *b* (cyt *b*) obtained by digestion with *Hinf*I are as follows: lane 2, rabbit; lane 3, sheep; lane 4, cattle; lane 5, cattle (forensic sample); lane 6, horse; lanes 7–9, horse (forensic samples). Molecular weight of restriction fragments was detailed on the right. An *Msp*I digest of pBR322 (New England BioLabs, Beverly, MA) was used as size marker (lane 1).

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