Species identification by polymerase chain reaction and restriction fragment length polymorphisms of mitochondrial DNA

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Abstract

Mitochondrial DNA is an important source for species or identity tests because the large numbers of copies of mitochondrial DNA in each cell significantly raise the sensitivity of the analysis. DNA was isolated from compact bones of cows, pigs, chickens, horses, an elephant, and dogs and blood of cows, buffaloes, goats, sheep, pigs, horses, dogs and a human by phenol/chloroform extraction and ethanol precipitation. Semi-nested polymerase chain reaction (PCR) amplified 359 base-pair (bp) fragments of the cytochrome *b* gene in mitochondrial DNA from various animal species. In the subsequent restriction fragment length polymorphism (RFLP) assay, 3 restriction endonucleases (*Hae*III, *Hinf*I and *Mbo*I) were applied to differentiate between those species. The sensitivity of semi-nested PCR amounted to 0.3 pg of elephant template DNA. Defining the species of origin of unknown animal and human materials are essential for the meat processing industry, animal conservation and forensic science.

Keywords: mitochondria, DNA, cytochrome *b*, species, differentiation.

Introduction

Establishing species of origin are one of the fundamental aims of analyses applied in the process of identifying biological materials in forensic laboratories. In court cases, where the only material evidence is a trace of animal or plant origin, defining its species attains a primary significance. Defining the species of origin is also becoming more and more significant in other fields such as the meat industry, fish processing and environment protection. Classical methods for species identification are mainly based on protein identifications either by electrophoresis (Bossezon *et al*., 1966) or agglutination techniques (Wiener *et al*., 1949). The methods have limited applications for the aged or degraded specimens, as protein molecules are subjected to time and environmental destruction. With the advance in molecular biology, new methods emerge, based on genetic differences among species.

For the aged and highly degraded specimens which are devoid of nuclear DNA, mitochondrial DNA (mtDNA) is the only source for species or identity testing. The large numbers of mtDNA copies present in each cell, significantly enhance the sensitivity of the analysis (Holland *et al*., 1993). One of the regions of the mtDNA used when establishing phylogenetic links among species and in species identification is a fragment of the gene encoding cytochrome *b* (*Cytb*). It has been shown that this region can be amplified in various species of animals using a single pair of universal primers in a polymerase chain reaction (PCR) (Kocher *et al*., 1989).

Bones contain few cells, therefore, only small amounts of DNA are available for study. Since only a small amount of target template DNA exists, efficient DNA extraction and amplification are crucial. We demonstrated a semi-nested PCR technique to increase sensitivity of the technique and used direct sequencing to authenticate PCR products. Here we report the suitability of the polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) techniques for identification of animal and human specimens.

Materials and methods

Sample. Surface materials were removed from the bones of three cows, three pigs, three chickens, three horses, an elephant and three dogs by washing with diluted bleach and distilled water. A 2x5 cm portion was cut from each compact bone. The clean bone fragments were mechanically ground into a fine meal in a sterile agate mortar. Peripheral blood mononuclear cells (PBMCs) were separated from whole EDTA blood of three cows, three buffaloes, three goats, three sheep, three pigs, three horses, three dogs and one human using Ficoll-Hypaque gradients. The specimens were kept at -70^{0} C until tested.

DNA extraction. DNA was extracted from 0.5 g bone powder or 100 µl PBMCs with proteinase-

K/SDS in Tris buffer, followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 30 µl sterile water and subjected to semi-nested PCR-based amplification. DNA extracts were measured by Gene Spec I (MiraiBio Inc, Japan) and the photometric values at 260 and 280 nm were used.

PCR primer design. Based on mitochondrial sequences available in the GenBank database (J01415, NC_001567, NC_002008, AF034253), we used the CLUSTAL X program (NCBI) to design a conserved primer, a reverse primer (CYT b3 5'TAGTTGTCTGGGTCTCCTAGGAGGTC3' position 15,488-15,513) and used the primers described by Kocher *et al.* (1989); CYT b1; 5'-CCATCCAACATCTCAGCATGATGAAA-3' position 14,816-14,841 and CYT b2; 5'-GCCCCTCAGAATGATATTTGTCCTCA-3' position 15,149-15,174 for the human mtDNA sequence (Anderson *et al.*, 1981).

DNA detection. DNA was amplified in an automated thermocycler (Perkin Elmer Cetus, Branchburg NJ, USA) using semi-nested primers of the *Cytb* gene. The PCR mixture (25 µl) contained 10 µl of MasterMix (2.5x) (1.5 mM Magnesium, 200 µM dNTPs and 1.25 U Taq DNA polymerase) (Eppendorf, Hamburg, Germany), 8 µl of distilled water, 60 pmol (1 µl) of each primer and 5 µl of mitochondrial DNA. The primary PCR mixture contained an upstream primer CYT b1 and a downstream primer CYT b3. The first amplification round consisted of an initial denaturation step at 94^0 C for 2 minutes, followed by 40 cycles comprising a 5 second denaturation step at 94^0 C, a 1 minute annealing step at 50^0 C and a 40 second extension step at 72^0 C, each. The amplification was concluded by a 4 minute elongation step at 72^0 C. The secondary PCR mixture (50 µl) contained 20 µl of MasterMix (2.5x) (Eppendorf, Hamburg, Germany), 23 µl of distilled water, 60 pmol (1 µl) of an upstream primer CYT b1 and of a downstream primer CYT b2 and 5 µl of primary PCR product. The conditions was the same as for the primary reaction. The secondary PCR products (359 bp) were analyzed on a 2% agarose gel made up in 1x TBE and containing 0.5 µg of ethidium bromide per ml.

Mitochondrial DNA sequencing. Secondary PCR products were purified for sequencing by the QIAquick Gel Extraction Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's specifications and subsequently subjected to electrophoresis on a 2% agarose gel in order to ascertain their purity. DNA was subjected to cycle sequencing by dye-labeled terminators which represents a rapid and convenient method for performing enzymatic extension reactions for subsequent DNA sequencing on the ABIPRISM™310 Genetic Analyzer (Perkin Elmer Cetus, Branchburg, NJ, USA). This round of amplifications was performed according to the manufacturer's specifications using the forward primer CYT b1 to amplify the particular DNA strand of interest for further sequencing. Cycle sequencing consisted of 25 cycles at 96^0 C for 10 seconds (denaturation), 50^0 C for 5 seconds (annealing), and 60^0 C for 4 minutes (extension). The reaction was terminated by cooling the thermal ramp to 4^0 C. We applied the

BLAST program (NCBI) to compare our sequences with sequences available in GenBank.

Species identification by RFLP. *Cytb* PCR products were used for species typing by RFLP technique. We further applied the restriction endonuclease most suitable for species identification by selecting from a table of enzymes using Webcutter, version 2 (www.ccsi.com/firstmarket/cutter). Computer predictions of restriction fragment sizes from PCR products indicated that *Hae*III, *Hin*fI and *Mbo*I should produce differentiable cleavage patterns of each species. Accordingly, we selected *Hae*III, *Hin*fI and *Mbo*I for species identification. Twenty units of enzyme were added to 20 µl of secondary PCR products and subsequently incubated at 37^0 C for 4 hours. The cluster products were subjected to electrophoresis on a 3% agarose gel and their respective sizes were compared to that of a suitable nucleotide size marker (100 bp DNA ladder, NEB, New England, USA). The expected sizes of the polymorphisms after RFLP are shown (Table 1).

Results

Based on the 260 nm/280 nm ratio, the DNA quantities from the compact bones of various species are shown (Table 2). A fragment of the *Cytb* gene (359 base pairs) from all compact bones was successfully amplified by semi-nested PCR. Likewise, all specimens were successfully amplified by semi-nested PCR using PBMCs as source materials.

Species identification was accomplished by restriction enzyme digestion. After digestion with *Hae*III, *Hin*fI and *Mbo*I and electrophoresis, each species yielded a characteristic pattern corresponding to that predicted by DNA sequence analysis as shown (Figs. 1-3), respectively.

We confirmed the positive PCR results by direct sequencing. Comparison applying the BLAST program (NCBI) search indicated that these sequences were similar to sequences of the cow, the buffalo, the goat, the sheep, the pig, the chicken, the horse, the elephant, the dog and the human stored in GenBank, accession numbers AY676873, AY521168, DQ089479, AY840104, AF034253, AY509649, AY819737, D50846, AF028136 and AY963583, respectively. All those sequences were submitted to the GenBank database with accession numbers DQ236088-DQ236097.

Discussion

The goal of efficient extraction is to obtain sufficient quantities of DNA to successfully amplify specific target sequences. Most of the DNA in compact bone is located in the osteocytes. There are approximately 20,000 to 26,000 osteocytes per cubic millimeter of calcified bone matrix (Frost, 1960). Fisher *et al.* (1993) reported that decalcification is not a necessary step in the DNA extraction process. Rankin *et al.* (1996) obtained an average of 73 µg DNA per gram of human compact bones while we

were able to extract between 575 and 2,470 µg DNA per gram of animal compact bones.

Insufficient amounts of DNA copies after PCR amplification lead to weak signals, therefore a semi-nested PCR amplification was adopted. We selected the universal primers CYT b1 and CYT b2 (Kocher *et al.*, 1989) and designed a reverse primer CYT b3 for amplification of a 359 bp fragment of various animal species to develop a PCR-RFLP assay for species identification. The sensitivity for the single-round PCR (Branicki *et al.*, 2003) was 10 pg of pig template DNA and 50 pg of cat template DNA whereas that for the semi-nested PCR in this study was 0.3 pg of elephant template DNA (data not shown).

The important points in PCR-RFLP analysis are the selection of endonucleases to allow maximum differentiation between the species under investigation, to ascertain the quality of endonucleases to facilitate complete digestion of the DNA fragments analyzed, and also to be easily available and low priced. Meyer *et al.* (1995) demonstrated a PCR-RFLP analysis for species identification in food products of animal origins (pigs, cattle, wild boars, buffaloes, sheep, goats, horses, chickens and turkeys) using four restriction endonucleases (*Alu*I, *Rsa*I, *Taq*I and *Hinf*I). To distinguish among 25 species, Wolf *et al.* (1999) used 11 restriction endonucleases. In this study, we were able to differentiate between nine animal species (cow, buffalo, goat, sheep, pig, chicken, horse, elephant and dog) and man using only three restriction endonucleases (*Hae*III, *Hinf*I and *Mbo*I) which were availabel in our research.

PCR-RFLP is simple and DNA samples from exotic animals can be analyzed quickly. Using specific primers for mitochondrial DNA and subsequent RFLP could be valuable to wildlife officers and responsible government bodies requiring identification of animal samples, particularly with regard to illegal poaching, trade of endangered animals, and forensic science when bones are the only samples left to be identified.

Table 1. Representations of experimental and theoretical PCR-RFLPs for specimens differentiation

Specimens	Size of DNA fragments (bp) after digestion with indicated endonuclease		
	*Hae*III	*Hinf*I	*Mbo*I
Cow	285	198	359
	74	117	
		44	
Buffalo	285	359	244
	74		115
Goat	230	198	213
	74	161	115
	55		31
Sheep	159	359	244
	126		115
	74		
Pig	153	359	244
	132		115
	74		
Chicken	159	188	359
	126	161	
	74	10	
Horse	159	234	359
	105	81	
	74	44	
Elephant	359	359	244
			115
Dog	233	203	244
	126	94	115
		44	
		18	
Human	232	198	192
	106	161	115
	21		52

Table 2. Photometric determination of DNA recovered from various specimens

Specimens	260 nm/280 nm	DNA (μg)/g samples
Cow	0.154	755
Pig	0.256	1,280
Chicken	0.494	2,470
Horse	0.135	675
Elephant	0.381	1,905
Dog	0.115	575

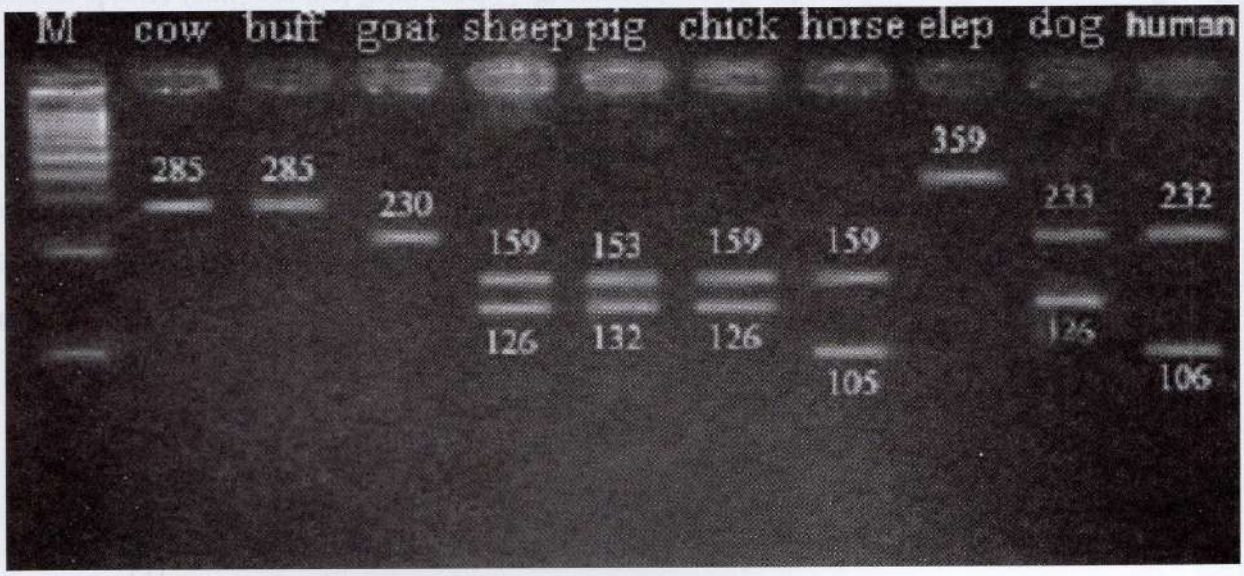

Fig. 1 RFLP patterns of *Cytb* gene of thecow, the buffalo, the goat, the sheep, the pig, the chicken, the horse, the elephant, the dog and thehuman, respectively, digested with endonuclease *Hae*III; M: 100-bp marker

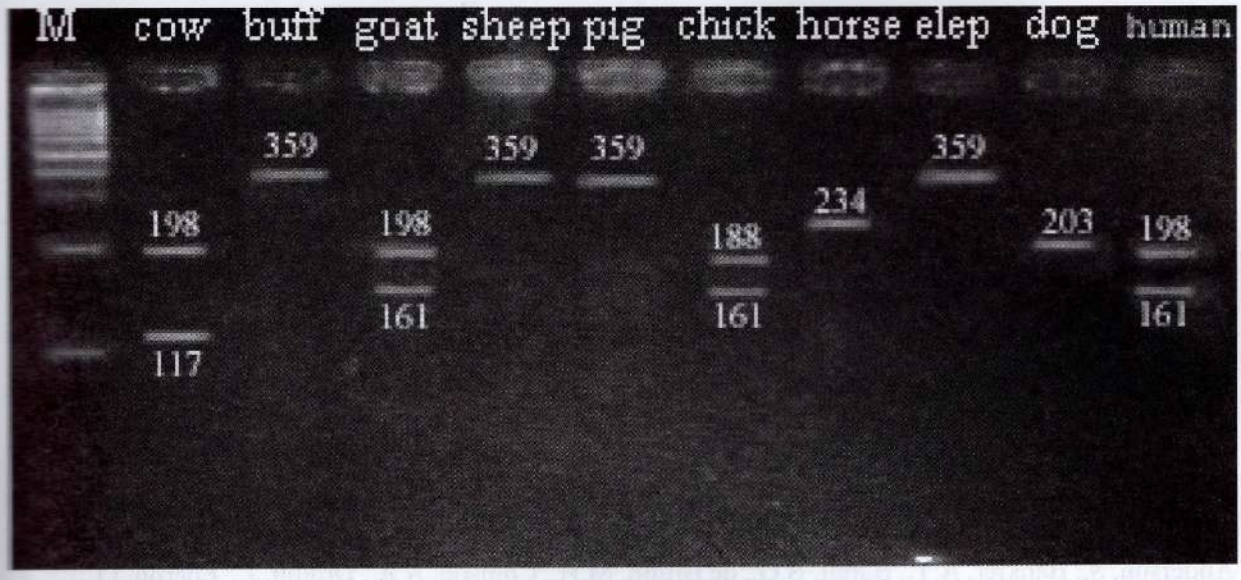

Fig. 2 RFLP patterns of *Cytb* gene of the cow, the buffalo, the goat, the sheep, the pig, the chicken, the horse, the elephant, the dog and the human, respectively, digested with endonuclease *Hinf*I; M: 100-bp marker

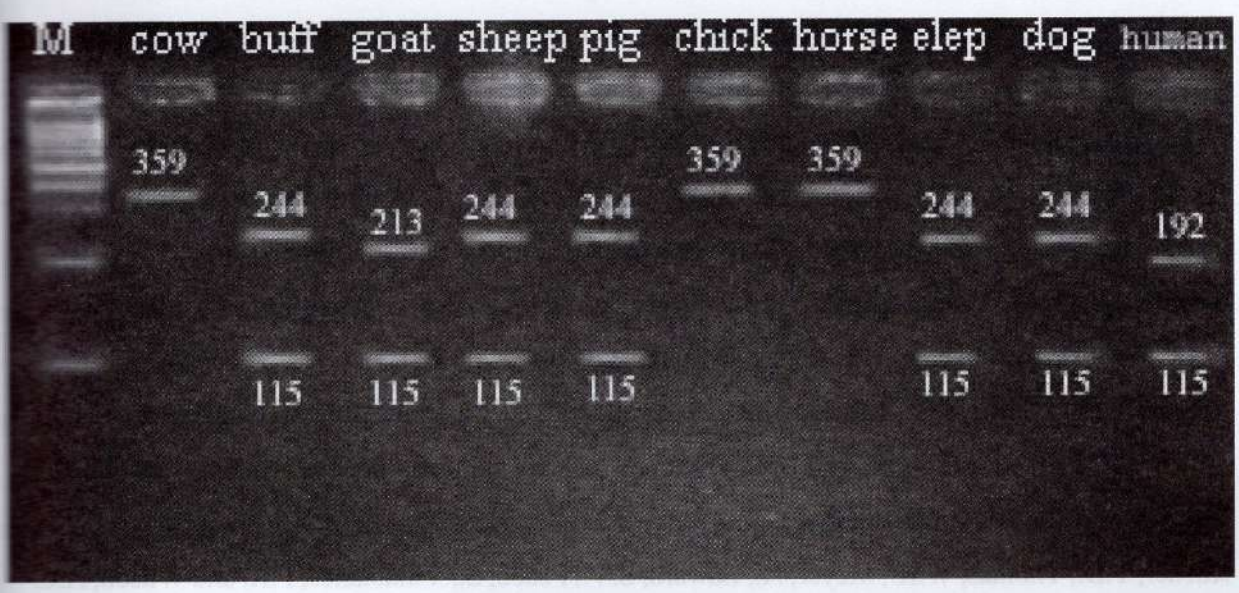

Fig. 3 RFLP patterns of *Cytb* gene of the cow, the buffalo, the goat, the sheep, the pig, the chicken, the horse, the elephant, the dog and the human, respectively, digested with endonuclease *Mbo*I; M: 100-bp marker

Acknowledgements

The authors would like to thank Department of Veterinary Anatomy, Faculty of Veterinary Science, Chulalongkorn University for collecting the specimens and to staff of Center of Excellence in Viral Hepatitis, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University for their assistance in the molecular laboratory. We would also like to thank Chulalongkorn University-Veterinary Science Research Fund RG 6/2547 and the Thailand Research Fund, Senior Research Scholar and the Molecular Research Project, Faculty of Medicine, Chulalongkorn University for supporting this research.

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การจำแนกชนิดสัตว์โดยอาศัยปฏิกิริยาลูกโซ่โพลีเมอเรสและลักษณะความยาวชิ้นส่วนที่แตกต่างกันของไมโตครอนเดรียดีเอ็นเอภายหลังตัดด้วยเอนไซม์

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บทคัดย่อ

ไมโตครอนเดรียดีเอ็นเอเป็นแหล่งสำคัญที่ใช้ในการตรวจหาชนิดสัตว์เนื่องจากมีจำนวนมากในแต่ละเซลล์ทำให้เพิ่มความไวของการตรวจวิเคราะห์ สกัดดีเอ็นเอจากตัวอย่างกระดูกของโค สุกร ไก่ ม้า ช้าง และสุนัข และเลือดของโค กระบือ แพะ แกะ สุกร ม้า สุนัขและมนุษย์โดยใช้ฟีนอล/คลอโรฟอร์มและตกตะกอนด้วยเอทานอล ทำการเพิ่มปริมาณดีเอ็นเอขนาด 359 เบสในยีนไซโตโครมบี ซึ่งเป็นส่วนหนึ่งของไมโตครอนเดรียดีเอ็นเอจากตัวอย่างที่ได้ จากนั้นใช้หลักการของเอนไซม์ *Hae*III, *Hinf*I และ *Mbo*I ที่ตัดชิ้นส่วนดีเอ็นเอได้หลากหลายในการจำแนกชนิดสัตว์ ความไวของการทำปฏิกิริยาลูกโซ่โพลีเมอเรสโดยใช้กระดูกช้างเป็นตัวอย่างในครั้งนี้เท่ากับ 0.3 พิโครกรัม การจำแนกชนิดสัตว์จากตัวอย่างที่ไม่ทราบชนิดที่แน่นอนมีความสำคัญกับอุตสาหกรรมที่เกี่ยวข้องกับการนำเนื้อมาเป็นอาหารและนิติวิทยาศาสตร์

คำสำคัญ: ไมโตครอนเดรีย ดีเอ็นเอ ไซโตโครมบี ชนิดสัตว์ การจำแนก