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Animal species identification by co-amplification of hypervariable region 1 (HV1) and cytochrome *b* in mitochondrial DNA

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요　약 : 미토콘드리아 DNA(mtDNA) 상의 조절부위(control region)에는 HV1과 HV2와 같은 과변이부위(hypervariable region)가 있으며, 이 부위에서 사람마다 차이가 나는 많은 SNPs(single nucleotide polymorphism)을 발견할 수 있다. mtDNA 염기서열 분석은 개인식별 및 백골화된 시신등의 신원확인에 유용하게 사용되어왔다. mtDNA상의 cytochrome b(*cyt*b) 유전자는 분자계통학(molecular phylogenetics) 분야에 널리 이용되고 있으며, 법과학 분야에서의 동물 종식별은 다양한 사건 현장 증거물의 인수식별 뿐 아니라 불법 유통되고 있는 각종 동물성 건강식품, 의약품의 원료 규명 및 보호 종의 밀렵 증명 등에 유용하게 적용될 수 있다. 본 연구에서는 광범위한 동물의 cytochrome b 유전자를 증폭할 수 있는 primer sets (H14724/L15149)와 사람에 특이적인 HV1 부위 primer set (H15997/L16236)를 이용한 동시 증폭을 통해 먼저 사람과 동물을 구별하였고, *cyt*b 증폭산물의 직접 DNA 염기서열 분석을 통해 종식별을 수행하였다. H14724/L15149 primer pair는 닭과 오리를 제외하고 사람, 소, 돼지, 개, 고양이, 생쥐, 쥐의 *cyt*b를 증폭할 수 있었으며, H14841/L15149 primer pair는 닭과 오리도 증폭할 수 있었다. 효모, 곤충 및 세균은 모두 증폭산물이 생산되지 않았으며, H15997/L16236의 경우 사람의 HV1만이 선택적으로 증폭되었다. 또한 실제 사건의 예에서와 같이 본 연구가 혈흔의 종식별에 매우 유용함을 보여주었다.

Abstract : Mitochondrial DNA (mt DNA) sequence analysis has been a useful tool for species identification of animals and human individuals. Two hypervariable regions (HV1 and HV2) in control region of mitochondrial genome were analyzed for human individual identification. In case of animal species identification, several genes on mt DNA such as cytochrome b (*cyt*b), RNAs, cytochrome oxidases (CO) were used. In this study, co-amplification of HV1 and *cyt*b was carried out in order to check the contamination of animal DNA and to verify the human DNA. The primer sets used in PCR were H15997/L16236 for HV1 and H14724/L15149 for *cyt*b. PCR products for HV1 and *cyt*b were 239 bp and 425 bp, respectively. The appearance of two bands on agarose gel implied the DNA came from human, however the single band of *cyt*b gene represented the non-human animal DNA.

Key words : Co-amplification, mitochondrial DNA, cytochrome b, HV1, species identification

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1. Introduction

The determination of the origin of a biological stains such as blood stains from crime scene evidences is very important in analysis of forensic casework. The commonly used methods of using STR (Short Tandem Repeat) markers are human-specific that non-human DNA do not produce any PCR product. Mitochondrial DNA (mtDNA) sequence analysis became an effective tool for animal species identification in addition to human identification. The identification of species using DNA technique has several advantages over protein analysis.[1] The most straightforward method for the determination of species is sequencing of the mitochondrial PCR product such as cytochrome *b* gene.[2,3] Cytochrome b (*cyt*b) have been considered as a good gene in species identification because it is a region with little variance within species but distinct differences between species.[4] Species identification using molecular biological approach became very useful tool for forensic casework analysis as well as poaching and smuggling of conservative and endangered animals. A lot of people are living with dogs and cats in their house. For that reason, hairs from pets could be related to the crime scene evidences. Futhermore, poaching and illegal trading of animals is increasing recently.

In this study, a segment of *cyt*b gene was co-amplified with hypervariable region 1 (HV1) and the species was determined by direct sequence analysis and web-based program of BLAST (Basic Local Alignment Search Tool) search of NCBI database. The two PCR products (two bands of 425 bp and 239 bp in size), one from *cyt*b and the other from HV1, mean that the sample originated from human. On the other hand, only one PCR product of cytochrome b (425 bp) designates that the sample come from non-human animal. Several non-human samples were tested to evaluate this method of species identification.

2. Materials and Methods

2.1. DNA samples

Reference DNA samples were purchased from Promega Co. (Madison USA) (*Table* 1). QIAamp DNA Mini kit (QIAGEN Inc. Valencia USA) was used for extraction of DNA from case work samples such as blood stains.

Table 1. DNA samples from various species

Species	Sources	Concentrations (mg/m*l*)
Shizosaccharomyces pombe	Promega G311A	0.16
Saccharomyces cerevisiae	Promega G310A	0.38
Drosophila melanogaster	Promega G314A	0.23
mouse	Promega G309A	0.13
rat	Promega G313A	0.12
pig	Promega G307A	0.17
chicken	Promega G312A	0.22
cat	Promega G306A	0.21
bovine	Promega G308A	0.17
dog	Promega G305A	0.25
Escherichia coli K12	ATCC 29425	0.25
Desulfovibrio desulfuricans	ATCC 27774	0.28
Human 1	Promega G773A	0.02
Human 2 (K562)	Promega DD208A	0.01

2.2. PCR primers and amplification conditions

Three sets of primer pairs were designed based upon the published GenBank sequences of *cyt*b gene and HV1 on mitochondrial genome (*Fig*. 1). H14841/L15149 and H14724/15149 were prepared for amplification of *cyt*b gene.[5] H15997/L16234 was selected for amplification of HV1 on human DNA.[6] DNA sequences of primers were shown in *Table* 2. Amplification were performed in GeneAmp PCR system 9700 (Perkin Elmer) and PCR products were photographed after agarose gel electrophoresis (1.5% agarose in 1× TBE buffer containing 1 μg/mL ethidium bromide). Approximately 1~10 ng of template DNA was added to each PCR reaction. The PCR reaction mix contained as follows; 0.5 μM each of forward and reverse primer for cytochrome b and HV1, 2.5 U of Gold-

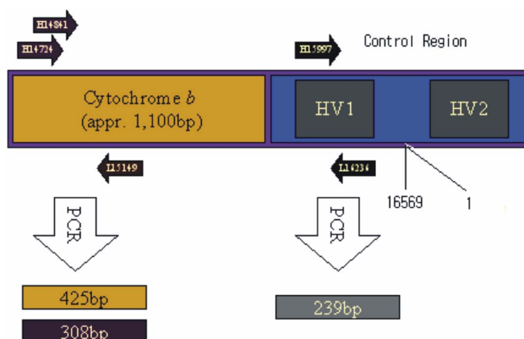

Fig. 1. Location of cytochrome b gene and HV1 in mitochondrial DNA.

Table 2. PCR and sequencing primers

Name	Sequences	Length
H14724	5'-CGAAGCTTGATATGAAAAACCATCGTTG-3'	28-mer
H14841	5'-AAAAAGCTTCCATCCAACCAACATCTCAGCATGATGAAA-3'	39-mer
L15149	5'-AAACTGCAGCCCCTCAGAAATGATATTTGTCCTCA-3'	35-mer
H15997	5'-CACCATTAGCACCCAAAGCT-3'	20-mer
L16236	5'-CTTTGGAGTTGCAGTTGATG-3'	20-mer

Taq DNA polymerase (1 U/μL), 0.2 μM each dNTP (2.5 mM each), 1.5 mM $MgCl_2$ (50 mg/μL), 4 μg of BSA, and 1× PCR buffer (PE). Sterile distilled water was added to bring the final volume of 25 μL. Cycling protocol for co-amplification of mt HV1 and cytochrome b was as follows; initial denaturation of 11 min at 94°C, 30 cycles of 20 s at 94°C, 30 s at 50°C and 45 s at 72°C, followed by a final extension for 7 min at 72°C.

2.3. DNA sequence analysis and determination of species

DNA sequence analysis was carried out using the same primers as PCR (*Table* 2). PCR products were purified by QIAGEN PCR product purification kit according to the manufacturer's guide. DNA sequencing was carried out using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit ver3.0 (Applied Biosystems) and ABI310 Genetic Analyzer (Applied Biosystems). The resulting DNA sequence data of cytochome b gene were checked by eye for quality and submitted to BLAST search in GenBank database (NCBI, USA).

3. Results and Discussion

3.1. Co-amplification of cytochrome b gene and HV1

In order to determine the origin of samples, cytochrome b gene and HV1 region in mitochondrial DNA were co-amplified. The DNA sequence of PCR products of cytochrome b gene was analyzed to identify the species. We used several DNA samples including human genome as target DNA for co-amplification (*Fig*. 2). Two sets of primer pair were used for amplification of *cyt*b, H14725/L15149 (425 bp) and H14725/L15149 (308 bp). DNA samples from mouse, rat, pig, cat, bovine, and dog DNA produced PCR product of *cyt*b only. Human DNA pro-

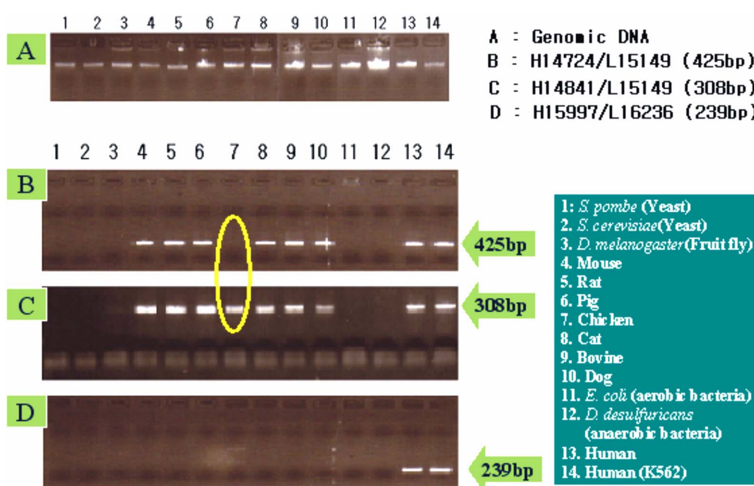

Fig. 2. PCR of cytochrome b gene and HV1. Plate A showed that the quantity and quality of the target DNA. Amplification of cytochrome b gene using two sets of primer pairs (H14724/L15149 and H14841/L15149) were shown on plate B and plate C, respectively. The human-specific amplification of HV1 was shown on plate D.

Fig. 3. Co-amplification products of various DNA samples. Primers for cytochrome b gene and HV1 were H14724/L15149 and H15997/L16236, respectively. The numbers in the figure indicated the same DNA samples as in Fig. 2.

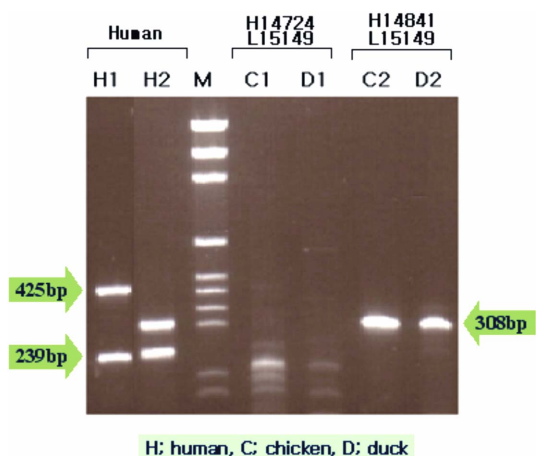

Fig. 4. Co-amplification of cytochrome b gene and HV1 using two different sets of primer pair producing 425 bp and 308 bp. Human DNA (H1 and H2) produced two PCR products with both primer pairs while chicken and duck DNA produced only 308 bp PCR product with H14841/L15149 primer pair.

duced PCR products of both cytochrome b and HV1 (*Fig*. 3). However, bacteria, yeasts and insect DNA didn't produce by primers for either cytochrome b or HV1. Chicken DNA produced cytochrome b products only when using H14841/L15149 primer pair. H14725/L15149 primer pair didn't work with chicken as well as duck DNA (*Fig*. 4). The two PCR products (two bands of 425 bp and 239 bp on agarose gel) of co-amplification of cytochrome b and HV1, one from cytochrome b and the other from HV1, designated that the sample originated from human. On the other hand, one band of *cyt*b PCR product (425 bp) on agarose gel indicated that the sample came from non-human animal. If there were no PCR products on agarose gel, there would be two possibilities; one is that the DNA sample would come from poultry or non-animals such as bacteria and insect, the other is that there is not enough quantity of DNA for PCR.

3.2. Determination of species by DNA sequence analysis

The PCR product of *cyt*b gene was directly used for DNA sequencing. The resulting DNA sequence of cytochrome b gene was compared with the sequences stored in the database through BLAST program. The matching results from BLAST search were 100% or slightly less.

3.3. Application to forensic casework

Blood stains on the coat of the intruder were submitted to laboratory for DNA typing. Most of the blood stains on the coat produced PCR products of AmpliFSTR profiler kit (Applied Biosystems) except one blood stain which didn't produce any PCR products. It was suspected to be a non-human blood stain. Co-amplification of *cyt*b gene and HV1 was carried out using the DNA samples extracted from the non-human blood stain (*Fig*. 5). Only one band of approximate size of 425 bp was shown on the agarose gel which was corresponding to the PCR prodcut of cytochrome b gene. The co-amplification poduct was directly used as template DNA for DNA sequencing. The analyzed DNA sequence data from the non-human blood stain was submitted to GenBank database to search the matching sequence using BLAST program. The *cyt*b sequence of dog showed perfect match to the submitted DNA sequence (*Fig*. 6). As a result, one blood stain on the coat of intruder

Fig. 5. Determination of animal or human origin by co-amplification of cytochrome b gene and HV1. The blood stain (lane 1 and 2) produced only 425 bp PCR product of cytochrome b gene after co-amplification with HV1 meaning that it is not human blood stain.

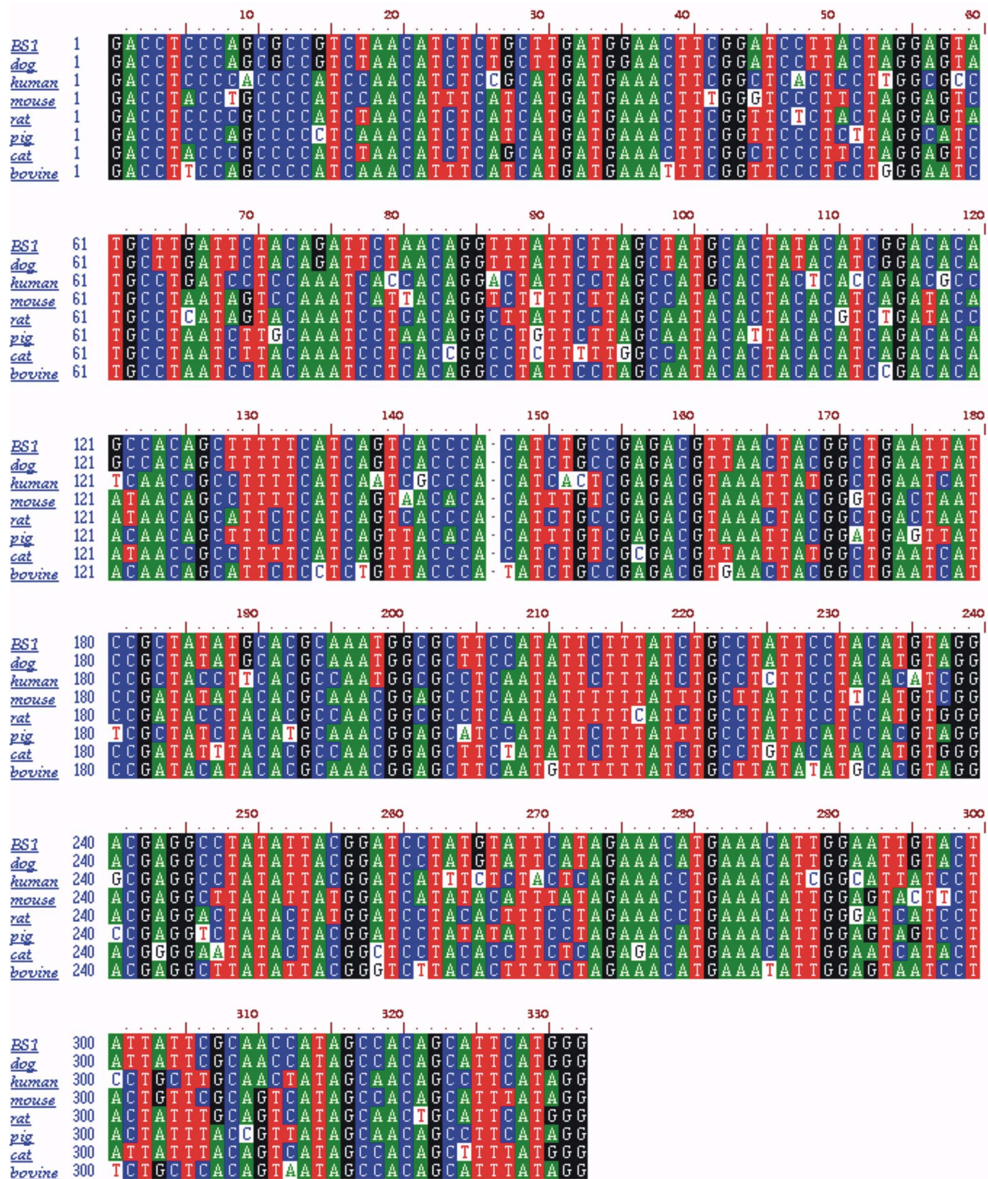

Fig. 6. Multiple alignment of the DNA sequences of cytochrome b gene from various species showing that the blood stain (BS1) matched exactly with dog.

was shown to come from dog. It was found that the intruder had been bitten by the dog in the house during fighting.

There was a threatening letter with bloody photograph to kill a very important person (*Fig*. 7). The blood stain on the photograph showed positive reaction to LMG (Leucomalichite Green) test. DNA extracted from the blood stain, however, didn't produce any PCR products by STR typing kit and mtDNA HV1. DNA sequencing and BLAST search were carried out using co-amplification product of *cyt*b gene and HV1. It was determined by DNA sequence data of *cyt*b gene that the origin of the blood stain was pig.

Fig. 7. Blood stain on the photograph. The blood stain was LMG positive but didn't product any PCR products by STR typing kit and mtDNA HV1.

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