

## Species Authentication of Dog, Cat, and Tiger Using *Cytochrome $\beta$* Gene

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### ABSTRACT

Adulteration of animal food products for economic reason has happened during the last decades. Species identification method development was needed to prevent falsification information. The objective of this research was to study species authentication (dog, cat, and tiger) to ensure animal origin in products using *cyt  $\beta$*  gene specific marker. DNA extraction and fragment amplification were conducted using phenol-chloroform and multiplex PCR (Polymerase Chain Reaction) method, respectively. This research showed that fragment length of amplification for species tested (dog, cat, and tiger) were 523, 331, 319 bp, respectively. Species specificity was also indicated by high reverse primers homology percentage. Multiplex PCR technique succeed to amplify DNA fragment from species tested, but has a limitation to amplify total DNA composite of mix DNA.

**Key words:** *cat, cytochrome  $\beta$  gene, dog, multiplex PCR, tiger*

### ABSTRAK

Upaya pemalsuan produk pangan asal ternak dengan alasan ekonomi masih sering terjadi hingga saat ini. Pengembangan metode identifikasi spesies diharapkan dapat melindungi konsumen dari pemalsuan informasi. Penelitian ini bertujuan untuk mempelajari pembuktian spesies anjing, kucing, dan harimau menggunakan marka spesifik berbasis gen sitokrom  $\beta$  (*cyt  $\beta$* ). Ekstraksi DNA dilakukan dengan metode fenol-kloroform, semetara amplifikasi fragmen DNA menggunakan metode multipleks PCR. Penelitian ini menunjukkan bahwa amplifikasi panjang fragmen pada spesies anjing, kucing, dan harimau adalah 523, 319, dan 319 pb. Selain itu, kespesifikan spesies juga ditunjukkan dengan persentase homologi primer reverse yang tinggi pada masing-masing spesies. Metode multipleks PCR berhasil mengamplifikasi fragmen DNA dari semua spesies yang diuji, namun mempunyai keterbatasan dalam mengamplifikasi gabungan DNA total semua spesies.

**Kata kunci:** *anjing, gen sitokrom  $\beta$ , harimau, kucing, multipleks PCR*

### INTRODUCTION

Today, many consumers are concerned by issues variety, such as food authenticity and adulteration (Aida *et al.*, 2005; Ahmed *et al.*, 2007; Abdel-Rahman *et al.*, 2009). The identity of species origin in processed or composite mixture is not always readily apparent and accurate (Aida *et al.*, 2005; Sakalar & Abasiyanik, 2012). Consumers rarely can identify the species in product that they purchase: fresh or frozen cuts, and processed meat such as sausage, jerky, and canned foods (Hsieh *et al.*, 2005; Ahmed *et al.*, 2007). This opens fraudulent adulteration and substitution possibility of expected species with less costly value (Che Man *et al.*, 2007; Rastogi *et al.*,

2007; Abdel-Rahman *et al.*, 2009). To protect consumer rights, the legislation of each country should impose an accurate labelling declared the species to prevent food fraud (Ahmed, 2007; Abdel-Rahman *et al.*, 2009; Ballin, 2010). The government has tried to protect consumers with the law (Law of the Republic Indonesia no. 8, 1999) and government regulation (Government Regulation no. 28, 2004, on safety, quality, and nutrition).

Most assays for species identification test only for husbandry species (Matsunaga *et al.*, 1999; Hsieh *et al.*, 2005; Martin *et al.*, 2007a; Ahmed *et al.*, 2007; Rastogi *et al.*, 2007), and only a few reports for detection pet species in commercial materials (Ilhak & Arslan, 2007; Martin *et al.*, 2007b). Even though cat and dog are not commonly used, their presence in food products occasionally occurs (Martin *et al.*, 2007b), such as the use of cat and dog meat in beef, lamb, and goat meat (Ilhak & Arslan, 2007).

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Fraudulent substitution of alternative meat species in meat product needs a reliable and specific methods to determine the species.

Beside meat falsification, banned trade of endangered animals may still exist (Fajardo, 2010). Protected animal such as tiger is usually used as a component of medical product (Traditional Chinese Medicines) (Kitpipit *et al.*, 2012; Wetton *et al.*, 2004). This required supervision to prevent falsification information to consumer, along with increased market demand and high prices (Wetton *et al.*, 2004).

Molecular technique development which can detect at DNA level are more accurate, although the samples had been processed. DNA sequence amplification from several species with a lot of primer (using same forward primer) in same reaction is one of the variation PCR (Polymerase Chain Reaction) called multiplex PCR (Matsunaga *et al.*, 1999; Markoulatos *et al.*, 2002; Jain *et al.*, 2007). Matsunaga *et al.* (1999) using multiplex PCR to identify six meats (cattle, pig, chicken, sheep, goat, and horse) processed. Multiplex PCR could be used as a routine method with highly sensitive, rapid, simple, and not expensive to distinguish species (Jain *et al.*, 2007). This research was to study species authentication (i.e. dog, cat, and tiger) to ensure animal origin in product using *cyt β* gene specific marker and multiplex PCR. Thus, if specific reverse primers of *cyt β* gene obtain, species identification will conduct at the same time for several species suspected.

*Cyt β* gene is one of gene in mitochondrial DNA (mtDNA). mtDNA have multiple presences in cell (Minarovic *et al.*, 2010). *Cyt β* gene was used for species identification, but in 2003, cytochrome c oxidase subunit 1 (CO1) gene 'barcoding' was introduced for species identification and taxonomy. The size of *cyt β* gene ranging from 1130 to 1149 bp (Tobe *et al.*, 2009) with average 1140 bp (Minarovic *et al.*, 2010), and CO1 ranging from 1537 to 1557 bp (Tobe *et al.*, 2009). CO1 had more conserve area (43.7% of 1557 bp) than *cyt β* (22.4% of 1149 bp). Hence, for smaller fragment in mammalian samples, *cyt β* gene will offer greater informative (Tobe *et al.*, 2009).

## MATERIALS AND METHODS

### Specific Primers

Specific primers of *cyt β* gene were used to amplify DNA fragment of goat, chicken, cattle, pig, and horse followed Matsunaga *et al.* (1999) method. DNA fragment amplification of sheep used a modified primer from Matsunaga *et al.* (1999), and rat primer followed the method of Nuraini *et al.* (2012). Forward primer used to amplify ten animals was same, and sequence of the primer as follows: 5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3' (Matsunaga *et al.*, 1999). DNA sequences of dog (GenBank JF342903), cat (GenBank AB194817), and tiger (GenBank EU184702) were aligned using MEGA 5 software, furthermore specific reverse primers of *cyt β* gene were designed manually (Table 1).

### DNA Extraction

Blood samples (goat, chicken, cattle, sheep, horse, cat, rat), cooked meat samples (pig and dog), feces sample (tiger) were used for DNA extraction. Meat samples were used about 25 mg and feces sample in 1 x STE solution about 500 μL Tiger feces normally contains some mucous. This mucous expected to contain epithelial tissue was kept in 1 x STE solution for DNA extraction process. DNA extraction process used phenol-chloroform method (Sambrook & Russel, 2001), included sample preparation, protein degradation, organic degradation, and DNA precipitation. Extraction process for meat and feces was started at protein degradation level. DNA concentration used for copying process in PCR was 50 μg/mL. Using sample with same concentration conducted to equate amplification (Nuraini *et al.*, 2012).

### DNA Genome Pool

Genomics DNA from ten animals which each species containing 100 ng were mixed in one tube. Furthermore, DNA sample from genome pool was taken 50 ng and distributed on three tube, i.e tube 1 mixed with ten primers (goat, chicken, cattle, tiger, sheep, pig,

Table 1. Specific reverse primers of *cyt β* gene

Species	Reverse (5'-3')	PCR product length
Goat <sup>a</sup>	CTC GAC AAA TGT GAG TTA CAG AGG GA	157 bp
Chicken <sup>a</sup>	AAG ATA CAG ATG AAG AAG AAT GAG GCG	227 bp
Cattle <sup>a</sup>	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG	274 bp
Tiger	TAG CCA TGA CCG TAA ACA ATA GC	319 bp
Sheep <sup>b</sup>	CTA TGA ATG CTG TGG CTA TTG TCG CAA AT	331 bp
Pig <sup>a</sup>	GCT GAT AGT AGA TTT GTG ATG ACC GTA	398 bp
Horse <sup>a</sup>	CTC AGA TTC ACT CGA CGA GGG TAG TA	439 bp
Dog	TTG CTA GAG CTG CGA TGA TGA AA	523 bp
Cat	AGG GGT TGT TAG ATC CTG TTT CA	568 bp
Rat <sup>c</sup>	GAA TGG GAT TTT GTC TGC GTT GGA GTT T	603 bp

Note: <sup>a</sup>Matsunaga *et al.* (1999); <sup>b</sup>modified Matsunaga *et al.* (1999); <sup>c</sup>Nuraini *et al.* (2012).

horse, dog, cat, rat), tube 2 with five primers (goat, cattle, sheep, horse, cat), and tube 3 with five primers (chicken, tiger, pig, dog, rat).

### Specific DNA Fragments Amplification Using Multiplex PCR

Specific DNA fragment amplification used PCR technique (polymerase chain reaction) with thermo cycler machine. PCR components used in total volume 15  $\mu$ L contained DNA sample (including DNA pool genome) 50 ng genomic DNA and PCR reaction (i.e. distillate water 9  $\mu$ L, forward primer 1.667 pmol, reverse primer 0.1667 pmol for each species, 1 x buffer reaction, dNTPs 0.267 mM, MgCl<sub>2</sub> 1.667 mM, and enzyme *taq fermentas* 1 unit). PCR reaction had different component volume with five primers (i.e. distillate water 9.5  $\mu$ L, forward primer 0.833 pmol, reverse primer 0.1667 pmol for each species, 1 x buffer reaction, dNTPs 0.267 mM, MgCl<sub>2</sub> 1.667 mM, and enzyme *taq fermentas* 1 unit). The condition of thermo cycler machine (Mastercycler Personal 22331, Eppendorf, Germany) consisted of predenaturation at 95 °C for 5 min, followed by 30 cycles of denaturation 95 °C for 30 s, annealing 60 °C for 45 s, extension 72 °C for 1 min, and the final extension step was at 72 °C for 5 min.

### Electrophoresis

PCR amplicons electrophoresis performed on 1.5% agarose gel and stained with EtBr (ethidium bromide) were visualized in UV transilluminator. Specific DNA fragment (goat, chicken, cattle, tiger, sheep, pig, horse, dog, cat, and rat) was analyzed by standard DNA size marker (100 bp).

## RESULTS AND DISCUSSION

### Similarity Degree of *Cyt β* Gene Sequences

Specific reverse primers homology percentage (Table 2) showed tracing reverse primers have a high

homology percentage in one particular species and low in other species, so it could be used as a specific primer (Nuraini *et al.*, 2012). Forward primer had high homology percentage about 84%-92% (38 nucleotides) among ten species, so it could be used as a general primer. *Cyt β* gene has some stable sequences which were used for suggestion of universal primers and some variable sequences used for animal identification (Minarovic *et al.*, 2010). Matsunaga *et al.* (1999) stated sheep primer mismatched with goat DNA only two nucleotides, however, 3' end mismatching was fatal for PCR amplification and resulted in no sheep band from goat template. In this research, only found one nucleotide mismatched with goat DNA (5'CTA TGA ATG CTG TGG CTA TTG TCG CA-3'), so sheep reverse primer was modified by adding three nucleotides in 3' end (5'-CTA TGA ATG CTG TGG CTA TTG TCG CAA AT-3'). Attachment reverse primers at specific sequence of certain animal were caused by: 1) mismatched 3' end on each reverse primer (Matsunaga *et al.*, 1999), 2) difference mismatched between reverse primers on every sequence DNA sample (about 9%-45%) resulted different melting temperature (T<sub>m</sub>) (Viljoen *et al.*, 2005).

### Specific Fragments Amplification of *Cyt β* Gene on Dog, Cat, and Tiger

Primer specificity was tested in cooked dog meat, cat blood, and tiger feces. Processed product of cat meat was still rare, so cat meat sample was not used in this study, but DNA fragment of cat was amplified successfully from blood. Similarly with tiger sample was amplified successfully from feces. Electrophoresis DNA fragment of *cyt β* gene amplification from dog, cat, and tiger was presented in Figure 1. Ilhak & Arslan (2007) successfully to amplified cat and dog meat by adding 5%, 2.5%, 1%, 0.5%, and 0.1% in beef, lamb, and goat meat. The number of PCR cycles used for amplification played an essential role in identification of meat in mixes < 0.5%. PCR was conducted at 30 cycles for mixtures at the 5%, 2.5%, 1%, 0.5% level, while at 35 cycles for mixture at the 0.1% level (Ilhak & Arslan,

Table 2. Specific reverse primers homology in ten animals

Specific primer	% Homology										
	<i>Capra hircus</i>	<i>Gallus gallus</i>	<i>Bos taurus</i>	<i>Bos indicus</i>	<i>Panthera tigris</i>	<i>Ovis aries</i>	<i>Sus scrofa</i>	<i>Equus caballus</i>	<i>Canis lupus</i>	<i>Felis catus</i>	<i>Rattus norvegicus</i>
Forward (38 nt)	92,105	89,474	92,105	89,474	88,889	92,105	92,105	86,842	86,842	84,211	89,474
Goat (26 nt)	96,154	65,385	73,077	73,077	69,231	84,615	73,077	73,077	73,077	73,077	69,231
Chicken (27 nt)	70,370	100,000	62,963	62,963	70,370	66,667	62,963	70,370	70,370	62,963	77,778
Cattle (29 nt)	72,414	62,069	100,000	100,000	68,966	75,862	72,414	79,310	68,966	68,966	75,862
Tiger (23 nt)	56,522	56,522	60,870	60,870	100,000	56,522	69,565	69,565	60,870	78,261	69,565
Sheep (29 nt)	86,207	55,172	72,414	72,414	72,414	100,000	75,862	68,966	86,207	72,414	75,862
Pig (27 nt)	81,481	77,778	77,778	77,778	-	70,370	100,000	81,481	74,074	74,074	81,481
Horse (26 nt)	80,769	69,231	73,077	73,077	-	80,769	76,923	100,000	69,231	69,231	88,462
Dog (23 nt)	78,261	56,522	65,217	65,217	-	82,609	69,565	73,913	100,000	73,913	78,261
Cat (23 nt)	86,957	78,261	78,261	78,261	-	86,957	78,261	91,304	82,609	100,000	82,609
Rat (28 nt)	71,429	67,857	78,571	78,571	-	64,286	64,286	67,857	71,429	78,571	96,429

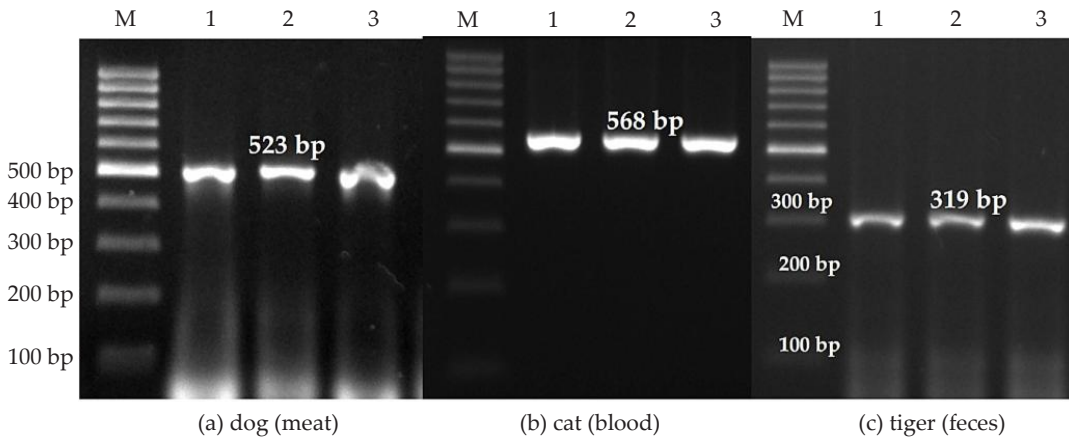


Figure 1. Specific fragments amplification on dog, cat, and tiger. M: marker 100 bp, (1) (2) (3): sample replication.

2007). Owing to the potential for degradation of samples found in a forensic context, nuclear DNA is unlikely to yield results, therefore, mitochondrial DNA maybe used an alternative means of species identification (Kitpipit *et al.*, 2012). Species identification of tiger and cat had been distinguished at the genus level using specific reverse primers.

**Specific Fragments Amplification of *Cyt β* Gene on Dog, Cat, and Tiger**

Reverse primers of *cyt β* gene successfully to amplified DNA fragment of ten animals with different length fragment. The amplification fragment length of goat, chicken, cattle, sheep, pig, horse were 157, 227, 274, 331, 398, and 439 bp, respectively (Matsunaga *et al.*, 1999), and fragment rat was 603 bp (Nuraini *et al.*, 2012), while tiger, dog, and cat amplified were 319, 523, 568 bp, respectively (Figure 2). Amplification target sequences

from several species simultaneously (using the same forward primer) including more than one pair of primers in the same reaction is a variant of PCR called Multiplex PCR (Matsunaga *et al.*, 1999; Markoulatos *et al.*, 2002; Jain *et al.*, 2007). Electrophoresis specific DNA fragment of *cyt β* gene was presented in Figure 3. Minarovic *et al.* (2010) successfully to identify species using PCR-RFLP with same primer for all species (i.e. *Mustela vison* (American mink), *Mustela putorius furo* (Ferret), *Sus scrofa domesticus* (pig), *Oryctolagus cuniculus* (Rabbit)), which were designed by Kocher *et al.* (1989). PCR products length did not different for all species, 359 bp, furthermore were cleaved by restriction enzyme *AluI*. Every animal has a unique combination of restriction fragments (Minarovic *et al.*, 2010). Species determination by PCR was affected by cooking temperature, time, and size of the DNA fragment to be amplified (Martinez & Yman, 1998; Matsunaga *et al.*, 1999; Arslan *et al.*, 2006).

	10	20	30	40	50	60	
Forward primer	GACCTCCCAG	CTCCATCAAA	CATCTCATCT	TGATGAAA			
Capra_hircus	-----A	-C-----	-----A	-----CT	TTGGATCCCT	CCTAGGAATT	
Gallus_gallus	-----C	-----C	-----TG	-----TT	TCGGCTCCCT	ATTAACAGTC	
Bos_taurus	-----T	-----C	-----A	-----TT	TCGGTCCCT	CCTGGGAATC	
Bos_indicus	-----T	-----C	-----T	-----A	-----TT	TCGGTCCCT	CCTGGGAATC
Panthera_tigris	-----	-----C	-----G	-----A	-----CT	TTGGCTCCTT	ACTAGGGGTG
Ovis_aries	--T-----	-----	T-----	-----A	-----CT	TTGGCTCTCT	CCTAGGCATT
Sus_scrofa	-----	-C-C-----	-----A	-----CT	TCGGTCCCT	CCTAGGCATC	
Equus_caballus	-----A	-C-C-----	-----T	-----A	-----CT	TCGGCTCCCT	CCTAGGAATC
Canis_lupus	-----	-G-G-T-----	-----TG	-----CT	TCGGATCCTT	ACTAGGAGTA	
Felis_catus	-----A	-C-----T	-----G	-----A	-----CT	TCGGCTCCCT	TCTAGGAGTC
Rattus_norvegicus	-----C	-C-----T	-----A	-----CT	TCGGTCTCT	ACTAGGAGTA	
	70	80	90	100	110	120	
Capra_hircus	TGCCTAATCT	TACAAATCCT	GACAGGCCTA	TTCCTAGCAA	TACTACTATAC	ATCCGACACA	
Gallus_gallus	TGCCTCATGA	CCCAAATCCT	CACCGGCCTA	CTACTAGCCA	TGCACTACAC	AGCAGACACA	
Bos_taurus	TGCCTAATCC	TACAAATCCT	CACAGGCCTA	TTCCTAGCAA	TACTACTACAC	ATCCGACACA	
Bos_indicus	TGCCTAATCC	TACAAATCCT	CACAGGCCTA	TTCCTAGCAA	TACTACTACAC	ATCCGACACA	
Panthera_tigris	TGCTTAATCT	TACAAATCCT	CACTGGCCTC	TTTCTAGCCA	TACTACTACAC	ATCAGACACA	
Ovis_aries	TGCTTAATTT	TACAGATCCT	AACAGGCCTA	TTCCTAGCAA	TACTACTATAC	ACCTGACACA	
Sus_scrofa	TGCCTAATCT	TGCAAATCCT	AACAGGCCTG	TTCTTAGCAA	TACATTACAC	ATCAGACACA	
Equus_caballus	TGCCTAATCC	TCCAAATCCT	AACAGGCCTA	TTCCTAGCCA	TACTACTACAC	ATCAGACACG	
Canis_lupus	TGCTTGATTC	TACAGATCCT	AACAGGTTTA	TTCTTAGCTA	TGCACTATAC	ATCCGACACA	
Felis_catus	TGCCTAATCT	TACAAATCCT	CACCGGCCTC	TTTTTGGCCA	TACTACTACAC	ATCAGACACA	
Rattus_norvegicus	TGCCTCATAG	TACAAATCCT	CACAGGCCTA	TTCCTAGCAA	TACTACTACAC	GTCTGATACC	

Continued

	130	140	150	160	170	180
Capra_hircus	ATAACAGCAT	TTCCCTCTGT	AACTCACATT	TGTCGAGATG	TAAATATGG	CTGAATCATC
Gallus_gallus	TCCCTAGCCT	TC-----C--	-G-C---C-	--C---GAACG	TACAATACGG	CTGACTCATC
Bos_taurus	ACAACAGCAT	TC-----	T--C--T--C	--C---ACG	TGAACACGG	CTGAATCATC
Bos_indicus	ACAACAGCAT	TC-----	T--C--T--C	--C---ACG	TGAACACGG	CTGAATCATC
Panthera_tigris	ATAACCGCTT	TC--A--A-	T--C-----	--C--C-ACG	TAAACTACGG	TTGATTATC
Ovis_aries	ACAACAGCAT	TC-----	--C-----	--C---ACG	TAAACTATGG	CTGAATTATC
Sus_scrofa	ACAACAGCTT	TC--A--A-	T--A-----	-----ACG	TAAATACGG	ATGAGTTATT
Equus_caballus	ACAACAGCTT	TC--A--A-	C-----C-	--C---ACG	TAAACTACGG	ATGAGTTATT
Canis_lupus	GCCACAGCTT	T--A--A-	C--C-----	--C---ACG	TAACTACGG	CTGAATTATC
Felis_catus	ATAACCGCTT	T--A--A-	T--C-----	-----C-ACG	TAAATATGG	CTGAATCATC
Rattus_norvegicus	ATAACAGCAT	TC--A--A-	C--C-----	--C---ACG	TAAACTACGG	CTGACTAATC

	190	200	210	220	230	240
Capra_hircus	CGATACATAC	ACGCAAACGG	A--A--A-A	----T----	-CC-A--CAT	ACATATCGGA
Gallus_gallus	CGGAATCTCC	ACGCAAACGG	CGCCTCATTC	TTCTTCATCT	GTATCTTCTC	TCACATCGGA
Bos_taurus	CGATACATAC	ACGCAAACGG	A--T--A-G	--T--T----	-CT-A-ATAT	GCACGTAGGA
Bos_indicus	CGATACATAC	ACGCAAACGG	A--T--A-G	--T--T----	-CT-A-ATAT	GCACGTAGGA
Panthera_tigris	CGATATCTAC	ATGCCAACGG	A-----CA-A	-----T----	-C-A-ACAT	GCACGTAGGA
Ovis_aries	CGATACATAC	ACGCAAACGG	A--A--A-A	--T--T----	-CC-A--TAT	GCATGTAGGA
Sus_scrofa	CGCTACCTAC	ATGCCAACGG	A--A--CA-G	----T--T-	-CC-A--CAT	CCACGTAGGC
Equus_caballus	CGCTACCTCC	ATGCCAACGG	A--A--A-A	--T--T----	-CC---CAT	TCACGTAGGA
Canis_lupus	CGCTATATGC	ACGCCAATGG	--T--CA-A	-----T----	-CC-A--CCT	ACATGTAGGA
Felis_catus	CGATATTTAC	ACGCCAACGG	A--T--TA-A	-----T----	-CC-G-ACAT	ACATGTAGGA
Rattus_norvegicus	CGATACCTAC	ACGCCAACGG	-----A-A	--T-----	-CC-A--CCT	CCATGTGGGA

	250	260	270	280	290	300
Capra_hircus	CGAGGTC---	-C--T--A-	A--T--C--	---AAACAT	GAAACATFGG	AGTAATC--C
Gallus_gallus	CGAGG-C---	-C-----C-	C---CTC-A-	AAG-AAACCT	GAAACACAGG	AGTAATC--C
Bos_taurus	CGAGGCTTAT	ATTACGGGTC	TTACACTTTT	CTAGAAACAT	GAAATATFGG	AGTAATC--T
Bos_indicus	CGAGGCTTAT	ATTACGGGTC	TTACACTTTT	CTAGAAACAT	GAAATATFGG	AGTAATC--T
Panthera_tigris	CGAGGAA---	-C-----C-	C-----C-C	TC--AAACAT	GAAATATCGG	GATTGTGCTA
Ovis_aries	CGAGG-C---	---T--A-	A--T--C--C	---AAACAT	GAAACATCGG	AGTAATC--C
Sus_scrofa	CGAGG-C---	-C-----A-	C--T--TA-C	---AAACAT	GAAACATFGG	AGTAGTC---
Equus_caballus	CGCGG-C-C-	-C-----C-	-----A-C	---AGACAT	GAAACATFGG	AATCATC---
Canis_lupus	CGAGG-C---	-----A-	C--TGTA-C	A--AAACAT	GAAACATFGG	AATTGTA---
Felis_catus	CGGGGAA---	-C-----C-	C-----C-C	TC--AGACAT	GAAACATFGG	AATCATA---
Rattus_norvegicus	CGAGGAC---	-C--T--A-	C-----C-	---AAACCT	GAAACATFGG	GATCATC---
Primer_Panthera	-----	-----	-----	-----	-----	-----GCTA

	310	320	330	340	350	360
Capra_hircus	CT-C-C*-*	**-----	-----	-GCTATGTTT	TACCATGAGG	ACAAATATCA
Gallus_gallus	CTCC-C-AC	---*---*	C--C--TG-G	-GCTATGTTC	TCCCATGGGG	CCAAATATCA
Bos_taurus	CT-C-C-A-	*---*---	-----T---	-GATACGTCC	TACCATGAGG	ACAAATATCA
Bos_indicus	CT-C-C-A-	*---*---	-----T---	-GATACGTCC	TACCATGAGG	ACAAATATCA
Panthera_tigris	-T-----	-----	-----C----	-GATATGTCT	TACCATGAGG	ACAAATATCA
Ovis_aries	CTATTTGCGA	CAATAGCCAC	AGCATTCATA	GCTATGTTT	TACCATGAGG	ACAAATATCA
Sus_scrofa	CT*---C-	T--*--A-	-----C-	-----	-GCTACGTCC	TGCCCTGAGG
Equus_caballus	CTT-C-A-	T--*--A-	-----G-	-GCTATGTCC	TACCATGAGG	CCAAATATCC
Canis_lupus	T*-C*-A*	*---*---	-----G-	-GCTATGTAC	TACCATGAGG	ACAAATATCA
Felis_catus	-T*---A-	*---*---	-----T--T--G	-GATACGTCC	TACCATGAGG	CCAAATGTCC
Rattus_norvegicus	CT*---A-	*---*--A-	T-----G-	-GCTATGTAC	TCCCATGAGG	ACAAATATCA
Primer_Panthera	TTGTTTACGG	TCATGGCTA-	-----	-----	-----	-----

	370	380	390	400	410	420
Capra_hircus	TTTTGAGGGG	CA--A----	---T-----T	--T-----AA	TCCCATATAT	TGGC--A-A-
Gallus_gallus	TTCTGAGGGG	CC--C--T--	-----C--	T-C-----AA	TTCCCTACAT	TGGACAC---
Bos_taurus	TTCTGAGGAG	CA--A----	---C--C--C	T-----AA	TCCCATACAT	CGGC--A-AT
Bos_indicus	TTCTGAGGAG	CA--A----	---C--C--C	T-----AA	TCCCATACAT	CGGC--A-AT
Panthera_tigris	TTCTGAGGGG	CA-----	-----	-----	-----	-----
Ovis_aries	TTCTGAGGAG	CA--A--T-	T--C--C--C	--T-----AA	TCCCATATGT	TGGC--A-A-
Sus_scrofa	TTCTGAGGAG	CTACGGTCAT	CACAAATCTA	CTATCAGCTA	TCCCTTATAT	CGGA--AGA-
Equus_caballus	TTTTGAGGAG	CA--A----	---G--C--C	-----AA	TTCCCTACAT	CGGFACTACC
Canis_lupus	TTTTGAGGAG	CA--T--A-	---T-----T	--C--T--CA	TCCCTTATAT	CGGA---GA-
Felis_catus	TTCTGAGGAG	CA--C--A-	---T--C--C	--G-----AA	TCCCATACAT	CGGG---GAA
Rattus_norvegicus	TTCTGAGGAG	C--A--A-	T-----C--	T-----TA	TCCCTTACAT	TGGG-----

	430	440	450	460	470	480
Capra_hircus	--A-----A-	-----G	GGGATTCTCA	GTAGACAAAG	CCACTCTCAC	CCGATTCTTC
Gallus_gallus	--A--A--	--GC-----G	GGGATTTTCA	GTCGACAACC	CAACCCTTAC	CCGATTCTTC
Bos_taurus	T-A-----A-	-----G	CGGATTCTCA	GTAGACAAAG	CAACCCTTAC	CCGATTCTTC
Bos_indicus	T-A-----A-	-----G	CGGATTCTCA	GTAGACAAAG	CAACCCTTAC	CCGATTCTTC
Panthera_tigris	-----	-----	-----	-----	-----	-----
Ovis_aries	--A-----A-	-----G	AGGATTCTCA	GTAGACAAAG	CTACCCTCAC	CCGATTTTTC
Sus_scrofa	-----A--A-	-----G	GGGCTTTTCC	GTCGACAACC	CAACCCTCAC	ACGATTCTTC
Equus_caballus	CTCGTCGAGT	GAATCTGAGG	TGGATTCTCA	GTAGACAAAG	CCACCCTTAC	CCGATTTTTT
Canis_lupus	T-A--A--A-	-G-----G	CGGCTTCTCA	GTGGACAAAG	CAACCCTTAC	ACGATTCTTT
Felis_catus	--A--A--A-	-G-----G	GGGCTTCTCA	GTAGACAAAG	CCACCCTTAC	ACGATTCTTC
Rattus_norvegicus	--A-----A-	-----G	AGGCTTCTCA	GTAGACAAAG	CAACCCTTAC	ACGCTTCTTC

Continued

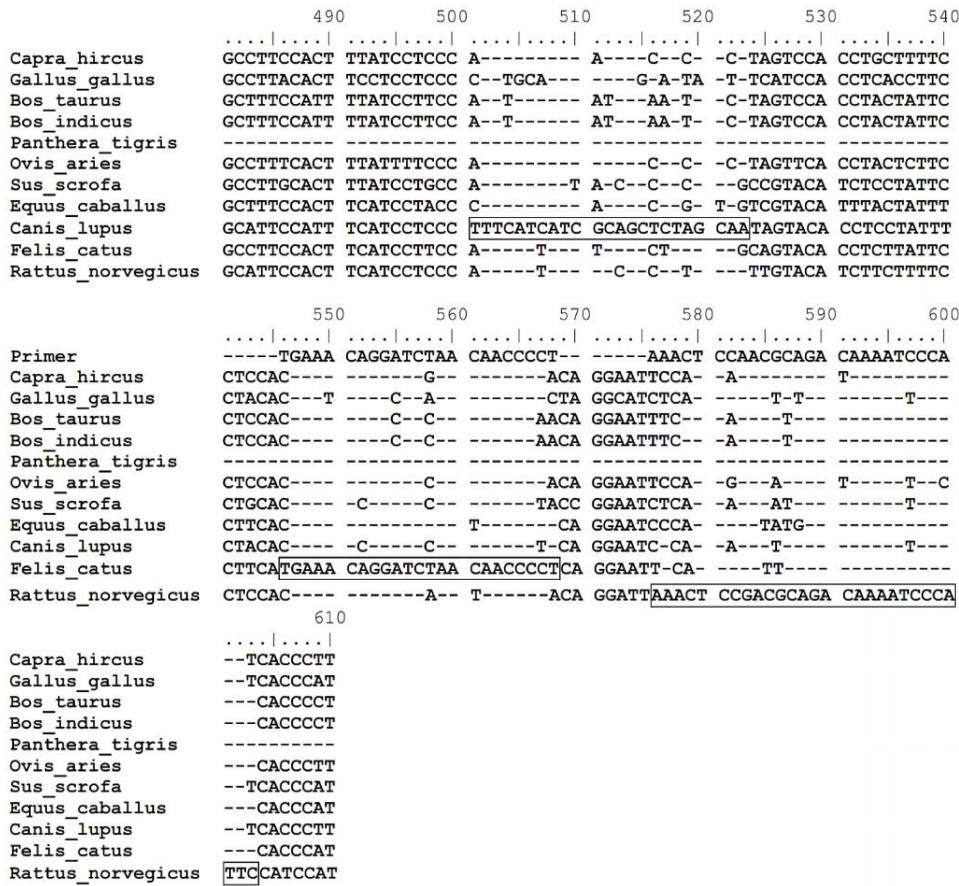


Figure 2. Primer sequences and target region on *cytochrome β* gene (boxes: forward and specific reverse primers, dash: identical nucleotides with primer sequence, open boxes and dash: identical nucleotides with tiger sequence, star symbol: identical nucleotides with sheep sequence).

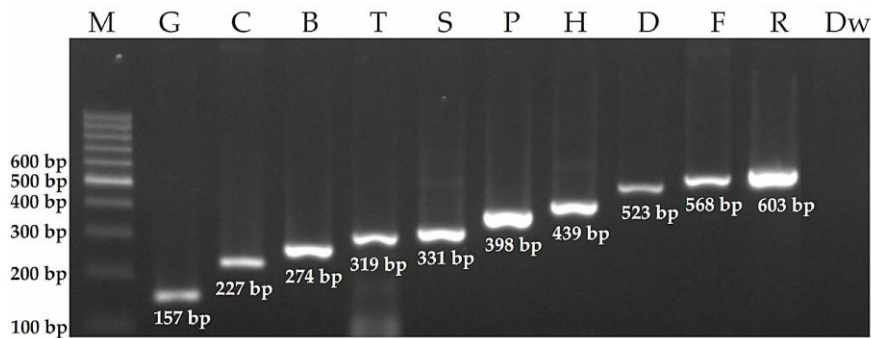


Figure 3. Specific fragments amplification on several animal. M: marker 100 bp, G: goat, C: chicken, B: cattle, T: tiger, S: sheep, P: pig, H: horse, D: dog, F: cat, R: rat, Dw: negative control.

**Specific Fragments Amplification of *Cyt β* Gene on DNA Genome Pool**

This research showed only six bands in tube 1 (i.e. goat, chicken, cattle, tiger, pig, cat) were amplified successfully at DNA mix from ten species (Figure 4). It was probably caused band overlapped between tiger (319 bp) and sheep (331 bp); dog (523 bp), cat (568 bp), and rat (603 bp), because they have adjacent fragment length. Large molecules migrate more slowly than smaller mol-

ecules (Sambrook & Russel, 2001). To ensure this, the test was carried out by separating overlapped band and adjacent fragment length. Tube 2 had five bands (goat, cattle, sheep, horse, cat), but tube 3 only had four bands (chicken, tiger, pig, rat) and no dog band (Figure 4). In general, quantitative PCR is difficult because of unequal efficiency of amplification. Amplification efficiency is affected by the difference primer sequences (Matsunaga *et al.*, 1999).

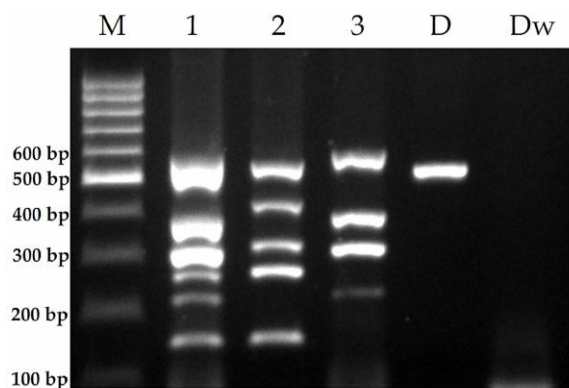


Figure 4. Specific fragments amplification on genome pool. M: marker 100 bp, 1: tube 1 (goat, chicken, cattle, tiger, pig, cat), 2: tube 2 (goat, cattle, sheep, horse, cat), 3: tube 3 (chicken, tiger, pig, rat), D: dog, Dw: negative control.

### CONCLUSION

Dog, cat, and tiger DNA are amplified successfully with fragment length of 523, 568, 319 bp, respectively. Species specificity of dog, cat, and tiger are indicated by high reverse primers homology percentage. Multiplex PCR technique success to amplify DNA fragment from species tested, but has a limitation to amplify total DNA composite of mix DNA.

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