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Species Authentication of Dog, Cat, and Tiger Using *Cytochrome β* 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 β* 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 β 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 *β* (*cyt β*). 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, 568, 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 β, 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 sequances 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[a]	CTC GAC AAA TGT GAG TTA CAG AGG GA	157 bp
Chicken[a]	AAG ATA CAG ATG AAG AAG AAT GAG GCG	227 bp
Cattle[a]	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG	274 bp
Tiger	TAG CCA TGA CCG TAA ACA ATA GC	319 bp
Sheep[b]	CTA TGA ATG CTG TGG CTA TTG TCG CAA AT	331 bp
Pig[a]	GCT GAT AGT AGA TTT GTG ATG ACC GTA	398 bp
Horse[a]	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[c]	GAA TGG GAT TTT GTC TGC GTT GGA GTT T	603 bp

Note: [a]Matsunaga *et al.* (1999); [b]modified Matsunaga *et al.* (1999); [c]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 µL contained DNA sample (including DNA pool genome) 50 ng genomic DNA and PCR reaction (i.e. distillate water 9 µL, forward primer 1.667 pmol, reverse primer 0.1667 pmol for each species, 1 x buffer reaction, dNTPs 0.267 mM, MgCl2 1.667 mM, and enzyme *taq fermentas* 1 unit). PCR reaction had different component volume with five primers (i.e. distillate water 9.5 µL, forward primer 0.833 pmol, reverse primer 0.1667 pmol for each species, 1 x buffer reaction, dNTPs 0.267 mM, MgCl2 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

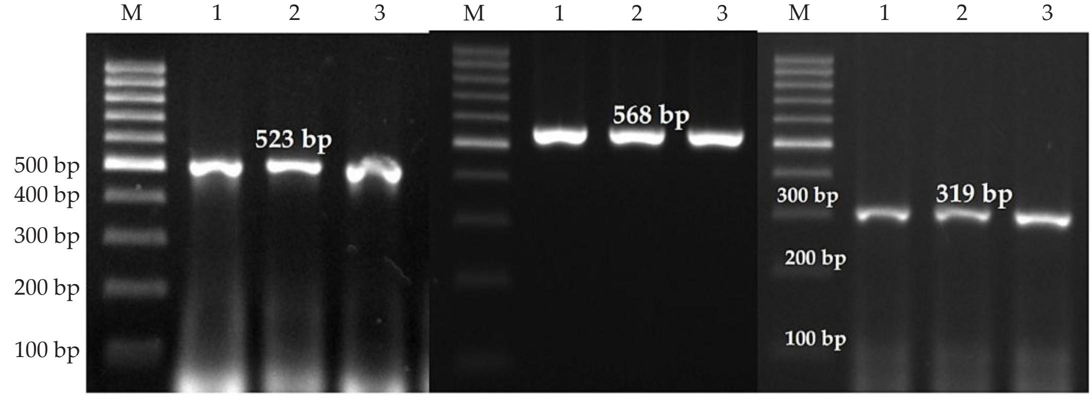
Specific reverse primers homology percentage (Table 2) showed tracing reverse primers have a high homology percentage in one particular species and low in other species, so it could be used as a specific primer (Nuraini *et al*., 2012). Forward primer had high homology percentage about 84%-92% (38 nucleotides) 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 (Tm) (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										
	Capra hircus	*Gallus gallus*	*Bos taurus*	*Bos indicus*	*Panthera tigris*	*Ovis aries*	*Sus scrofa*	*Equus caballus*	*Canis lupus*	*Felis catus*	*Rattus norvegicus*
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



(a) dog (meat) (b) cat (blood) (c) tiger (feces)

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). Ampilification 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).

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                             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 TCGGTTCCCT CCTGGGAATC
Bos_indicus          -----T---- -C-------- ---T-----A --------TT TCGGTTCCCT CCTGGGAATC
Panthera_tigris      ---------- -------C-- -------G-A --------CT TTGGCTCCTT ACTAGGGGTG
Ovis_aries           --T------- ---------- T--------A --------CT TTGGCTCTCT CCTAGGCATT
Sus_scrofa           ---------- -C--C----- ---------A --------CT TCGGTTCCCT CTTAGGCATC
Equus_caballus       -----A---- -C--C----- ---T-----A --------CT TCGGCTCCCT CCTAGGAATC
Canis_lupus          ---------- -G--G--T-- ------TG-- --------CT TCGGATCCTT ACTAGGAGTA
Felis_catus          -----A--C- -C-----T-- -------G-A --------CT TCGGCTCCCT TCTAGGAGTC
Rattus_norvegicus    --------C- -C-----T-- ---------A --------CT TCGGTTCTCT ACTAGGAGTA

                             70         80         90        100        110        120
                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Capra_hircus         TGCCTAATCT TACAAATCCT GACAGGCCTA TTCCTAGCAA TACACTATAC ATCCGACACA
Gallus_gallus        TGCCTCATGA CCCAAATCCT CACCGGCCTA CTACTAGCCA TGCACTACAC AGCAGACACA
Bos_taurus           TGCCTAATCC TACAAATCCT CACAGGCCTA TTCCTAGCAA TACACTACAC ATCCGACACA
Bos_indicus          TGCCTAATCC TACAAATCCT CACAGGCCTA TTCCTAGCAA TACACTACAC ATCCGACACA
Panthera_tigris      TGCTTAATCT TACAAATCCT CACTGGCCTC TTTCTAGCCA TACACTACAC ATCAGACACA
Ovis_aries           TGCTTAATTT TACAGATCCT AACAGGCCTA TTCCTAGCAA TACACTATAC ACCTGACACA
Sus_scrofa           TGCCTAATCT TGCAAATCCT AACAGGCCTG TTCTTAGCAA TACATTACAC ATCAGACACA
Equus_caballus       TGCCTAATCC TCCAAATCTT AACAGGCCTA TTCCTAGCCA TACACTACAC ATCAGACACG
Canis_lupus          TGCTTGATTC TACAGATTCT AACAGGTTTA TTCTTAGCTA TGCACTATAC ATCGGACACA
Felis_catus          TGCCTAATCT TACAAATCCT CACCGGCCTC TTTTTGGCCA TACACTACAC ATCAGACACA
Rattus_norvegicus    TGCCTCATAG TACAAATCCT CACAGGCTTA TTCCTAGCAA TACACTACAC GTCTGATACC
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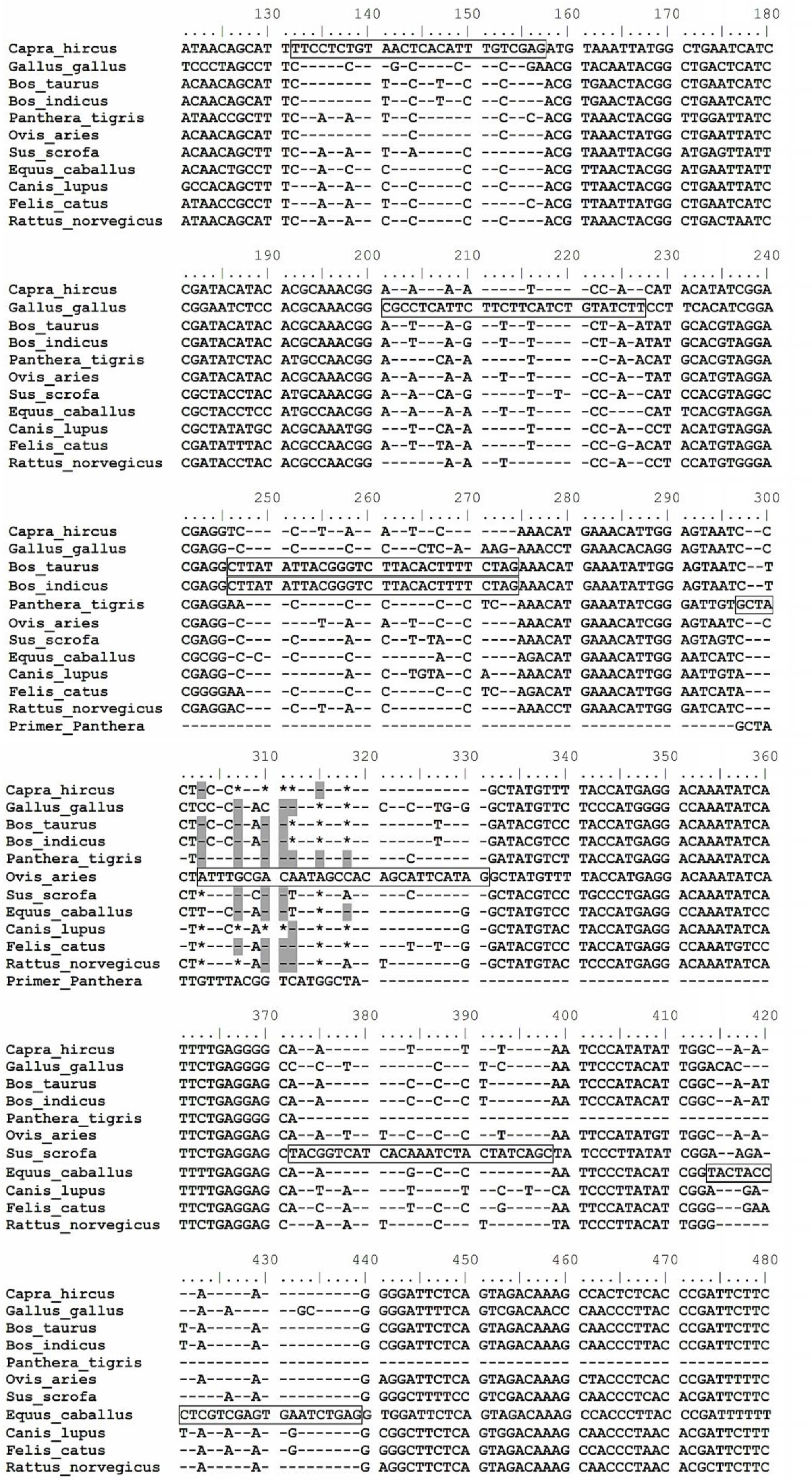
Continued



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                          130        140        150        160        170        180
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Capra_hircus       ATAACAGCAT TTTCCTCTGT AACTCACATT TGTCGAGATG TAAATTATGG CTGAATCATC
Gallus_gallus      TCCCTAGCCT TC-----C-- -G-C----C- --C--GAACG TACAATACGG CTGACTCATC
Bos_taurus         ACAACAGCAT TC-------- T--C--T--C --C----ACG TGAACTACGG CTGAATCATC
Bos_indicus        ACAACAGCAT TC-------- T--C--T--C --C----ACG TGAACTACGG CTGAATCATC
Panthera_tigris    ATAACCGCTT TC--A--A-- T--C------ --C--C-ACG TAAACTACGG TTGGATTATC
Ovis_aries         ACAACAGCAT TC-------- ---C------ --C----ACG TAAACTATGG CTGAATTATC
Sus_scrofa         ACAACAGCTT TC--A--A-- T--A-----C -------ACG TAAATTACGG ATGAGTTATT
Equus_caballus     ACAACTGCCT TC--A--C-- C--------C --C----ACG TTAACTACGG ATGAATTATT
Canis_lupus        GCCACAGCTT T---A--A-- C--C-----C --C----ACG TTAACTACGG CTGAATTATC
Felis_catus        ATAACCGCCT T---A--A-- T--C-----C -----C-ACG TTAATTATGG CTGAATCATC
Rattus_norvegicus  ATAACAGCAT TC--A--A-- C--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 GTATCTTCCT 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 ATGCAAACGG 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 ACGCAAATGG ---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

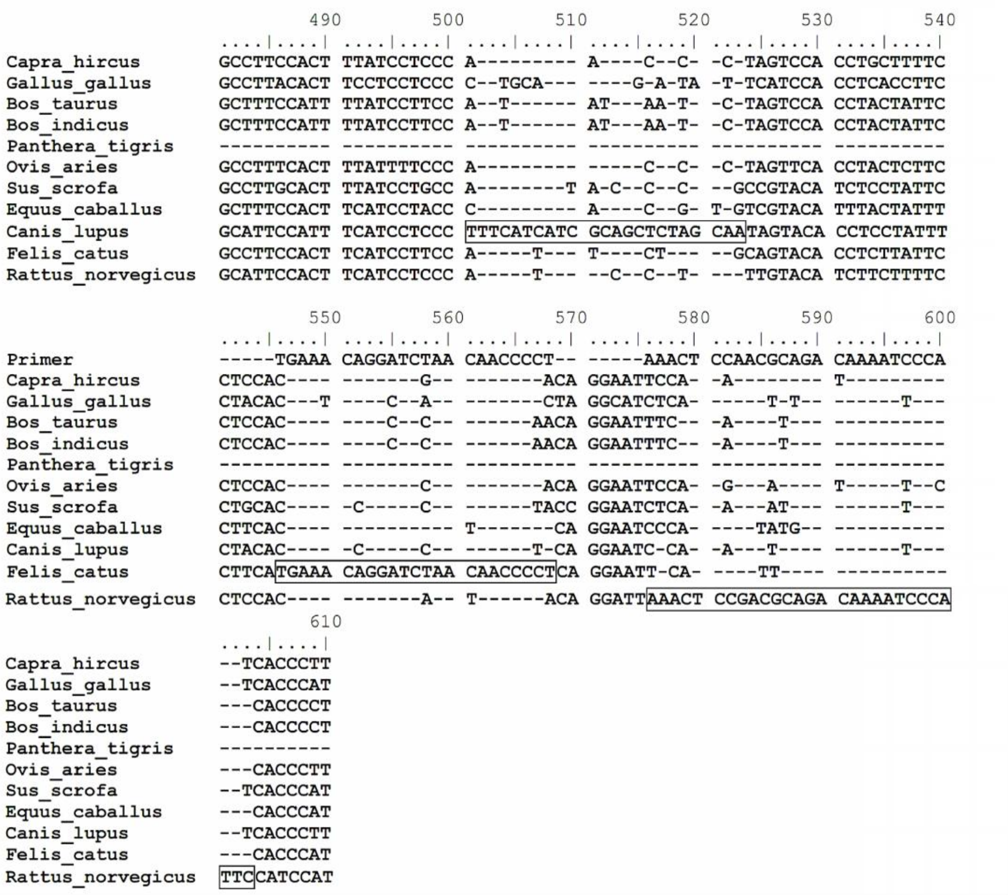
                          250        260        270        280        290        300
                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Capra_hircus       CGAGGTC--- -C--T--A-- A--T--C--- ----AAACAT GAAACATTGG AGTAATC--C
Gallus_gallus      CGAGG-C--- -C-----C-- C---CTC-A- AAG-AAACCT GAAACACAGG AGTAATC--C
Bos_taurus         CGAGGCTTAT ATTACGGGTC TTACACTTTT CTAGAAACAT GAAATATTGG AGTAATC--T
Bos_indicus        CGAGGCTTAT ATTACGGGTC TTACACTTTT CTAGAAACAT GAAATATTGG 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 GAAACATTGG AGTAGTC---
Equus_caballus     CGCGG-C-C- -C-----C-- ------A--C ----AGACAT GAAACATTGG AATCATC---
Canis_lupus        CGAGG-C--- -------A-- C--TGTA--C A---AAACAT GAAACATTGG AATTGTA---
Felis_catus        CGGGGAA--- -C-----C-- C-----C--C TC--AGACAT GAAACATTGG AATCATA---
Rattus_norvegicus  CGAGGAC--- -C--T--A-- C--------C ----AAACCT GAAACATTGG 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 GGCTATGTTT TACCATGAGG ACAAATATCA
Sus_scrofa         CT*-----C- -T--*--A-- ---C------ -GCTACGTCC TGCCCTGAGG ACAAATATCA
Equus_caballus     CTT--C--A- -T--*----- ---------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 TTCCATATGT TGGC--A-A-
Sus_scrofa         TTCTGAGGAG CTACGGTCAT CACAAATCTA CTATCAGCTA TCCCTTATAT CGGA--AGA-
Equus_caballus     TTTTGAGGAG CA--A----- ---G--C--C --------AA TTCCCTACAT CGGTACTACC
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 TTCCATACAT 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 GTCGACAAAG CAACCCTCAC ACGATTCTTC
Equus_caballus     CTCGTCGAGT GAATCTGAGG TGGATTCTCA GTAGACAAAG CCACCCTTAC CCGATTTTTT
Canis_lupus        T-A--A--A- -G-------G CGGCTTCTCA GTGGACAAAG CAACCCTAAC ACGATTCTTT
Felis_catus        --A--A--A- -G-------G GGGCTTCTCA GTAGACAAAG CCACCCTAAC ACGATTCTTC
Rattus_norvegicus  --A-----A- ---------G AGGCTTCTCA GTAGACAAAG CAACCCTAAC ACGCTTCTTC
```

Continued



```
                      490        500        510        520        530        540
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Capra_hircus        GCCTTCCACT TTATCCTCCC A--------- A----C--C- -C-TAGTCCA CCTGCTTTTC
Gallus_gallus       GCCTTACACT TCCTCCTCCC C--TGCA--- ----G-A-TA -T-TCATCCA CCTCACCTTC
Bos_taurus          GCTTTCCATT TTATCCTTCC A--T------ AT---AA-T- -C-TAGTCCA CCTACTATTC
Bos_indicus         GCTTTCCATT TTATCCTTCC A--T------ AT---AA-T- -C-TAGTCCA CCTACTATTC
Panthera_tigris     ---------- ---------- ---------- ---------- ---------- ----------
Ovis_aries          GCCTTTCACT TTATTTTCCC A--------- -----C--C- -C-TAGTTCA CCTACTCTTC
Sus_scrofa          GCCTTGCACT TTATCCTGCC A--------T A-C--C--C- --GCCGTACA TCTCCTATTC
Equus_caballus      GCTTTCCACT TCATCCTACC C--------- A----C--G- T-GTCGTACA TTTACTATTT
Canis_lupus         GCATTCCATT TCATCCTCCC TTTCATCATC GCAGCTCTAG CAATAGTACA CCTCCTATTT
Felis_catus         GCCTTCCACT TCATCCTTCC A-----T--- T----CT--- --GCAGTACA CCTCTTATTC
Rattus_norvegicus   GCATTCCACT TCATCCTCCC A-----T--- --C--C--T- ---TTGTACA TCTTCTTTTC

                      550        560        570        580        590        600
               ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Primer              -----TGAAA CAGGATCTAA CAACCCCT-- -----AAACT CCAACGCAGA CAAAATCCCA
Capra_hircus        CTCCAC---- -------G-- -------ACA GGAATTCCA- -A-------- T---------
Gallus_gallus       CTACAC---T ----C--A-- -------CTA GGCATCTCA- -----T-T-- ------T---
Bos_taurus          CTCCAC---- ----C--C-- ------AACA GGAATTTC-- -A----T--- ----------
Bos_indicus         CTCCAC---- ----C--C-- ------AACA GGAATTTC-- -A----T--- ----------
Panthera_tigris     ---------- ---------- ---------- ---------- ---------- ----------
Ovis_aries          CTCCAC---- -------C-- -------ACA GGAATTCCA- -G---A---- T-----T--C
Sus_scrofa          CTGCAC---- -C-----C-- ------TACC GGAATCTCA- -A---AT--- ------T---
Equus_caballus      CTTCAC---- ---------- T-------CA GGAATCCCA- ----TATG-- ----------
Canis_lupus         CTACAC---- -C-----C-- ------T-CA GGAATC-CA- -A---T---- ------T---
Felis_catus         CTTCATGAAA CAGGATCTAA CAACCCCTCA GGAATT-CA- ----TT---- ----------
Rattus_norvegicus   CTCCAC---- -------A-- T------ACA GGATTAAACT CCGACGCAGA CAAAATCCCA
                      610
               ....|....|
Capra_hircus        --TCACCCTT
Gallus_gallus       --TCACCCAT
Bos_taurus          ---CACCCCT
Bos_indicus         ---CACCCCT
Panthera_tigris     ----------
Ovis_aries          ---CACCCTT
Sus_scrofa          --TCACCCAT
Equus_caballus      ---CACCCAT
Canis_lupus         --TCACCCTT
Felis_catus         ---CACCCAT
Rattus_norvegicus   TTCCATCCAT
```

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.

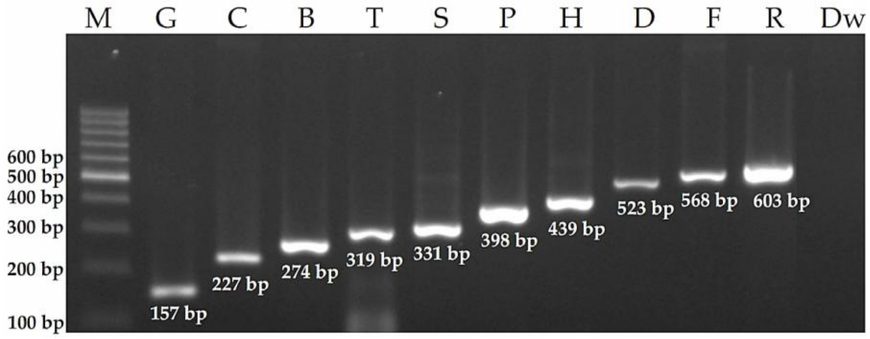


Figure 3. Specific fragments amplification on several animal. M: marker 100 bp, G: goat, C: chicken, B: cattle, T: tiger, S: sheep, P: pig, H: horse, D: dog, F: cat, R: rat, Dw: negative control.

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 molecules (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 amplication. Amplification efficiency is affected by the difference primer sequences (Matsunaga *et al*., 1999).

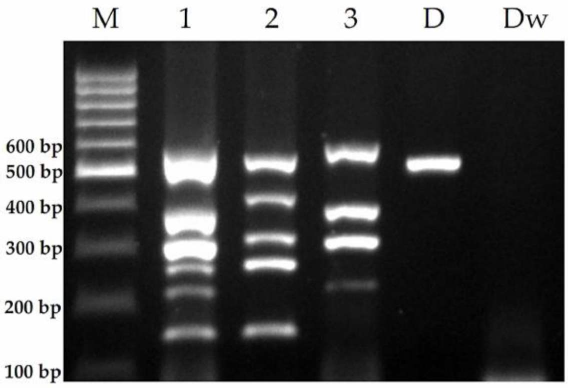


Figure 4. Specific fragments amplification on genome pool. M: marker 100 bp, 1: tube 1 (goat, chicken, cattle, tiger, pig, cat), 2: tube 2 (goat, cattle, sheep, horse, cat), 3: tube 3 (chicken, tiger, pig, rat), D: dog, Dw: negative control.

CONCLUSION

Dog, cat, and tiger DNA are amplified successfully with fragment length of 523, 568, 319 bp, respectively. Species specifity of dog, cat, and tiger are indicated by high reverse primers homology persentage. 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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