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

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).
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*  | CTC GAC AAA TGT GAG TTA CAG AGG GA | 157 bp |
| Chicken† | AAG ATA CAG ATG AAG AAG AAT GAG GCG | 227 bp |
| Cattle§ | CTA GAA AAG TGT AAG ACC CGT AAT ATA AG | 274 bp |
| Tiger | TAG CCA TGA CCG TAA ACA ATA GC | 319 bp |
| Sheep∥ | CTA TGA ATG CTG TGG CTA TTG TCG CAA AT | 331 bp |
| Pig* | GCT GAT AGT AGA TTT GTG ATG ACC GTA | 398 bp |
| Horse§ | 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‡ | GAA TGG GAT TTT GTC TGC GTT GGA GTT T | 603 bp |

Note: *Matsunaga et al. (1999); †modified Matsunaga et al. (1999); §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 TGG CTA TTG TCG CA-3’), so sheep reverse primer was modified by adding three nucleotides in 3' end (5’-CTA TGA ATG 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 mixture at the 0.1% level (Ilhak & Arslan, 2012).

Table 2. Specific reverse primers homology in ten animals

| Specific primer | 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     | 69,231     | 88,889          |
| Chicken (27 nt) | 70,370        | 100,000       | 62,963     | 62,963     | 66,667        | 62,963     | 70,370     | 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     | 66,667     | 75,862          |
| Tiger (23 nt)   | 56,522        | 56,522        | 60,870     | 60,870     | 100,000       | 56,522     | 69,565     | 69,565        | 69,565     | 69,565     | 76,923          |
| 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        | 77,777        | 77,777     | 77,777     | -             | 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          |

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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 as 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).
| Species            | GenBank Accession Numbers | Base Pairing | Comments |
|-------------------|---------------------------|--------------|----------|
| Capra hircus      | ATGAGAGAGAGA                | 1-9          | Yucatan  |
| Gallus gallus     | ATTATTATTATTATTATTATTATTTAT | 1-9          | Tucana   |
| Bos taurus        | TTGGTTGGTTGGTTGGTTGGTTGG   | 1-9          | Indicus  |
| Bos indicus       | TTTTTTTTTTTTTTTTTTTTTTTT   | 1-9          | Taurus   |
| Panthera tigris   | ATGAGAGAGAGA                | 1-9          | Bengal   |
| Ovis aries        | ATGAGAGAGAGA                | 1-9          | Pecora   |
| Sus scrofa        | ATGAGAGAGAGA                | 1-9          | Equidae  |
| Equus caballus    | ATGAGAGAGAGA                | 1-9          | Camelid  |
| Felis catus       | ATGAGAGAGAGA                | 1-9          | Feline   |
| Rattus norvegicus | ATGAGAGAGAGA                | 1-9          | Rodent   |

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