African Journal of Biotechnology Vol. 10(29), pp. 5670-5676, 22 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2432
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Identification of species (meat and blood samples) using nested-PCR analysis of mitochondrial DNA

Sasimanas Unajak[1], Piyachat Meesawat[1], Komsan Anyamaneeratch[1], DuangtipAnuwareepong[1], Kornsorn Srikulnath[2,3] and Kiattawee Choowongkomon[1,3]*

[1]Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkhen, Bangkok, 10900, Thailand.
[2]Department of Genetics, Faculty of Science, Kasetsart University, Bangkhen, Bangkok, 10900, Thailand.
[3]Center for Advanced Studies in Tropical Natural Resources, National Research University, Kasetsart, Chatuchak, Bangkok, 10900, Thailand.

Accepted 3 June, 2011

Crocodile meat product is an alternative protein source. Although, crocodile meat is more expensive, its taste is similar to that of chicken and fish. The authentication of commercial meat species is important for consumer's confidence. In this study, sensitive and specific method multiplex nested-PCR was applied to identify commercial meat species. Dried blood was used as an alternative DNA source for detection. The detection sensitivity was enhanced by primers specifically designed to encompass the mitochondrial *Cytochrome b* and *NADH dehydrogenase 5/6* genes. The specificity and sensitivity of multiplex PCR system were tested. Different lengths of specific nested-PCR products were detected to be 350, 570, 750 and 1000 bp for chicken, pig, cow, and crocodile, respectively. The system allowed detection with as little as 5 nanogram of DNA from either meat or blood sample. Detection sensitivity of individual species was improved, enabling the detection of DNA with as little as 1 picogram. Cross reaction was not detected among the tested species. It was shown that the multiplex-PCR assay enhanced the sensitivity of routine species identification and allowed the use of blood as an alternative DNA source for detection.

Key words: *Cytochrome b*, *NADH dehydrogenase*, mitochondrial DNA, meat, blood, species identification, nested-PCR, crocodile.

INTRODUCTION

Animal or meat species identification has been developed to address different concerns. Authentication of food ingredients is important for consumers because of food fraud. The traceability of meat component in food improves consumer's confidence in food products. The substitution of expensive meat with cheaper one is a major concern. For some consumer groups, such as vegetarians, the contamination of food with meat residue is strictly prohibited. Another good example of meat identification is the Halal food for the Muslim consumers, who are prohibited from consuming pork.

To date, the crocodile luxury leather industry is dramatically growing. Besides leather, crocodile meat is also a good source of low cholesterol and low lipid content protein for human consumption (Hoffman, 2008). The taste of crocodile meat is similar to that of chicken and fish, although it is more expensive. Thus, crocodile meat is usually sold in sophisticated markets. Besides meat and hide, blood, a byproduct of the crocodile leather industry, is also consumed as food supplement (Chaeychomsri et al., 2009). The price of crocodile meat and blood is superior but more expensive than chicken's. The authenticity of crocodile meat and blood in the market would raise consumer's confidence.

Several genetics-based and protein-based analytical methods approaches were applied for meat identification

*Corresponding author. E-mail: fsciktc@ku.ac.th. Tel: (+66)2-562-5555. Ext: 2051. Fax: (+66)2-561-4627.

Abbreviations: NADH dehydrogenase, reduced nicotinamide adenine dinucleotide dehydrogenase; **mtDNA,** mitochondrial DNA; **PCR,** polymerase chain reaction.

(Ballin et al., 2009). In genetics based analysis, polymerase chain reaction (PCR) based methods were widely used for identifying eat species,including traditional PCR method (Saez et al., 2004; Aida et al., 2005; Kesmen et al., 2007; Tanabe et al., 2007 and Haunshi et al., 2009), PCR restriction fragment length polymorphism (PCR-RFLP) (Verkaar et al., 2002; Ong et al., 2007; Murugaiah et al., 2009), a multiplex-PCR (Bai et al., 2009; Ghovvati et al., 2009), loop mediated isothermal amplicons (LAMP) (Ahmed et al., 2009), and real-time PCR assay (Chisholm et al., 2005; Kesmen et al., 2009). Protein based analysis techniques were also used to identify animal species, including radioimmunoassay (Lowenstein et al., 2006) and liquid chromatography (Chou et al., 2007). The genetics based analytical methods have higher detection sensitivity and specificity than protein based methods (Ballin et al., 2009).

Mitochondrial DNA (mtDNA) is a good target for phylogenic reconstruction at several taxonomic levels. Phylogenic approaches normally use sequences from a single gene such as *Cyt b* and *COI*, which are utilized for species and family level analysis (Hebert et al., 2004; Kartavtsev and Lee, 2006) as well as for resolution of taxonomic controversies. The increase in mutation rate of mtDNA is 5-10 times relative to a single copy nuclear gene which resulted in an accumulation of base substitutions over a long period of time. The ordering of mitochondrial genes often remains unchanged over long periods of evolutionary time (Boore, 1999). mtDNA is suitable for phylogenic applications due to its very low recombination level and its abundance in small size compared with genomic DNA make (Elson and Lightowlers, 2006). In authenticating food products, a number of mtDNA genes are used as target for detecting or isolating different animal species. *Cytochrome b* (*Cyt b*) gene region is one of the conserved regions used as a molecular marker for this purpose (Hsieh et al., 2001). Several NADH dehydrogenase genes are also used for species identification (Lopez-Andreo et al., 2005; Kesman et al., 2009).

To identify authentic meats and bloods, especially from the expensive and exotic crocodile products, we developed the sensitive nested-PCR method for very low DNA content products like meats and bloods. In this study, a multiplex-PCR was used to differentiate commercial meat species. The amplification targeted *NADH dehydrogenase 5/6* genes with flanking by *Cyt b* and *tRNA^{leu}* gene. The method also enabled sample DNA identification from dried animal blood. The mixture of raw materials and of DNA marker was subsequently determined to verify the specificity and the sensitivity of our method.

MATERIALS AND METHODS

Animal specimens and DNA extraction

Fresh chicken, cow, pig and crocodile meats were collected from a local market in Thailand. Blood samples were directly withdrawn from live animals by using 0.5 M ethylene diamine tetra-acetate (EDTA) solution as an anti-coagulant. Blood mixture was stored in sterile 50 ml centrifuge tube, subsequently freeze dried to obtain dried blood powder, and stored at 4°C until used. Total genomic DNAs were isolated from the meat and the dried blood as described previously by Sambrook et al. (2001). The isolated DNAs were analyzed for quality and concentration using 1% agarose gel electrophoresis and spectrophotometric method and then stored at -20°C until used.

Primer design and nested-PCR amplification

The nucleotide sequences of mitochondrial *Cyt b*, *NADH dehydrogenase 5/6 (ND5/6)* and tRNA^{leu} genes from chicken, cow, pig, and crocodile stored in Genbank (www.ncbi.nlm.nih.gov) were taken and aligned using the default parameters of ClustalW (Table 1). The universal primer sets were designed based on the conserved region of the mtDNA sequence, whereas all pairs of species-specific primers were designed based on the distinctive sites of the mtDNA sequence belonging to the species. All nucleotide sequences of the primers used are shown in Table 2. Ten nanograms of genomic DNA from each specimen was taken in 20 µl of 1×DreamTaq buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM specific primers and 0.5 U of Dream*Taq* DNA polymerase (Fermentas, USA). All of second round of PCR was used: 1:100 dilution of the first round PCR product. Nested-PCR was performed under the following condition. The first round with the universal primer: an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30s, at 50°C for 45s, at 72°C for 2 min, and finally at 72°C for 10 min for the final extension. The second round with the species-specific primers: an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, at 54°C for 45s, at 72°C for 1 min, and at 72°C for 10 min for the final extension. Then, the PCR products were visualized on 1% agarose gel by UV transilluminator.

Multiplex nested-PCR assay

To test the sensitivity of species specific primer, serial dilution of the DNA template ranging from 0.001ng to 5 ng were subjected to the reaction containing the individual species-specific primer. The specificity of PCR was analyzed by using 5 ng of each animal species from either meat or blood, and added to a mixture of species-specific primer set. Alternatively, the mixture of animal DNAs was subjected to an individual species-specific primer set. The effectiveness of the multiplex nested-PCR system was determined by adding DNA mixture from each animal species in a mixture of species-specific primer set. The amplification was performed as described earlier. The sensitivity of the detection was analyzed using 1% agarose gel by UV transilluminator.

RESULTS

Amplification using nested-PCR

To determine the specificity of the primer set, the universal primer designed based on the conserved region of the mitochondrial *Cyt b* and *ND5/6* gene, was initially used to amplify in the first PCR reaction. Amplicons with approximately 2,000 bp were detected in pig, cow, and crocodile DNA, whereas 1,400 bp amplicon was only observed in chicken DNA (Figure 1). To increase detection

Table 1. The targeted sequence position for each primer pair in the mitochondrial genome and the predicted size of the amplicons.

Species	Accession number (NCBI)	Sequence				Predicted product Size (bp)
		Universal primer		Species specific primer		
		Forward	Reverse	Forward	Reverse	
Chicken						
Gallus gallus	AB086102	13509 - 13528	14917 - 14940	14094 - 14113	14742 - 14766	352
Gallus lafayettei	AP003325	13565 - 13584	14973 - 14996	14150 -14169	14481 - 14502	352
Gallus sonneratii	AP006741	13565 - 13584	14973 - 14996	14150 - 14169	14481 - 14502	352
Gallus varius	AP003324	13506 - 13525	14914 - 14937	14091 - 14110	14422 - 14443	352
Cow						
Bosgrunniens	EF494179	13067 - 13086	15058 - 15080	14012 - 14037	14742 -14760	748
Bosindicus	AY126697	12538 - 12557	14529 - 14552	13482 - 13508	14213 - 14231	749
Bostaurus	AB074965	12534 - 12553	14525 - 14548	13479 - 13504	14209 - 14227	748
Crocodile						
Alligator mississippiensis	Y13113	12256 - 12275	14248 - 14271	12986 - 13007	14010 - 14029	1043
Alligator sinensis	AF511507	12272 - 12290	14255 - 14278	13002 - 13023	14017 - 14036	1043
Caiman crocodilus	AJ404872	12489 - 12508	14476 - 14499	13219 - 13240	14231 - 14250	1031
Crocodylus niloticus	AJ810452	12289 - 12308	14298 - 14321	13019 - 13040	14043 - 14062	1043
Crocodylus porosus	AJ810453	12297 - 12316	14307 - 14330	13027 - 13048	14051 - 14070	1043
Crocodylus siamensis	DQ353946	12292 - 12311	14302 - 14325	13022 - 13043	14046 - 14065	1043
Crocodylus siamensis	EF581859	12270 - 12289	14280 - 14303	13000 - 13021	14024 - 14043	1043
Gavialis gangeticus	AB079596	12247 - 12266	14254 - 14277	12977 -12998	14001 - 14020	1043
Pig						
Sus scrofa	AY337045	13331 - 13350	15325 - 15348	14198 - 14215	14742 - 14766	568
Human						
Homo sapiens	NC 001807	12764 - 12783	14760 - 14783	-	-	-

Table 2. Primers used in this study.

Primer name	Forward primer (5′ → 3′)	Reverse sequence (5′ → 3′)
Universal primers	GGHTGAGARGGMGTNGGMAT	TTTTADDAGKGGGTGKGATTTTCG
Species specific primers		
Chicken	TTATTCCTRTGCTCYGGCCT	CGTAYGATTGGGAGRATGGCTG
Cow	CTAATYGGAAGCCTCTTCGCAGGRTA	AACGGCTATGGCTACRGAA
Crocodile	CHGGHTTYTWYTCMAAAGACGC	GRRGGHGGVAGTTTTATRCC
Pig	TTCCTAGGGCAATCACGT	TTGATCATTGCTTATTGGAGTTGT

specificity and sensitivity, nested-PCRs were conducted using species-specific primers as indicated in Table 2. The distinctive sizes of the second PCR products were determined according to the predicted species-specific amplicons as follows: 350 bp for chicken, 570 bp for pig, 750 bp for cow and 1,000 bp for crocodile (Figure 2).

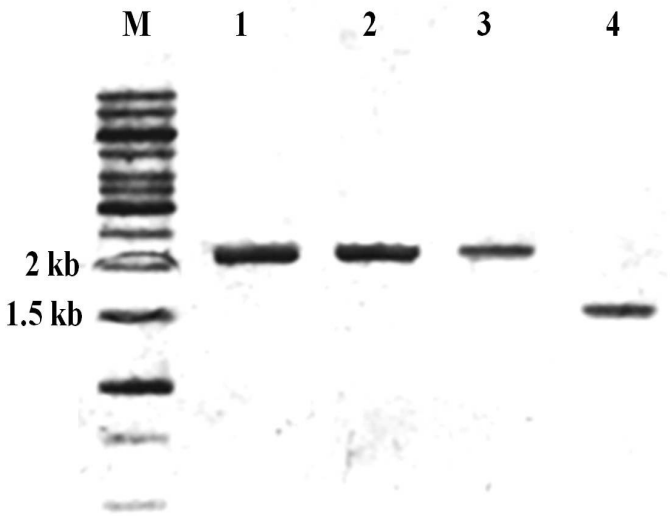

Figure 1. Agarose gel eletrophoresis of PCR products using the universal primers. M: DNA marker, 1: crocodile, 2: pig, 3: cow, and 4: chicken.

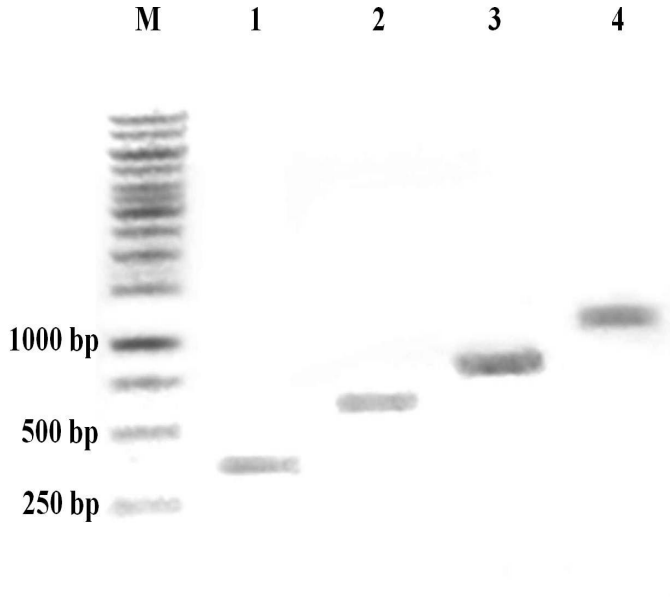

Figure 2. Agarose gel eletrophoresis of PCR products using the species specific primers. M: DNA marker, 1: chicken, 2: pig, 3: cow and, 4: crocodile.

Sensitivity of the PCR assay

The sensitivity of the PCR assay of each species specific primer was confirmed by detecting the limitation of those specific primers in the amplification of the serially diluted DNA templates ranging from 0.001 ng to 5 ng. The results showed that the species specific nested-PCR primers can detect DNA at 0.001, 0.01, 0.5, and 1 ng level of cow, crocodile, chicken, and pig meats, respectively. These nested-primer sets could also detect at 0.001, 0.05, 0.5, and 5 ng level of cow, pig, crocodile, and chicken blood DNA templates as well (Figure 3).

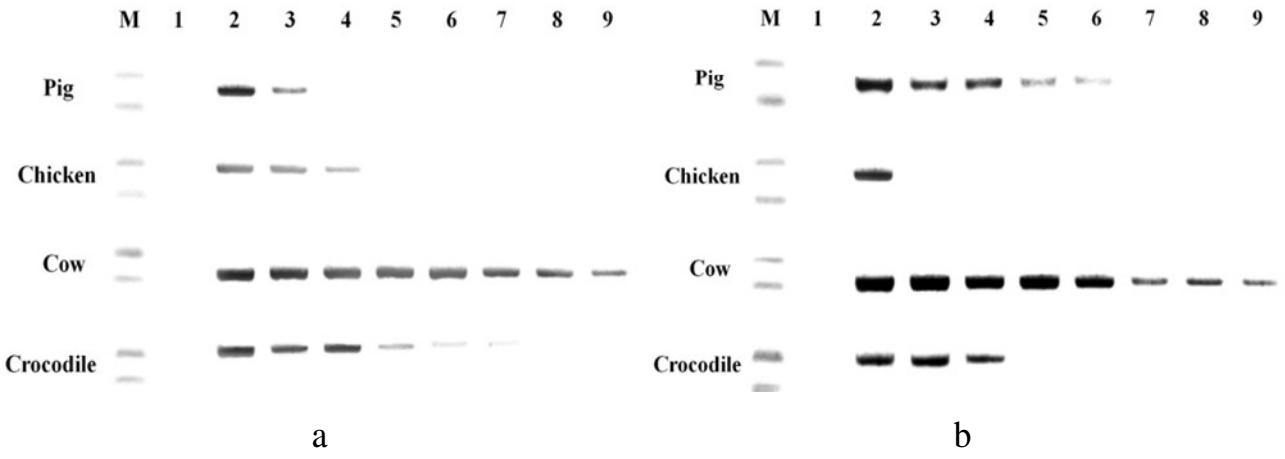

Figure 3: The result of sensitivity of PCR assays from meat (a) and blood (b) for species specific primers. M: marker, 1: negative control, 2: 5 ng, 3: 1 ng, 4: 0.5 ng, 5: 0.1 ng, 6: 0.05 ng, 7: 0.01 ng, 8: 0.005 ng, and 9: 0.001 ng of DNA templates.

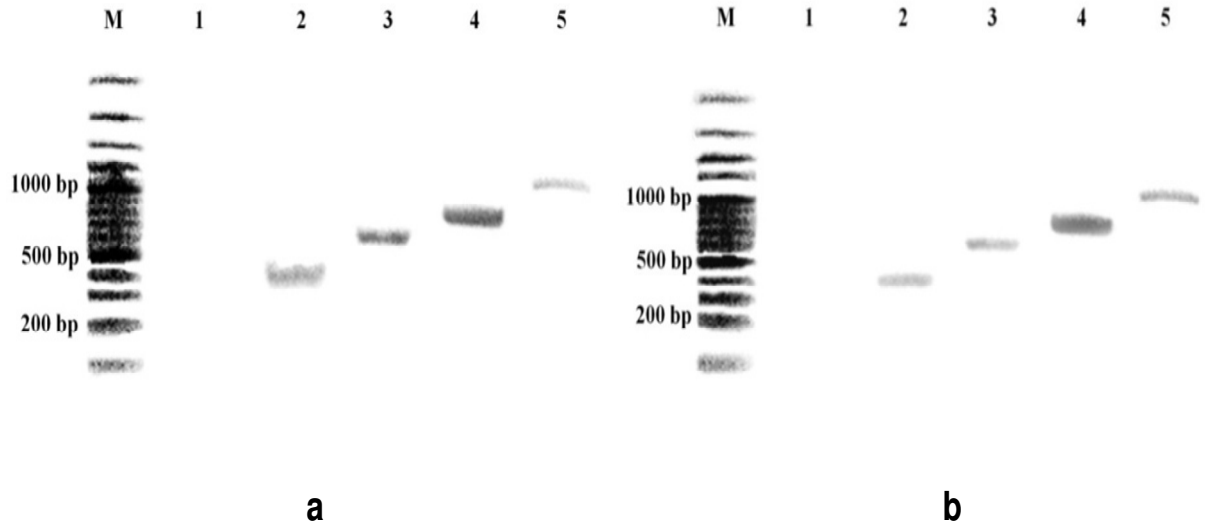

Figure 4. Specificity of multiplex PCR assays of specific primer mixtures (a: DNA template from meat and b: DNA template from blood). M: Maker 100 bp ladder, 1: negative control, 2: chicken template, 3: pig template, 4: cow template, and 5: crocodile template.

Multiplex PCR assay

To validate the specificity of the nested-primer sets in a single reaction, a multiplex PCR-mediated assay of individual animal species was conducted using four species-specific primer mixtures. The results showed that the specific fragments of 350, 570, 750 and 1,000 bp for chicken, pig, cow, and crocodile, respectively, were predominantly amplified from both meat and blood samples. Moreover, cross-reaction was not detected in all primer sets (Figure 4). Mixture of DNA samples was also analyzed in individual specified specific primer sets. The responsive size for each animal species from either meat or blood was detected to correspond to each animal species without cross-contamination (Figure 5). The identification of animal species from interspecies DNA mixed from meat or blood in multiplex PCR approach was performed. The result indicated that DNAs from all species were specifically detected (Figure 6).

DISCUSSION

The adulteration or substitution of meat is a serious concern for different groups of consumers. The identify-cation of the meat source is a great challenge for

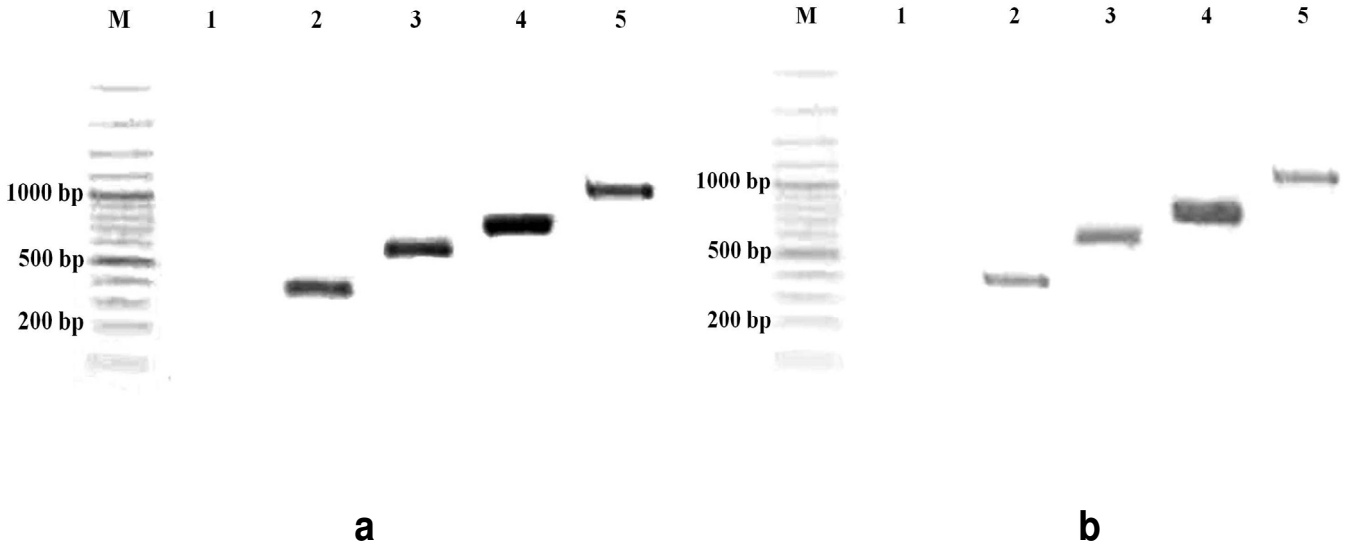

Figure 5. Specificity of multiplex PCR assays of DNA mixtures from meat (a) and blood (b). M: Maker 100 bp ladder, 1: negative control, 2: chicken primer, 3: pig primer, 4: cow primer, and 5: crocodile primer.

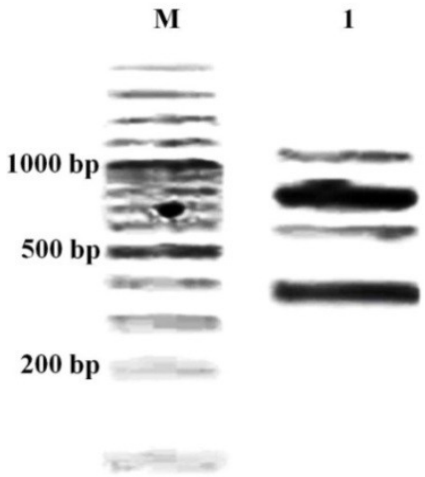

Figure 6. Specificity of multiplex PCR assays of DNA mixtures and specific primer mixtures. M: Maker 100 bp ladder, 1: PCR product detected specific of 4 species of animals (chicken, pig, cow, and crocodile).

molecular biologist in the past years. Here, we successfully developed an interspecies-specific multiplex-PCR assay to examine four kinds of commercial animal species that are common ingredient in Thai food and widely distributed in local and hypermarkets: chicken, pig, cow, and crocodile. Generally, the target source for animal species identification comes from fresh or cooked meat. Here, we demonstrated an alternative source of detection derived from blood. This alternative detection source may enhance the possibility of species identification without damaging the meat; in fact, the system could be modified for blood stain identification in forensic science.

Multiplex PCR approach has been applied successfully in several animal species. The universal specific primers in combination of species-specific primers vastly increase the specific sensitivity. The primers were designed using *Cyt b* and *ND5*/6 genes to obtain specific size of each animal species. The most often source of DNA template comes from either fresh or cooked meat (Ballin et al., 2009). In this study, four economically important animal species, pig, cow, chicken and crocodile were subjected for identification. The method can individually detect animal species with at least 1 ng of pig DNA, 5 ng of chicken DNA, 0.001 ng of cow DNA, and 0.5 ng of crocodile DNA. This method has better sensitivity than that of previous methods (Bai et al., 2009; Matsunaga et al., 1999; Aida et al., 2005; Murugaiah et al., 2009; Yan et al., 2005; Saez et al., 2004; Mane et al., 2009). Therefore, there was no cross-reactivity with other species. However, detection sensitivity using blood DNA was lower than when using meat DNA. It has been suggested that the use of EDTA, a *Taq* polymerase inhibitor, as an anticoagulant, inhibits the amplification of blood DNA. However, this is the first analysis that verifies the use of blood DNA as an alternative source for animal species detection, and with sufficient modification may also be applicable for other purposes.

A multiplex PCR system was developed to easily perform the detection reaction. All animal specific-primer sets were assembled and the target DNA was only added

prior to the time PCR analysis was conducted. This multiplex PCR system can produce the amplicon of each species specific fragment at a time without cross-contamination. The system assumes that approximately 1 ng of meat DNA or 5 ng of blood DNA from the respective species can be determined by using this single-tube multiplex PCR system. Hence, this approach is suitable for species determination of economically important meat.

In conclusion, this study suggests an accurate anaytical technique for chicken, pig, cow and crocodile meat identification by nested PCR analysis of the cyt b and NADH dehydrogenase 5/6 genes of mtDNA. This technique was used to detect and trace meat adulteration and to differentiate species pre-sent in meat mixture. The test could be used and applied by researchers and quality control laboratories for verification and control of industrial meat products, such as Halal authentication and raw material origin certification.

ACKNOWLEDGEMENTS

This study was financially supported by Thailand Research Fund (IRPUS I151A02003) and by a research grant from the Faculty of Science, Kasetsart University.

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