Partial Sequence Analysis of *Cytochrome b* Gene by FINS Technique Reveals Fraud Sambar Meat in Wild Food Restaurant

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ABSTRACT

Species identification of animals in the Cervidae by sequence comparison of the Cytochrome b (Cyt b) gene using FINS method is focused in this study. The Cyt b fragments of seven species in the Cervidae, including rusa deer (Cervus timorensis), sambar deer (C. unicolor), sika deer (C. nippon), hog deer (C. porcinus), axis deer (C. axis), Eld's deer (C. eldi) and barking deer (Munticacus muntjak) were amplified by our own designed primers. The amplicons (322 bp) were sequenced and their partial sequences (263 bp) were then analyzed. Barking deer showed the highest value of genetic diversity within species (π = 0.0364; h = 0.9). The phylogenetic analysis had shown that the partial sequence of Cyt b gene can be used to classify most studied species in Cervidae accurately, except for sambar and rusa deer that cannot be differentiated. Hence, species identification of unknown meat samples was then performed by this method. Referenced Cyt b sequences of wild boar (Sus scrofa), favorite meat in wild food restaurant, and the Cyt b sequence of known sambar tissue samples were additionally compared. The genetic distances indicated that unknown meat samples were presumably wild boar. Although this method cannot differentiate sambar from rusa deer, this study

will be useful for wildlife forensic particularly when screening examination of irrelevant samples and fraud sambar meats identification are necessary.

Keywords: *Cytochrome b* gene, DNA variation, Cervidae, FINS

INTRODUCTION

Nowadays, large numbers of endangered wildlife animals listed in the CITES appendices are smuggled for illegal trades. In this case, wildlife forensic science has been used as a significant tool to prosecute the smuggler. Studies on wildlife forensic science have been reviewed (Johnson et al., 2014). Most of them have focused on species identification, for which methods based on DNA analysis are more advantageous than those of traditional morphological identification because they can be applied on different types of samples, for example, processed animal parts, derivatives within Traditional Chinese Medicine (TCMs) and objects made from animal parts (Iyengar, 2014) DNA markers used for species identification must be species-specific. These sequences can be found in both nuclear and mitochondrial DNA. However, mitochondrial DNA is more preferable because it contains high copy numbers and hardly degrades.

Many studies have shown that species-specific sequences of mitochondrial DNA, such as *ND1* (Kitpipit et al., 2012; Welton et al., 2013), *COI* (Wilson-Wilde et al., 2010), *16S rRNA* (Imaizumi et al., 2007), *Cyt b* (Jun et al., 2011), D-loop (Fumagalli et al., 2009; Gupta et al., 2011) and ITS-2 (Clarke et al., 2006) can be used to identify animal species. Molecular techniques like PCR-RFLP have been conducted to discriminate DNA polymorphisms of a target sequence for species identification, for example *Cyt b* for identification of fish species in the Cyprinidae family (Chen et al., 2011) and *16S rRNA* for sea cucumber identification (Wen et al., 2010). However, the questioned species may not be revealed when the PCR-RFLP profile of an unknown sample does not match with any of those reference species. Hence, a technique called FINS (Forensically Informative Nucleotide Sequencing) has been introduced. This technique identifies species by using phylogenetic analysis in which DNA sequence of a sample is compared with known DNA sequences in the database (Li et al., 2011)). The FINS technique has been reported as a successful method for determination of authentic seafood ingredients including, octopus (Espineira et al., 2010), jelly fish (Armani et al., 2013) and ling fish (Santaclara et al., 2014). This technique has also been applied for mislabeling investigation of pet canned food (Armani et al., 2015).

In wildlife forensic science, species identification based on DNA analysis can be performed by PCR amplification of target DNA using species-specific primer, i.e. examination of a shawl woven from Tibetan antelope hair, a protected species listed in the CITES appendix I (Lee et al., 2006) and identification of protected buffalo meat in Sri Lanka (Rajapaksha et al., 2003). Nevertheless, species identification by PCR amplification with species-specific primer cannot differentiate samples of different subspecies, in which case determination of genetic variation by FINS will cope with this limitation as shown by Gupta et al. (2013) who employed the technique to distinguish wild pigs from domestic ones. However, species identification by FINS needs reference DNA data from related species. The lack of reference DNA may cause misinterpretation. Unknown samples of a viverrid were misidentified as coming from hyena due to the insufficient data of *Veverricula indica 16S rRNA* sequences in the NCBI database (Sahajpal and Goyal, 2010).

In Thailand, large numbers of livestock belong to the Cervidae family, i.e. sika, rusa, hog and sambar deer. The latter is a protected species; however, due to the popularity of its meat, farming sambar deer is allowed albeit only through legal permission. Since its farming practice is subject to tight regulation, the samba deer meat is highly valued and costly. Owing to this, meats of other animals are sometimes deceitfully advertised as the meat of sambar deer, necessitating proper identification of the meats. Although DNA barcoding (*COI*) has been successfully used to identify sambar deer meat, the accuracy of this method depends on the quality of the database (Kumar et al., 2012). Moreover, for species identification by DNA barcoding, a target size of 5' *COI* region is approximately 650 bp (Trivedi et al., 2016). This may be troublesome when highly degraded DNA samples are performed. Hence, an alternative method for sambar deer meat identification by comparing *Cyt b* sequences using FINS technique is focused in this study. According to our results, the FINS technique has proved a useful tool for species identification enabling us to forensically trace the origins of the meats sold in wildlife restaurants across Thailand.

MATERIALS AND METHODS

Samples

Blood samples of five individuals per species including *C. timorensis*, *C. unicolor*, *C. nippon*, *C. porcinus*, *C. axis*, *C. eldi* and *M. muntjak* were kindly provided by Khon Kaen Zoo under the permission from the Animal Ethics Committee (ACUC-KKU-31/2559). Ten milliliters of blood was taken into a test tube containing 500 µl of 0.5M EDTA. Blood samples were then kept at

4°C until DNA extraction was performed. For unknown meats, five samples (100 g each) were taken from wild food restaurants in Thailand (two from Prajeenburi, two from Phra Nakorn Sri Ayuttaya and one from Nakhon Nayok province). The meats were kept at 4°C until they were used. For reference, meat (MC) and liver (LC) of *C. unicolor* and *C. timorensis* antler (HC) were also provided by Khon Kaen Zoo.

DNA isolation

DNA extraction from the blood samples was conducted using QIAamp® DNA Blood Mini Kit (QIAGEN, Germany). Briefly, 200 µl of each sample was incubated with proteinase K at 56°C for 10 min. Then, ethanol was added and the mixture was transferred onto QIAamp mini spin column. This column was centrifuged to get rid of the liquid. The column was subsequently washed twice. The DNA was then eluted from the column. For meat and antler samples, DNA extraction using NucleoSpin® kit was performed according to the manufacturer's instruction. Twenty-five mg of each tissue sample was incubated with proteinase K at 56°C for 3 h. Then, 100% ethanol was added and the mixture mixed before being transferred to NucleoSpin® tissue column. The liquid was removed from the column by centrifugation. The column was then washed and DNA was subsequently eluted. The quantity and quality of DNA solution were examined by NanoDrop spectrophotometer.

Primer design

The nucleotide sequences of *Cyt b* genes from animals in the Cervidae were obtained from GenBank database (http://www.ncbi.nlm.nih.gov/genbank). *Cyt b* sequences of *C. porcinus* (DQ379301.1), *C. axis* (AY607040.1), *C. eldi* (AY157735.1), *C. nippon* (D32192.1), *C. timorensis* (AF423200.1), *C. unicolor* (AF423201.1) and *M. muntijak* (AF042718.1) were multiple aligned using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primers were designed from conserved regions by OligoAnalyzer 3.1 (https://www.idtdna.com/calc/analyzer).

PCR amplification

The PCR reaction containing 0.2 ng DNA, 0.4 µM of each primer and 1xPCR Master Mix (Vivantis, Malysia) was set up. A total volume of 25 µl was made by adding deionized water. The amplification was then performed in a Thermal Cycler with the following cycling conditions: initial denaturation step at 94°C for 2 min followed by 35 cycles of 94°C 30 sec, 57°C 30 sec, 72°C 30 sec and a final extension at 72°C 5 min. The PCR product was

subsequently analyzed on 1.5% agarose gel. The size of the amplicon was estimated from the 100 bp DNA marker (VIVANTIS, Malaysia).

DNA sequencing and haplotype analysis

The PCR products were sent to Macrogen (South Korea) for DNA sequencing with our designed primers. The nucleotide sequences of forward and reverse strands from each sample were aligned by BioEdit 7.0 program (Hall, 1999). Fine adjustment was manually performed with visual inspection. These sequences were then assembled. The sequences of all samples were multiple aligned. Fault insertion and deletion of the bases were manually edited. Nucleotide sequences were trimmed at both ends to produce the equal lengths of the sequences. From the result by multiple alignment, the polymorphic sites of nucleotide were examined using DnaSP 5.0 (Librado and Rozas, 2009). Then, nucleotide diversity (π) and haplotype diversity (h) were determined.

Phylogenetic analysis

Thirty-five sequences of the *Cyt b* gene obtained from this study were aligned with seven sequences of species belonging to the Cervidae familyretrieved from the GenBank database, using MEGA version 6. A fragment of 263 bp *Cyt b* gene was selected for comparison by means of neighbor-joining (NJ) tree. The genetic distances were also computed using K2-parameter model with 2000 bootstrap re-samplings.

Species identification by FINS methods

The DNA extraction from unknown meat samples, reference samples of *C. unicolor* (meat, liver) and *C. timorensis* (antler) were conducted. PCR amplification of the *Cyt b* gene was then performed followed by DNA sequencing of amplicon as described earlier. The DNA sequences were compared with reference sequences of the *Cyt b* gene of known cervids.

RESULTS

Primer design

Based on the result from the alignment of *Cyt b* gene sequences from seven species of the Cervidae, primers were designed from conserved regions. Then, nucleotide positioned 182-200 and 481-503 were chosen for forward and reverse primers, respectively (Figure 1). As a result, a DNA fragment with a length of 322 bp was amplified.

```
MM 1   ATGACAAATATTCGAAAAACCCACCCACTAATAAAAATTGTAAACAATGCATTCATTGAT 60
CE 1   ATGACCAATATCCGAAAAACCCACCCATTAATAAAAATTGTGAACAACGCATTTATTGAC 60
CN 1   ATGACTAATATCCGAAAAACCCACCCATTAATAAAAATTGTAAACAACGCATTCATTGAC 60
CT 1   ATGACCAATATCCGAAAAACCCACCCATTAATAAAGATTGTAAACAACGCATTCATTGAC 60
CU 1   ATGACCAATATCCGAAAAACCCACCCATTAATAAAGATTGTAAACAACGCATTCATTGAC 60
CP 1   ATGATCAATATCCGAAAAACCCACCCATTAATAAAAATTGTAAACAACGCATTCATTGAC 60
CA 1   ATGATCAACCTCGGAAAAACCTCCCCATTAATAAAAATTGTAAACAACGCATTCATTGAC 60
       **** ** * ******** **** ******* ***** ***** ***** *****

MM 61  CTCCCAGCCCCATCAAATATCTCATCTTGATGAAACTTTGGCTCCCTACTAGGAATCTGC 120
CE 61  CTCCCAGCCCCATCAAATATTTCATCCTGATGAAATTTCGGCTCCTTGCTAGGAGTTTGC 120
CN 61  CTCCCCGCCCCATCAAATATTTCATCCTGATGAAATTTCGGCTCCCTACTAGGAATTTGT 120
CT 61  CTCCCAGCCCCATCAAATATTTCATCCTGATGAAATTTCGGCTCCTTACTAGGAATTTGT 120
CU 61  CTCCCAGCCCCATCAAATATTTCATCCTGATGAAATTTCGGCTCCTTACTAGGAATTTGT 120
CP 61  CTCCCAGCACCATCAAATATTTCATCCTGATGGAACTTCGGCTCTCTGCTAGGAGTCTGC 120
CA 61  CTCCCAGCCCCATCAAATATTTCATCCTGATGGAACTTCGGCTCTTTGCTAGGAGTCTGC 120
       ***** ** ********** ***** ***** ** ** *****  * ****** * **

MM 121 TTAATTCTACAAATCCTCACAGGCCTATTTCTAGCAATGCACTACACATCCGACACAATA 180
CE 121 CTAATTCTACAAATCCTCACAGGCCTATTTCTAGCAATACACTACACATCTGATACAATA 180
CN 121 CTAATCCTACAAATCCTTACAGGCCTATTCCTAGCAATACACTATACATCTGACACAATA 180
CT 121 CTAATCCTACAAATCATCACAGGCCTGTTCCTAGCAATACACTATACATCCGATACAATA 180
CU 121 CTAATCCTACAAATCATCACAGGCCTATTCCTAGCAATACACTATACATCCGATACAATA 180
CP 121 TTAATTCTACAAATCCTCACAGGCCTATTCTTGGCAATACACTATACATCCGACACAATA 180
CA 121 TTAATTCTACAAATTCTCACGGGCTTATTCTTAGCAATACACTATACATCTGATACAATA 180
       **** ********  * ** *** * **  * ***** ***** ***** ** ******

       5'-CAGCATTTTCCTCTGTTAC-3'
MM 181 ACAGCATTCTCCTCGGTCACCCATATCTGCCGAGACGTCAACTATGGCTGAATCATCCGA 240
CE 181 ACAGCATTTTCCTCTGTCACCCATATCTGTCGAGATGTCAACTATGGCTGAATTATTCGA 240
CN 181 ACAGCATTTTCCTCTGTCACCCATATCTGTCGAGATGTCAACTATGGTTGAATTATCCGA 240
CT 181 ACAGCATTTTCCTCTGTTACCCATATCTGCCGAGATGTCAATTATGGCTGAATCATTCGA 240
CU 181 ACAGCATTTTCCTCTGTTACCCATATCTGCCGAGATGTCAATTATGGCTGAATCATTCGA 240
CP 181 ACAGCATTCTCCTCTGTTACCCATATTTGCCGAGACGTCAATTACGGCTGAATTATTCGA 240
CA 181 ACAGCATTCTCCTCTGTCACTCATATCTGTCGAGACGTCAACTACGGCTGAATTATTCGA 240
       ******** ***** ** ** ***** ** ***** ***** ** ** ***** ** ***

MM 241  TATATACACGCAAACGGAGCATCAATATTTTTCATTTGCTTATTTATTCATGTAGGACGA  300
CE 241  TACATACACGCAAACGGAGCATCAATATTTTTCATCTGTCTATTCATACATGTAGGACGA  300
CN 241  TACATACACGCAAACGGGGCATCAATATTTTTCATCTGCCTATTCATACATGTAGGACGA  300
CT 241  TATATACACGCAAACGGGGCATCAATATTTTTCATCTGTCTATTTATACACGTAGGACGA  300
CU 241  TATATACACGCAAACGGGGCATCAATATTTTTCATCTGTCTATTTATACACGTAGGACGA  300
CP 241  TATATGCACGCAAATGGAGCATCAATATTTTTTATCTGCCTATTTATACATGTAGGACGA  300
CA 241  TATATGCACGCAAATGGAGCATCAATATTTTTTATTTGCTTATTTTTACATGTAGGACGA  300
        ** ** ******** ** ************** ** **  ****  * ** *********

MM 301 GGCCTATACTATGGATCATACACCTTCCTAGAAACATGAAACATTGGAGTGATTCTTTTA 360
CE 301 GGCCTGTACTACGGATCATATACCTTTCTAGAGACATGAAACATTGGAGTAATTCTCCTA 360
CN 301 GGCCTGTACTACGGATCATATACTTTTCTAGAGACATGAAACATCGGAGTAATTCTCCTA 360
CT 301 GGCCTGTACTACGGATCATACACTTTTCTAGAGACATGAAACATCGGAGTAATCCTCCTA 360
CU 301 GGCCTGTACTACGGATCATACACCTTTCTAGAGACATGAAACATCGGAGTAATCCTCCTA 360
CP 301 GGCCTGTACTACGGATCATACACCTTTCTAGAAACATGAAACATTGGAGTAATCCTCCTA 360
CA 301 GGTCTGTATTACGGATCATATACCTTTTTAGAAACATGAAACATTGGAGTAATCCTCCTA 360
       ** ** ** ** ******** ** **  **** ********** ***** ** **  **
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MM  361  TTTACAGTTATAGCCACGGCATTCGTAGGATATGTTTTACCATGAGGACAAATATCATTT  420
CE  361  TTTACAGTTATAGCAACAGCATTCGTAGGGTATGTCTTACCATGAGGACAGATATCATTC  420
CN  361  TTTACAGTTATAGCCACAGCATTCGTAGGATATGTCCTACCATGAGGACAAATATCATTC  420
CT  361  TTTACAGTTATAGCCACAGCATTCGTAGGGTATGTCCTACCATGAGGACAAATATCATTC  420
CU  361  TTTACAGTTATAGCCACAGCATTCGTAGGGTATGTTCTACCATGAGGACAAATATCATTC  420
CP  361  TTCACGGTTATAGCCACAGCATTCGTAGGATATGTCCTACCATGAGGACAAATATCATTC  420
CA  361  TTTACAGTTATAGCCACAGCATTTGTGGGATACGTCCTACCATGAGGACAGATATCATTC  420
         ** ** ******** ** ***** ** ** ** **  ************ ********

MM  421  TGAGGAGCAACAGTCATCACTAACCTCCTTTCAGCAATTCCATATATTGGCACAAACTTA  480
CE  421  TGAGGAGCAACAGTCATCACCAACCTCCTCTCAGCAATTCCATACATCGGCACAAATCTA  480
CN  421  TGAGGAGCAACAGTCATTACCAACCTCCTCTCAGCAATTCCATATATTGGCACAAACCTA  480
CT  421  TGAGGAGCAACAGTCATTACCAAACTTCTCTCAGCAATTCCATATATTGGTACAAACCTA  480
CU  421  TGAGGAGCAACAGTCATTAACAACCTTCTCTCAGCAATTCCATATATTGGTACAAACCTA  480
CP  421  TGAGGGGCAACAGTTATTACCAACCTCCTCTCAGCAATCCCTTACATCGGCACAAATCTA  480
CA  421  TGAGGAGCAACAGTTATTACCAATCTCCTCTCAGCAATCCCTTACATTGGTACAAATCTA  480
         ***** ******** ** *  ** ** ** ******** ** ** ** ** *****  **

   3'-CAGCTTACTTAGACTCCTCCGAA-5'
MM  481  GTCGAATGAATCTGAGGAGGCTTTTCAGTTGATAAAGCAACCCTCACCCGATTCTTTGCC  540
CE  481  GTCGAATGAATCTGAGGGGGCTTTTCAGTAGATAAAGCAACCCTGACCCGATTTTTCGCT  540
CN  481  GTCGAATGGATCTGAGGGGGCTTCTCAGTAGATAAAGCAACCCTAACCCGATTTTTCGCT  540
CT  481  GTCGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCCACCCTAACCCGATTCTTTGCT  540
CU  481  GTCGAATGAATCTGAGGAGGCTTTTCAGTAGATAAAGCCACCCTAACCCGATTTTTTGCT  540
CP  481  GTCGAATGAATCTGAGGGGGCTTTTCAGTAGACAAAGCAACCTTAACCCGATTCTTCGCT  540
CA  481  GTCGAATGAATTTGAGGAGGCTTTTCAGTAGATAAAGCAACCCTAACCCGATTTTTCGCT  540
         ******** ** ***** ***** ***** ** ***** *** * ******** ** **
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Figure 1. Multiple sequence alignment of the *Cyt b* gene from 7 species of the Cervidae including *M. muntijak* (MM), *C. eldi* (CE), *C. nippon* (CN), *C. timorensis* (CT), *C. unicolor* (CU), *C. porcinus* (CP) and *C. axis* (CA). Forward and complementary sequences of reverse primers are shown in bold texts. Asterisk (*) represents identical nucleotides.

PCR results

DNAs extracted from the animal blood and meat samples were quantified and diluted to 50 ng/ul for PCR amplification. Optimal melting temperature for PCR was also investigated. As expected from the primer design result, the amplicon was 322 bp long in all samples (Figure 2), indicating successful primer design.

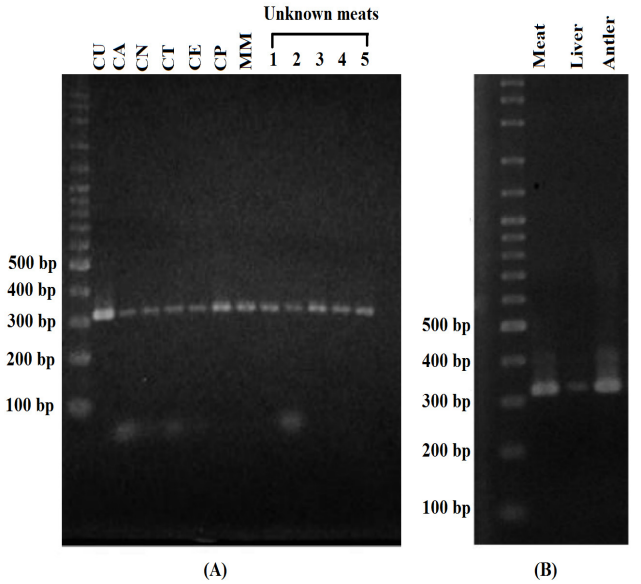

Figure 2. PCR amplification of the *Cyt b* gene on DNA extracted from animal blood samples of 7 cervids and from unknown meat samples **(A)** meat, liver of *C. unicolor* and antler of *C. timorensis* **(B)** using the designed primer.The size of the amplicon is compared with 100 bp DNA ladder (M). N represents negative control.

Haplotype diversity

The 263 bp nucleotide sequences of 322 bp PCR products from each species were analyzed for haplotype and nucleotide diversity. Unexpectedly, the *Cyt b* gene from *C. unicolor*, *C. timorensis*, *C. nippon* and *C. axis* samples had no sequence variation as indicated by one haplotype in each species (Table 1). On the contrary, the DNA sequences of the *Cyt b* gene from *M. muntjak* samples showed highest variation (h = 0.9; $\boldsymbol{\pi}$ = 0.03640) in which four haplotypes were identified. For *C. eldi* and *C. porcinus*, two haplotypes were found.

Table 1. Number of haplotypes, haplotype diversity and nucleotide diversity in the *Cyt b* gene (263 bp) of the cervids.

Species	Sample no.	Number of haplotype	Haplotype diversity (h)	Nucleotide diversity (π)
C. unicolor	1-5	1	0	0
C. axis	6-10	1	0	0
C. nippon	11-15	1	0	0
C. timorensis	16-20	1	0	0
C. eldi	21-25	2	0.5	0.00190
A. porcinus	26-30	2	0.4	0.00152
M. muntjak	31-35	4	0.9	0.3640

Phylogenetic analysis

The 263 bp nucleotide sequences of the *Cyt b* gene from 35 samples were compared and analyzed whether or not they could be used for species identification**.** In order to prove this, *Cyt b* reference sequences of the cervids retrieved from the GenBank database were also included in the NJ tree (Figure 3). Noteworthy, samples of 5 out of 7 species were classified in the same group as their reference species indicating that the sequence of this *Cyt b* region could be used as DNA target for species identification. Although, the two species of *C. unicolor* and *C. timorensis* could not be differentiated, most species in the Cervidae could be identified**.** Moreover, sample no.12 was grouped as *C. axis* instead of *C. nippon*. Although, this sample was subjected to repeated analyses, the same result was achieved indicating the possibility of mislabeling.

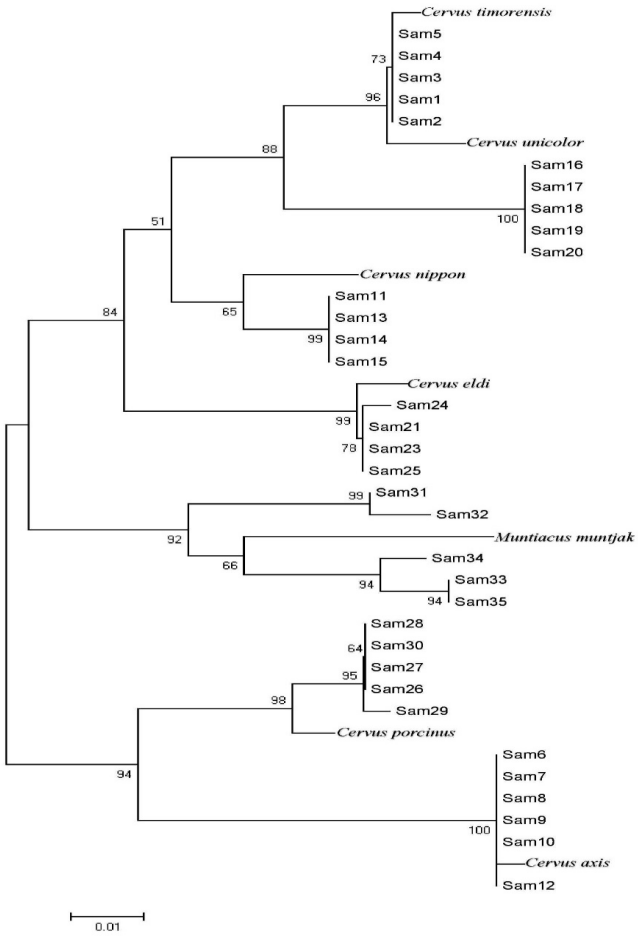

Figure 3. The neighbor joining (NJ) tree of the *Cyt b* gene from the cervid samples compared with those of reference sequences from the database. Bootstrap values were indicated on each node.

Species identification

For FINS technique, the DNA sequences (263 bp) from unknown meats were compared with the reference sequences of *Cyt b* from seven species of the Cervidae and known *C. unicolor* samples. Additionally, wild boar (*S. Scrofa*), a popular meat ingredient in wild food restaurants was also included to represent a fraud sambar meat occurrence. Phylogenetic analysis revealed that unknown meats (Unk1-Unk5) were clustered in the same group as wild boar

(Figure 4). The pairwise distances are shown in Table 2. The variable nucleotide positions of *Cyt b* sequences from sambar deer and wild boar are shown in Table 3.

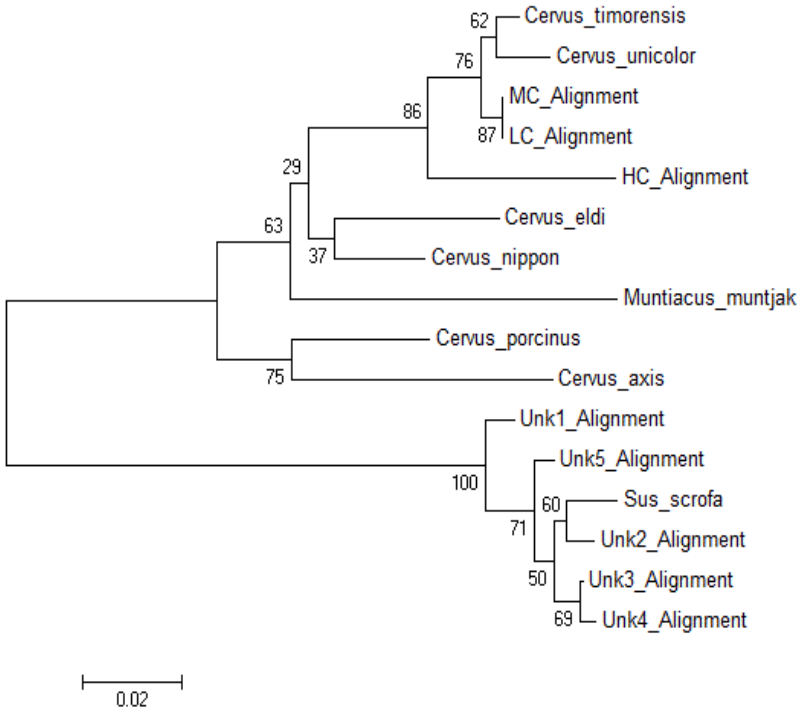

Figure 4. The neighbor joining **(**NJ**)** showing the relationships among unknown meats (Unk1-Unk5), wild boar (*S. Scrofa*), reference cervid species with known *C. unicolor* samples; meats (MC), liver (LC) and *C. timorensis* antlers (HC) based on the alignment of *Cyt b* gene sequences.

Table 2. Pairwise distances among reference species and samples (below diagonal) and values of the corresponding standard error computed by bootstrap (above diagonal).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 *C. porcinus*	0	0.016	0.018	0.018	0.017	0.017	0.019	0.023	0.023	0.022	0.023	0.023	0.023	0.017	0.019	0.017
2 *C. axis*	0.076	0	0.018	0.018	0.019	0.019	0.019	0.022	0.022	0.022	0.023	0.023	0.022	0.020	0.019	0.020
3 *C. eldi*	0.095	0.103	0	0.013	0.015	0.015	0.018	0.022	0.021	0.022	0.022	0.022	0.022	0.016	0.018	0.016
4 C. *nippon*	0.099	0.106	0.049	0	0.014	0.014	0.017	0.022	0.022	0.022	0.022	0.022	0.022	0.014	0.016	0.014
5 *C. timorensis*	0.087	0.122	0.076	0.057	0	0.007	0.018	0.023	0.023	0.023	0.023	0.024	0.023	0.007	0.014	0.007
6 *C. unicolor*	0.087	0.125	0.076	0.065	0.015	0	0.018	0.023	0.023	0.023	0.024	0.024	0.023	0.008	0.014	0.008
7 *M. muntjak*	0.114	0.122	0.095	0.091	0.103	0.095	0	0.024	0.024	0.024	0.024	0.024	0.024	0.018	0.019	0.018
8 *S. scrofa*	0.179	0.179	0.175	0.183	0.186	0.194	0.202	0	0.010	0.007	0.007	0.008	0.009	0.023	0.022	0.023
9 Unk1	0.161	0.169	0.157	0.165	0.169	0.176	0.192	0.031	0	0.010	0.007	0.008	0.009	0.023	0.022	0.023
10 Unk2	0.164	0.172	0.176	0.183	0.179	0.187	0.198	0.015	0.031	0	0.007	0.008	0.009	0.023	0.022	0.023
11 Unk3	0.172	0.179	0.168	0.176	0.179	0.187	0.198	0.015	0.015	0.015	0	0.004	0.005	0.024	0.022	0.024
12 Unk4	0.176	0.183	0.164	0.172	0.183	0.191	0.195	0.019	0.019	0.019	0.004	0	0.006	0.024	0.023	0.024
13 Unk5	0.172	0.179	0.160	0.168	0.172	0.179	0.198	0.023	0.023	0.023	0.008	0.011	0	0.023	0.022	0.023
14 MC	0.091	0.129	0.080	0.061	0.011	0.019	0.106	0.186	0.176	0.187	0.187	0.191	0.179	0	0.013	0
15 HC	0.014	0.125	0.103	0.076	0.057	0.065	0.129	0.171	0.169	0.172	0.172	0.176	0.164	0.046	0	0.013
16 LC	0.091	0.129	0.080	0.061	0.011	0.019	0.106	0.186	0.176	0.187	0.187	0.191	0.179	0	0.046	0

Table 3. Polymorphic sites in the partial sequences of the *Cyt b* gene from five unknown meats (Unk1-Unk5), reference *C. unicolor* samples (MC, LC), reference *C. timorensis* samples (HC), reference sequences of seven species in the Cervidae and wild boar (*S. Scrofa*). The position of variable nucleotide is shown as three digit numeric values at the top. Identical nucleotides are represented by **"."**.

	042	048	051	105	152	176	179	185	192	214	223	226	234	238
C. porcinus	T	T	G	C	C	A	C	A	C	C	A	A	C	C
C. axis	.	.	.	T	.	.	T	.	.	.	.	.	.	T
C. eldi	.	C	A	.	T	.	.	G	T	.	.	.	.	.
C. nippon	C	C	A	.	T	.	.	.	.	.	.	.	.	.
C. timorensis	.	.	A	.	.	.	.	G	.	.	.	.	.	A
C. unicolor	.	.	A	.	.	.	.	G	.	.	.	.	A	.
M. muntjak	C	.	A	.	T	.	.	.	T	.	.	.	.	.
S. scrofa	.	C	A	T	.	C	.	C	.	T	T	G	.	T
Unk1	.	C	A	.	.	C	.	T	.	.	T	G	.	T
Unk2	.	.	A	T	.	C	.	C	.	.	T	G	.	T
Unk3	.	C	A	.	.	C	.	C	.	.	T	G	.	T
Unk4	.	C	A	.	T	C	.	C	.	.	T	G	.	T
Unk5	.	C	A	.	.	C	.	C	.	.	T	.	.	T
MC	.	.	A	.	.	.	.	G	.	.	.	.	.	.
HC	.	.	A	.	.	G	.	.	.	.	.	.	.	.
LC	.	.	A	.	.	.	.	G	.	.	.	.	.	.

DISCUSSION

Species identification of sambar deer (*C. Unicolor*), a member of the Cervidae which is listed in Thailand Wildlife Protection, by FINS technique had been examined in this study. A species-specific sequence of *Cyt b* gene was focused because it has been reported as the locus most frequently used for species identification in forensic science (Parson et al., 2000; Hsieh et al., 2001). Hence, a pair of primers were designed from the *Cyt b* sequences of seven species of the Cervidae. PCR amplification of *Cyt b* by these primers was performed on 35 DNA samples of the cervids (5 samples/species). These primers were appropriate to this study as an amplicon of *Cyt b* (322 bp) was produced in all samples (Figure 1). The sequence analysis of these amplicons was then conducted. Interestingly, the *Cyt b* sequences of *M. muntjak* showed highest DNA variations, followed by *C. eldi* and *C. porcinus*, respectively (Table 1). However, no DNA variations were observed among sequences of the rest of the species. Low genetic variation in this case may be explained by the inbreeding because they have been raised in an enclosed zoo. Studies on species identification by *Cyt b* sequence has been reported earlier but the larger amplified DNA fragments of 358 bp (Parson et al., 2000), 402 (Hsieh et al., 2003) and 421 (Gupta et al., 2013) were investigated.

The NJ tree was then constructed to analyze whether these *Cyt b* sequences could determine the species in the Cervidae (Figure 2). The partial sequences of *Cyt b* (263 bp) were compared with those of reference cervids. Noticeably,samples were classified in the same group as their source species except for samples from *C. unicolor* and *C. timorensis* that cannot be differentiated. These results indicated that the *Cyt b* sequences (263 bp) could be used as a marker for most species identification in the Cervidae. Hence, unknown meats from wild food restaurants were determined for their species using FINS analysis on these sequences (Figure 3). Surprisingly, we found that all unknown meats were classified in the same group as wild boar**.** To confirm the results that the unknown meats were not belong to Cervidae, controlled samples of *C. unicolor* samples were also investigated and they were clustered in the same group as *C. unicolor* and *C. timorensis*. Three forensically informative nucleotide variations were observed between unknown meats and *S. scrofa* (Table 3). In addition, BLAST analysis was also performed in order to ensure that our

investigations were correct. We found that the sequences of unknown meats (Unk1-5) were close to wild boar with similarity at 94%, 96%, 96%, 96% and 96%, respectively. These evidences clearly confirm that the analysis of *Cyt b* (263 bp) by the FINS method can identify the source species of meat.

This study has shown that comparison of *Cyt b* partial sequence (263 bp) by the FINS technique is useful for species identification not only for cervids but also other species (if reference sequences are available). Even though, the differentiation between *C. unicolor* and *C. timorensis* cannot be made, this method still has the advantage for wildlife forensic in which it can be used to exclude the irrelevant evidence from consideration. Moreover, as the shorter targeted *Cyt b* had been investigated in this study, DNA analysis for species identification on degraded samples could be achieved (Lopez-Oceja et al., 2016).

CONCLUSION

Comparison of *Cyt b* partial sequence (263 bp) by FINS method can be used for species identification of animals in the Cervidae. However, *C. unicolor* and *C. timorensis* cannot be discriminated by this method. For wildlife forensic application, this study may be useful for preliminary examination to exclude irrelevant evidence.

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