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Research article

A screening assay based on PCR amplification of *Cytochrome b* for species identification of some animals in Cervus

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Abstract

Sambar deer *(Cervus unicolor)* is a protective animal in Thailand. Sambar deer meat has become popular among consumer. Therefore, the smuggling of sambar deer has increased. For the success of prosecution, species identification of meat origin is necessary. An easy-to-perform screening assay for species identification of sambar deer meat is described in this study. The detection of three species of cervids including sambar deer, sika deer *(Cervus nippon)* and rusa deer *(Cervus timorensi)* was achieved by using newly designed primers targeting sambar deer cytochrome b *(Cyt b)* region producing an amplicon of approximately 350 base pairs (bp). The possibility of cross-amplification was prevented by testing with other species of cervids including eld's deer *(Cervus eldi)*, muntjac *(Muntiacus spp.)*, chital deer *(Cervus axis)*, hog deer (Axis porcinus) and popular consumed meats (pork, beef, chicken, seabass). The limit of detection (LOD) on sambar deer DNA was 31.25 pg. Sensitivity of detection of possible sambar deer in mixed meat species was 2%. Suitability of the screening assay was confirmed on processed meats like frozen meats (-20 ºC, -80 ºC) and cooked meats including boiled, steamed, autoclaved, fried, microwave cooked and grilled. Hence, this screening assay can effectively be used for preliminary examination of sambar deer origin in questioned evidence

Keywords: Cervus unicolor, Cytochrome b, Species identification, Wildlife forensic

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INTRODUCTION

In Thailand, six native deer species in Cervidae are found. These species include sambar deer (*Cervus unicolor*), eld's deer (*Cervus eldi*), fea's muntjac (*Muntiacus feae*), northern red muntjac (*Muntiacus vaginalis*), southern red muntjac (*Muntiacus muntjak*), and hog deer (*Axis porcinus*). Sambar deer is a protected animal listed in the wildlife preservation and protection act B.E.2562 (Wildlife Preservation and Protection Act, 2019). As sambar deer meat has become popular among consumer, the rearing of sambar deer meat for meat production is allowed but legal permission for farming must be first approved. Wildlife forensic investigation of the species has rapidly increased as sambar deer are frequently illegally poached for their meat, which is often fraudulently sold at market (Vorajinda et al., 2019). Although a physiological characterization may be adequate to confirm sambar deer identification, in the case of portioned meat or small volumes of animal parts, it is extremely difficult to identify species. Therefore, species identification based on DNA analysis is conducted. The DNA target on mitochondrial DNA (mtDNA) is widely the focus for species identification due to its closed circular structure, small size and high copy number which is more resistant to destruction in the environment than nuclear DNA. Species identification of wildlife animals based on mtDNA gene sequences had been described (Linacre and Tobe, 2011; Poommouang et al., 2022).

The first report of deer species identification from mtDNA was performed by PCR-RFLP analysis of *Cyt b* gene (Matsugana et al., 1998). Later, techniques based on PCR analysis of 16S rDNA (Zha et al., 2011), D-loop (Kim et al., 2012; Parkanyi et al., 2014) were successfully introduced. A method grounded on nucleotide comparison of targeted DNA sequence e.g., D-loop (Wu et al., 2005), *COI* (Kumar et al., 2012) has also been described for deer identification. In addition, the species of the questioned animal can be confirmed by phylogenetic analysis of targeted nucleotide sequence with those of related animal species, known as forensically informative nucleotide sequencing or FINS (Bartlett and Davidson, 1992). Wild animal species have successfully been identified by FINS (Sahajpal and Goyal, 2010; Kitpipit et al., 2013; Singh et al., 2020).

Recently, FINS analysis of *Cyt b* (Vorajinda et al., 2019) and *D-loop* (Munsuwan et al, 2021) were capable of discriminating most animal species in Cervidae. Although this technique could not identify sambar deer, it could be used for preliminary screening in order to exclude irrelevant samples or fraudulent sambar meat. As DNA sequencing is included in FINS, it may be difficult for some laboratories to use this method for sambar deer screening assay. For this reason, a simpler and cheaper screening assay for sambar deer identification would be helpful. This work aimed to investigate a screening method for sambar deer identification based on PCR amplification of *Cyt b* gene using our newly designed primers. Screening assay on meat samples in various conditions is also described in this study.

MATERIALS AND METHODS

Samples

Blood samples from each of sambar deer, rusa deer, sika deer, eld's deer, chital deer, hog deer and Muntiacus spp. used in this study were kindly provided by Khon Kaen Zoo under permission from the Animal Ethics Committee (ACUC-KKU-59/62). Fresh meats including pork, beef, chicken, and seabass were retrieved from fresh food market. Unknown meats were selected from five local wild food restaurants located in five different areas, which are: Pak Chong district, Nakhon Ratchasima province (NR); Wang tong district, Phitsanulok (PL); Cha-am district, Phetchaburi (PB); Kamphaeng Saen district, Nakhon Pathom (NP) and Phuphan district, Sakon Nakhon (SN). The blood samples of cervids and fresh meats (pork, beef, chicken and seabass) will be used for specificity and sensitivity of PCR assays and the detection of deer's DNA in mixed meat. Unknown meats will be used for screening assays for the detection of sambar deer and the robustness of PCR assays on processed and mixed meats.

DNA isolation and quantification

DNA extraction from blood and meat samples was conducted by DNeasy® Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. Briefly, blood (50 µl) or meat (25 mg) samples were incubated with proteinase K at 56ºC. The incubation time for blood sample was 10 min, but can take several hours (1-3 hrs) or overnight for meat samples depending on meat condition. The amount of extracted DNA was subsequently measured based on the absorption at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific).

Primer design

Nucleotide primers were designed based on the Cyt b specific region of sambar deer. Nucleotide sequences of Cyt b gene from seven deer species in Cervidae; rusa deer (AF423200), sambar deer (AF423201), sika deer (D32192), hog deer (DQ379301), axis deer (AY607040), Muntiacus spp. (AF042718), eld's deer (AY157735) retrieved from NCBI were aligned by ClustalW2 program (Larkin et al., 2007). These seven cervid species were chosen based on their habitat in which native (eld's deer, hog deer, sambar deer and Muntiacus spp.) and alien (rusa deer, sika deer and chital deer) species were considered. In addition, rusa and sika deer meats sold in the market are not illegal. The nucleotide regions specific to sambar deer were chosen for primer designation. Primers were designed by OligoAanlyzer 3.1 (Owczarzy et al., 2008). The designed primers were subsequently analyzed to confirm their specificity by Primer-BLAST (Ye et al., 2012).

PCR amplification

The designed primers were tested for PCR amplification of sambar deer DNA. A total volume of 25 µl PCR reaction supplemented with template DNA (1 ng), forward and reverse primers (10 µM each), 2xTaq master mix and deionized water was prepared. The PCR reaction was then placed in a

Thermal Cycler (Bioer Technology) with cycling conditions as follows; initial denaturation 94ºC for 2 min continued with 30 cycles of 95ºC 30 sec, 43ºC 30 sec, 72ºC 30 sec and a final extension at 72ºC for 5 min. Agarose gel electrophoresis was then carried out in order to determine the PCR product of amplification.

Specificity and sensitivity of PCR assays

Specificity or cross-amplification was tested for sambar-deer specific PCR against DNA from both cervids (sambar, rusa, sika, Muntiacus spp., hog, axis, elds) and non-cervids selected from the most commonly consumed meats in the market (beef, pork, chicken, seabass). The PCR reactions were set as described earlier. For the sensitivity, two-fold serial dilutions of sambar deer DNA were prepared starting with 1 ng i.e., 1.0000 ng, 0.5000, 0.2500, 0.1250, 0.0625 and 0.0312 ng. The PCR was performed with diluted template DNA per reaction.

Screening assays for the detection of sambar deer in unknown meats

Five unknown samples (NR, PL, PB, NP, SN) were examined for their source species. DNA extraction and PCR amplifications using our designed primers were conducted using the same condition as mentioned above. The PCR products were subsequently determined by agarose gel electrophoresis. The origin species of sambar deer will be examined by an amplicon with the size of 366 bp.

PCR assays on processed and mixed meat

The robustness of PCR assays was tested with raw, thermally processed, and mixed meat samples. Raw and thermally processed preparations of five unknown meat samples were examined. Fresh and frozen (-20ºC, -80ºC) meats were used as raw meats. Thermally processed meat samples were, boiled, grilled, fried, microwave cooked (600 watts, 5 min), steamed and autoclaved prior to investigation. The mixed meat samples were prepared by mixing either unknown meats NR or SN with the mixture of pork: beef: chicken: seabass (1:1:1:1) at different percentage (2%, 4%, 6%, 8%, 10% w/w). PCR amplification of raw, thermally processed and mixed meat DNA was subsequently performed, using designed primers followed by agarose gel electrophoresis.

RESULTS

DNA isolation and sambar deer specific primers

Mean recovery of DNA from blood samples was 10.10-32.14 ng/µl of blood. For meat samples, raw meats showed higher DNA recovery (22.03-51.80 ng/µl) than that of thermally processed meats (4.72-20.64 ng/µl). The DNA quality of thermally processed meats was also lower than those of raw and frozen meats, as indicated by the fragmented DNA sizes revealed by agarose gel electrophoresis. The sambar deer specific primers were designed based on a specific region of *Cyt b* gene from base

position 686-711 and 1028-1051 (Accession number AF423201) as shown in Figure 1. The nucleotide sequences of forward (Cytb2F) and reverse (Cytb2R) primers are 5'-TCTTAGGCATCTTACTTATAGTACTC-3' and 5'-CAATAATAATAAAGGGGTATTCAA-3', respectively. This pair of primers yielded a sambar deer-specific amplicon of 366 bp in length. Primer-BLAST results showed no primer binding with non-target DNA (Table 1).

Table 1 The parameters of designed primers retrieved from Primer-BLAST results.

Primers	Strand	Length	Tm	GC%	Self complement	Self 3' complement
Cytb2F	Plus	26	55.46	34.62	6.00	4.00
Cytb2R	Minus	24	50.75	25.00	4.00	3.00

Assessment of specificity and sensitivity of PCR assays

The specificity of PCR assays on sambar deer DNA was tested on DNA samples from seven species of cervids and four popularly consumed meats at the market. An amplification of amplicon with approximately 350 bp in length was observed in the PCR assays of DNA samples from sambar, sika and rusa deer (Figure 2). Hence, this PCR assay was not specific to sambar deer as sika and rusa deer samples also showed positive results. However, other samples showed negative results of PCR assays. Therefore, it would be useful to utilize this PCR assay as a screening method for sambar deer detection. The sensitivity of PCR assay was subsequently determined; it was able to detect at least 31.25 pg of sambar deer's DNA (Figure 3).

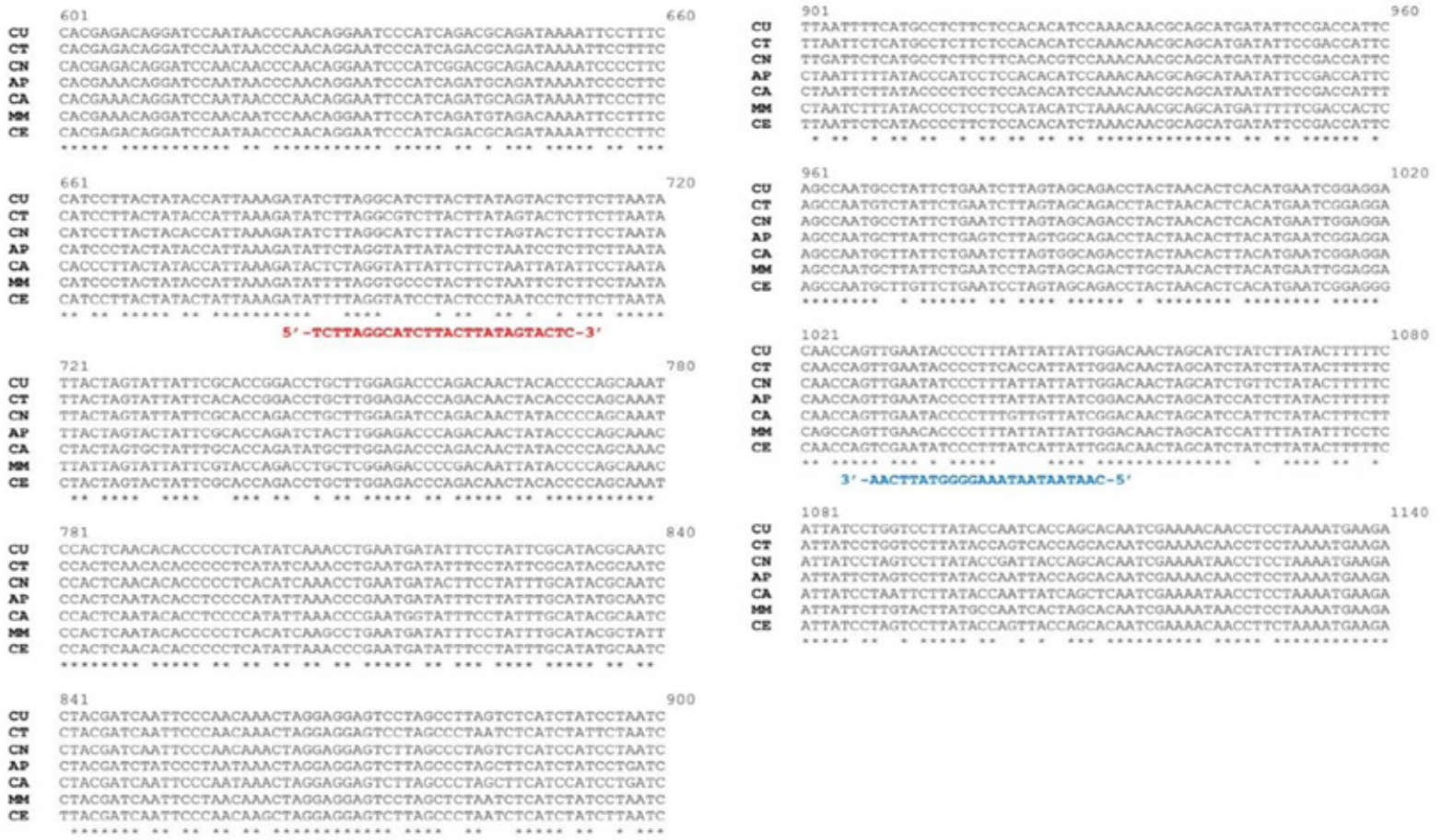

Figure 1 Multiple sequence alignment of the *Cyt b* genes from seven species including sambar deer (CU), rusa deer (CT), sika deer (CN), hog deer (AP), chital deer (CA), *Muntiacus* spp. (MM) and eld's deer (CE). The nucleotide sequences of forward and reverse primers are shown in red and blue. Identical nucleotide is represented by asterisk (*).

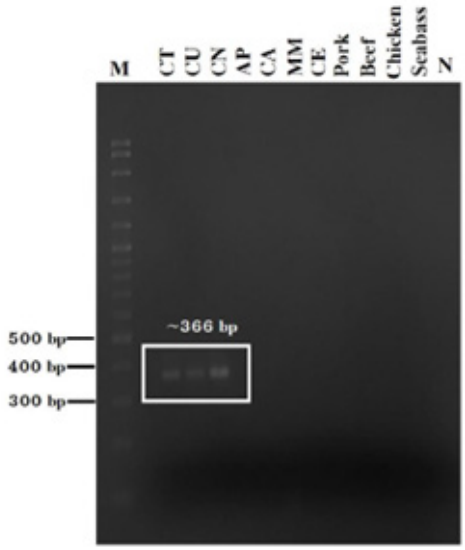

Figure 2 Specificity of PCR assays on the DNA samples of seven cervids species including rusa deer (CT), sambar deer (CU), sika deer (CN), hog deer (AP), chital deer (CA), Muntiacus spp. (MM) and eld's deer (CE) compared with DNA samples from commonly consumed meats (pork, beef, chicken, seabass). Lane M and N represent100 bp DNA ladder and the negative control, respectively.

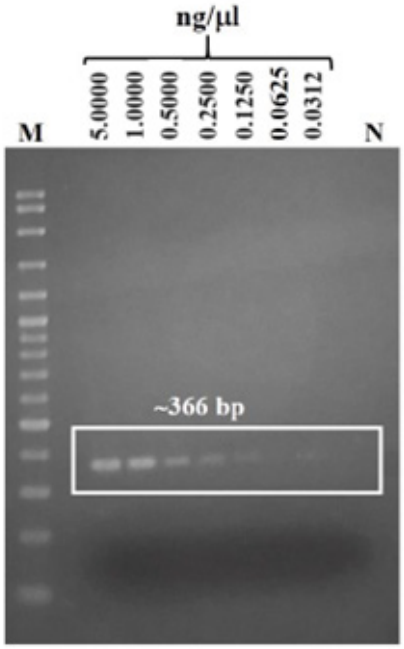

Figure 3 PCR assays on different amounts of sambar deer DNA. The size of PCR products was compared with 100 bp DNA ladder (M). Negative control is presented by N.

Screening assays of unknown meats

The species origin of sambar deer in five unknown meats were preliminarily examined by PCR assay. The results showed that an amplicon sizing around 350 bp was produced in the assay of unknown samples NR, PL, PB and NP (Figure 4). Therefore, it could be assumed that unknown NR, PL, PB and NP may have originated from sambar, rusa or sika deer. On the contrary, unknown SN did not show an amplicon sizing around 350 bp mirroring non-sambar deer origin of meat species.

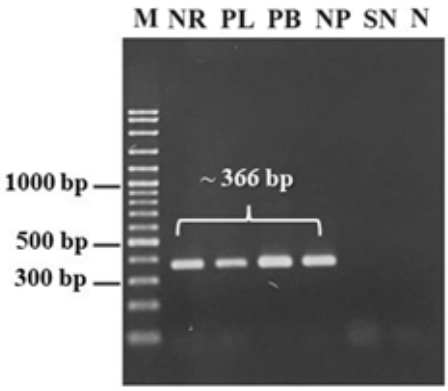

Figure 4 The screening PCR assays for the detection of sambar deer origin in five unknown meat samples (NR, PL, PB, NP and SN). The size marker (M) and negative controls (N) are included.

Effects of processed and mixed meat samples on PCR assays

The capability of PCR assay on the detection of sambar deer species in processed and mixed meat samples was investigated. For processed meats, thermally (boiled, fried, grilled, microwave cooked, steamed, autoclaved) and non-thermally (fresh, frozen) processed unknown meat samples were examined. Interestingly, unknown meat samples (NR, PL, PB and NP) whose origin had previously been detected as possible sambar deer still produced around 350 bp amplicon in all thermally and non-thermally processed meats (Figure 5). These results indicated that PCR assay had a capability to detect possible sambar deer origin in various conditions of meat samples. Despite the absence of PCR product in mixed meat samples of non-sambar deer meat origin (SN), the PCR assays showed the ability to detect the adulteration of possible sambar deer meat in all ranges (2-10%) of unknown NR in the mixture (Figure 6). These results confirmed the efficiency of PCR assays on the detection of possible sambar deer origin in mixed meat samples.

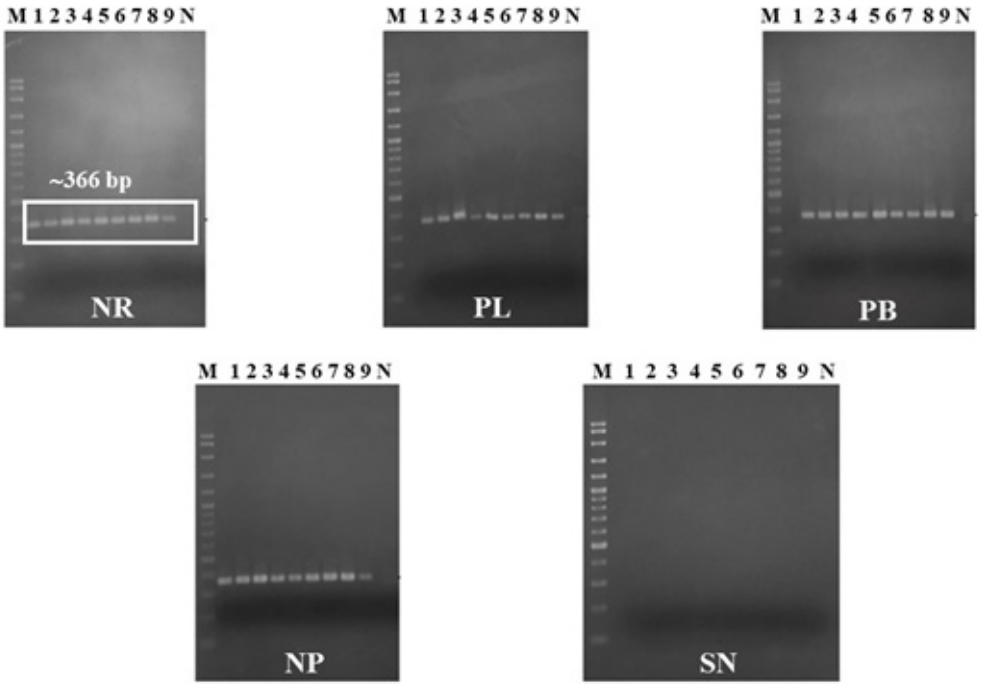

Figure 4 The screening PCR assays for the detection of sambar deer origin in five unknown meats (NR, PL, PB, NP and SN) in various conditions including fresh (1), frozen at -20ºC (2), -80ºC (3), boiled (4), steamed (5), autoclaved (6), fried (7), microwave cooked (8) and grilled (9). Lane M and N are 100 bp DNA ladder and negative control, respectively.

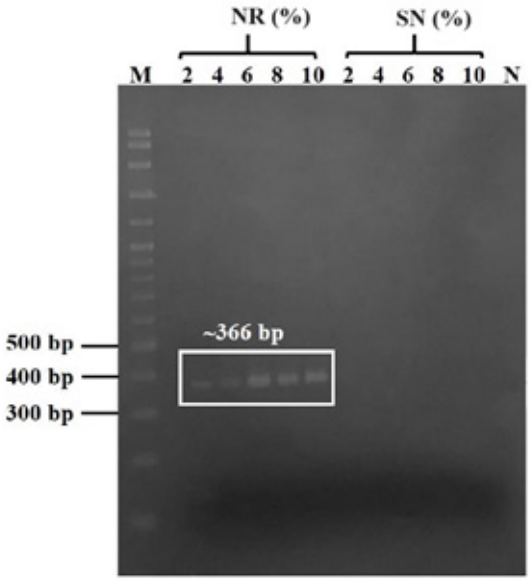

Figure 5 The screening PCR assays for the detection of sambar deer origin in mixed meat samples of unknown NR or unknown SN presented at different percentage (2%, 4%, 6%, 8%, 10% w/w) in a mixture of pork: beef: chicken: seabass (1:1:1:1). Lane M and N are 100 bp DNA ladder and negative control, respectively.

DISCUSSION

This study has described a method of forensic wildlife investigation for the determination of sambar deer species in meat of unknown origin. The *Cyt b* gene was used as a molecular target for this study according to the success of species identification in different varieties of animals by means of *Cyt b* gene (Parson et al., 2000). For the assay, PCR amplification by species-specific primer was selected as the result can be easily interpreted by the presence of amplicon (Rahman et al., 2014). Sambar deer specific primers targeting the *Cyt b* gene were designed based on the specific region of sambar *Cyt b* nucleotide sequence when compared with those of other deer species (sika, rusa, *Muntiacus* spp., elds, hog, chital). The specificity examination of designed primers on both cervid and non-cervid DNA showed that the primers were not specific to sambar deer as they also amplified the DNA templates of rusa and sika deer. Although the PCR conditions were verified, the amplifications of rusa and sika deer's DNA still occurred (data not shown). This could be explained by these three species sharing a close genetic relationship; previous reports have discussed the close genetic relationship between sambar deer and sika deer in which the genetic difference on *Cyt b* between sambar and sika deer was only 1-2% (Nagata et al., 1995) and the genetic distance based on partial *Cyt b* between sambar deer and rusa deer was 0.007 (Vorajinda et al., 2019).

Although the designed primers were not specific to the *Cyt b* of sambar deer, we found that this PCR assay could discriminate sambar, sika and rusa deer from other species. Hence, it will be useful to use this easy-to-perform PCR assay as a screening method to exclude irrelevant samples of non-sambar deer origin. Previous study had reported the case of fraud sambar deer meat (wild boar origin species) in the wild food restaurant (Vorajinda et al., 2019). The method validation of this PCR assay was then introduced. The lowest DNA quantity of possible sambar deer species that could be detected by this PCR assay was 31.25 pg, which was similar to previous reports on the detection of species-specific *Cyt b* gene in which the DNA quantity limit of species detection was 10 pg in rabbit (Santos et al., 2012) and 40 pg in dog (Rahman et al., 2014). Further, the extent of capability of PCR assays on the detection of possible sambar deer origin of meat was examined. Non-thermally processed (fresh, frozen) and thermally processed (boiled, grilled, steamed, autoclaved, microwave cooked) meats of five unknown samples were evaluated. The presence of an amplicon sizing approximately 350 bp had been observed in the PCR assays of non-thermally and thermally processed meat samples of possible sambar deer origin (unknown NR, PL, PB and NP). In contrast, no PCR products were detected in those of meat sample of non-sambar deer origin (unknown SN). These results revealed that this PCR assay had an efficacy on the detection of possible sambar deer origin in thermally processed meat. As *Cyt b* gene is on mitochondrial DNA which is closed circular and high copy, it is little degraded by the environment (Kowalczyk et al., 2021). In addition, the target size of PCR product is smaller than those of previous studies that reported the success of PCR assay on the detection of species origin in thermally processed meat in which the appearance of 404 bp (Karabasanavar et al., 2011) and 513 bp (Mane et al., 2012) amplicons mirrored the meat origin species of sheep and cow, respectively. The advantage of the smaller size of target amplicon for the

species identification is that the highly degraded DNA samples can be analyzed (Spencer et al., 2010).

We investigated the efficiency of PCR assay on the detection of possible sambar deer meat in mixed meat samples. The possible sambar deer meat (unknown NR) or non-sambar deer meat (unknown SN) mixed with commonly consumed meat (pork, beef, chicken, seabass) in different ranges (2-10% w/w) were examined. PCR products were found in all ranges of unknown NR mixed meat samples indicating the capability of this PCR assay to detect possible sambar deer meat in mixed samples. Similar studies of specific PCR assay on targeted species in mixed meat samples showed a detection limit of meat adulterant detection e.g. 0.1% beef in pork (Karabasanavar et al., 2014) and 0.2% mutton in other meat species (Karabasanavar et al., 2011). Additionally, no amplicon was detected in non-sambar deer mixed meat of unknown SN confirming that the primers used in this PCR assay are not specific to DNA from pork, beef, chicken and seabass.

From our study, it can be seen that PCR assay can detect possible sambar deer DNA in various types of meat samples. Although this PCR assay cannot definitively identify the sample as being of sambar origin, it could be used as a screening test as positive results would indicate a sample origin as one of three deer species, and warrant further investigation. In comparison to the nucleotide sequencing based species identification method, this screening assay is simple, easy-to-perform and cost-effective. Therefore, it can be introduced as a first step to determine possible sambar deer origin of questioned evidence before proceeding to the confirmatory identification method. To absolutely identify sambar deer species, the method to differentiate sambar from sika and rusa deer must be highly focused. In this case, further analysis of SNP markers on *Cyt b* should be considered. Several techniques can be introduced for SNP markers analysis such as allele-specific PCR (Hou and Gao, 2019; Zhao et al., 2019), pyrosequencing (Hu et al., 2015), SNapShot (Jiang et al., 2020).

CONCLUSIONS

A screening method for sambar deer identification based on PCR amplification of Cyt b gene has successfully been reported in this study. Possible sambar deer meat origin will produce an amplicon sizing around 350 bp. The limit of detection (LOD) on sambar deer DNA was 50 pg. Sensitivity of detection of possible sambar deer in mixed meat species was 2%. The screening assay is capable of detecting processed meats like frozen meats (-20ºC, -80ºC) and cooked meats including boiled, steamed, autoclaved, fried, microwave cooked and grilled reflecting its efficiency to use as preliminary examination of sambar deer meat origin.

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AUTHOR CONTRIBUTIONS

Preedaporn Surapak: Investigation, Formal analysis.
Chavin Chaisongkram: Conceptualization, Formal analysis.
Khemika Lomthaisong: Conceptualization, Investigation, Formal analysis, Writing, Funding acquisition, Supervision. All authors contributed to manuscript revisions. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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