

# Confirmatory assay for sambar deer species identification by pyrosequencing analysis of SNP markers on cytochrome b gene

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**ABSTRACT:** Sambar deer (*Rusa unicolor*) is a protected species in Thailand. An assay based on PCR amplification of cytochrome b (*Cyt b*) gene has recently been reported for screening to determine sambar deer origin from specimens. As rusa deer (*Rusa timorensis*) and sika deer (*Cervus Nippon*) were also detected, species origin confirmation of sambar deer needs to be further analyzed and refined. Thus, this study aims to differentiate sambar deer from rusa and sika deer through SNPs analysis on partial *Cyt b* gene (366 bp) using pyrosequencing. The amplified *Cyt b* genes from all three species were sequenced and compared to determine the informative SNP markers. The sequencing primer was subsequently designed and used for species identification through pyrosequencing. The results showed that species origin could be accurately determined via four bases. Remarkably, sambar deer origin specimens could be discriminated from those of rusa deer and sika deer. The pyrosequencing assay for species identification of sambar deer is reproducible and robust for use with mixed meat, cooked and uncooked meat samples. In addition, the sambar deer origin of positive samples from screening assay could be indicated by this method, and consequently can be used as a confirmatory assay for sambar deer origin in specimens.

**KEYWORDS:** cytochrome b, species identification, *Rusa unicolor*, pyrosequencing, SNP marker

## INTRODUCTION

Laws involving conserved or protected wildlife require scientific analysis, also known as wildlife forensics, to support law enforcement. Mostly, this focuses on species identification of questioned evidence which can be either animal parts (e.g. horn, ivory, bone, skin, shell) or processed animal products (e.g. traditional medicine, food, carving, fur). To overcome the limitations of morphological based techniques, methods based on DNA analysis have been established for species identification of evidence. The study of wildlife species identification mainly concerns the analysis of DNA markers on mitochondrial DNA, for example cytochrome b (*Cyt b*), NADH dehydrogenase 4 (*ND4*), cytochrome oxidase subunit I (*COI*), D-loop [1]. Among these mitochondrial DNA markers, the *Cyt b* has been most extensively studied and validated for species identification within the family Cervidae, with comprehensive reference sequences available in public databases such as GenBank [2, 3]. The *Cyt b* gene contains informative single nucleotide polymorphisms (SNPs) that enable reliable discrimination between closely related cervid species [4]. Species identification by these DNA markers has successfully been reported in various wildlife [5]. Beyond animal-based evidence, DNA-based species identification has also been applied to plant materials, particularly for protected or endangered species where morphological

identification is insufficient. Chloroplast barcoding markers, including *rbcL* and *matK*, as well as the nuclear internal transcribed spacer (ITS) region, serve as standard molecular tools for plant species identification, analogous to mitochondrial DNA markers used in animal forensics. The application of such an approach has recently been demonstrated for identification of the endangered water-onion (*Crinum thaianum*) in Thailand using DNA barcoding [6].

The family Cervidae, commonly known as deer, belongs to the order Artiodactyla and comprises approximately 55 species distributed across a wide range of habitats, from tropical rainforests to arctic tundra, across Asia, Europe and the Americas. Among the Cervidae sambar (*Rusa unicolor*), sika (*Cervus nippon*) and rusa deer (*Rusa timorensis*) are ecological significant species distributed across Asia and the Indo-Pacific region. These species hold substantial commercial value, with their meat widely consumed as a delicacy and actively traded in both domestic and international markets, however, increasing demand has raised concerns regarding illegal hunting, unsustainable trade, and food fraud. In Thailand, sambar deer is a protected animal listed in the wildlife preservation and protection act, B.E. 2562 (2019). As a result of the popularity of deer meat consumption, this species has been moved to the protected wildlife list for species whose commercial breeding is permitted. However, permission for farming must be initially gained. As

a result, the smuggling of sambar deer has increased. In addition, due to its premium value, incidences of fraudulently selling deer meat mislabeled as sambar have been found. To enforce the law, the means to identify sambar deer species is indispensable. Based on nucleotide sequence comparison, forensically informative nucleotide sequencing (FINS) has been introduced for species identification of sambar deer. This method, utilizing a partial sequence of *Cyt b* [7] and D-loop [8], had a potential to identify most of animals in Cervidae. However, it cannot differentiate sambar deer from rusa and sika deer. As a result, a method based on PCR assay with specific primer has been introduced. Recently, PCR amplification of *Cyt b* gene using sambar deer specific primers served as a screening assay for preliminary examination of sambar deer with origin in question [9]. Therefore, a technique that confirms which of those screening assay with positive results is truly sambar deer, would be helpful.

SNPs are genetic variations of single bases among individuals. In wildlife forensic science, SNP markers have successfully been used for species identification e.g. tiger [10], Mongolian wolf [11], Caucasian tur [12]. SNPs detection can be performed by DNA sequencing and restriction fragment length polymorphism (RFLP). However, these techniques are time consuming. Pyrosequencing is a DNA sequencing method for short stretches of targeted DNA which detects light emitted from pyrophosphate during synthesis, allowing for faster and cheaper analysis compared to traditional DNA sequencing. SNPs detection by pyrosequencing has successfully been described for species identification of specimens in forensic case-work [13, 14]. Hence, to confirm species origin, this study aimed to differentiate sambar deer from rusa and sika deer by detection of SNPs markers on targeted sequence of *Cyt b* gene using pyrosequencing. The reproducibility and robustness of this method was also investigated, gauging its suitability for application in wildlife forensic science as a confirmatory assay for sambar deer species identification.

## MATERIALS AND METHODS

### Samples

Three blood samples from each species: sambar deer (*R. Unicolor*), rusa deer (*R. timorensis*) and sika deer (*C. nippon*), were kindly provided by Khon Kaen Zoo. Unknown meat samples were also retrieved from previous study [9]. These unknown meat samples were taken from local wild food restaurants located in Pak Chong district, Nakhon Ratchasima (Unknown 1); Wang tong district, Phitsanulok (Unknown 2); Cha-am district, Phetchaburi (Unknown 3); Kamphaeng Saen district, Nakhon Phanom (Unknown 4) and Phuphan district, Sakon Nakhon (Unknown 5). This study was carried out with the approval of the Animal Ethics Committee (ACUC-KKU-68/65).

### DNA isolation

DNA was extracted from meat (25 mg) and blood (50  $\mu$ l) samples using the DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol with a final elution volume of 50  $\mu$ l. DNA concentration was determined by UV absorption at 260 nm on a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). Agarose gel electrophoresis (1.5%) was used to examine the integrity of DNA. Prepared DNA samples were diluted to 20 ng/ $\mu$ l and stored at  $-20^{\circ}\text{C}$  for later use.

### Investigation of targeted SNPs

The *Cyt b* genes were amplified from sambar deer, sika deer, and rusa deer using the method described by Suraphak et al [9]. The PCR products were checked and then sent to Macrogen, South Korea for nucleotide sequence analysis. Forward and reverse nucleotide sequences were verified, assembled and aligned using the BioEdit version 7.2.5 program [15]. The species origin of nucleotide sequences was confirmed by BLASTn. These nucleotide sequences were aligned by Clustal Omega [16]. SNP markers for sambar deer species were subsequently examined. For pyrosequencing analysis, the sequencing primer was designed based on the nucleotide sequence beside the targeted SNP.

### Amplification

A 366 bp-fragment of mitochondrial *Cyt b* gene was analyzed. The forward primer was 5'-TCTTAGGCATCTTACTTATAGTACTC-3', and reverse primer was Biotin-5'-CAATAATAATAAAGGGGTATTCA A-3'. A total volume of 25  $\mu$ l PCR reaction containing 20 ng template DNA, 2 $\times$  PyroMark PCR master mix (Qiagen), 10 $\times$  CoralLoad Concentrate (Qiagen), 0.2  $\mu$ M of each primer, and RNase-free water was set up. Amplification was carried out in a Thermal Cycler TC-25H (BIOER Technology, China), at 95  $^{\circ}\text{C}$  for 15 min followed by 50 cycles of 30 s at 94  $^{\circ}\text{C}$ , 30 s at 50  $^{\circ}\text{C}$ , 30 s at 72  $^{\circ}\text{C}$ , with a final extension at 72  $^{\circ}\text{C}$  for 10 min.

### Pyrosequencing assay

Pyrosequencing analysis of amplified *Cyt b* gene (366 bp) from screening assay was performed on PyroMark Q48 Autoprep platform (Qiagen) with PyroMark Q48 advanced reagent kit according to the manufacturer's instructions. The assay and run were created using the PyroMark Q48 Autoprep Software (version 2.4.2). The sequencing primer was 5'-CCAACAACTAGGAGGAGT-3'. Reagents including sequencing primer (10  $\mu$ M), dNTPs, denaturation solution, substrate, enzyme, binding buffer, and annealing buffer were loaded into the appointed injector. The magnetic beads (3  $\mu$ l) biotinylated PCR products (10  $\mu$ l) were pipetted into each well of the PyroMark

**Table 1** Four targeted SNPs on *Cyt b* gene for sambar, rusa, and sika deer identification by pyrosequencing assay. The SNPs variations were confirmed by comparing with the nucleotide sequences of *Cyt b* from three deer species in the NCBI.

Species	Targeted SNPs			
	SNP1	SNP2	SNP3	SNP4
Sambar deer	C	G/A	T/C	A
Rusa deer	T	A	T	A
Sika deer	T	G	C	G

Q48 Disc. The incorporated deoxyribonucleotide during the sequencing was monitored by light emission resulting from pyrophosphate conversion.

**Reproducibility and robustness tests**

For reproducibility, ten meat samples were retrieved from ten individual sambar deer. The meat samples of sambar deer in various conditions including uncooked (frozen meats at  $-4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ ), cooked (boiled, grilled, fried, steamed, autoclaved) and mixed with the mixture of pork:beef:chicken:fish:wild boar (1:1:1:1:1) at different percentages (0.5–10% w/w) were examined for robustness. All the samples were examined using the developed pyrosequencing method.

**Pyrosequencing assay of unknown meat samples**

The species origin of five unknown meat samples (Unknown 1–5) retrieved from previous study [9] were examined by developed pyrosequencing assay. The DNA was first extracted from meat samples and PCR amplification of  $\sim 366$  bp *Cyt b* using screening primer was then performed. Next, the four targeted SNPs on PCR products were examined by developed pyrosequencing assay. Species origins of the unknown meats were revealed by species- specific SNPs.

**RESULTS**

**SNPs for sambar deer identification**

The PCR products of partial *Cyt b* gene (366 bp) amplified from sambar, sika, and rusa deer as shown in Fig. 1A were sequenced. Then, the nucleotide sequences (268–289 bp) were blasted with NCBI database to confirm the species origin. Results showed 100% similarity to partial *Cyt b* gene of sambar deer (FJ556575.1), rusa deer (MF279248.1) and sika deer (AH007723.2) confirming their precise species origins. The nucleotide sequences of partial *Cyt b* gene from sambar, sika, and rusa deer were subsequently compared. Analysis revealed ten SNPs among these sequences (Fig. 1B). The sequencing primers were subsequently designed for pyrosequencing. For the efficacy of pyrosequencing, four SNPs (SNP1–SNP4) next to sequencing primers were targeted for species identification (Table 1). The variations of these four SNPs were also scrutinized with the *Cyt b* sequences of three

**Table 2** Four targeted SNPs on *Cyt b* gene examined by pyrosequencing from ten individual sambar deer meat samples.

Sambar deer sample no.	Targeted SNPs			
	SNP1	SNP2	SNP3	SNP4
1	C	G	C	A
2	C	G	T	A
3	C	G	T	A
4	C	G	C	A
5	C	G	T	A
6	C	G	C	A
7	C	G	C	A
8	C	G	C	A
9	C	G	C	A
10	C	G	C	A

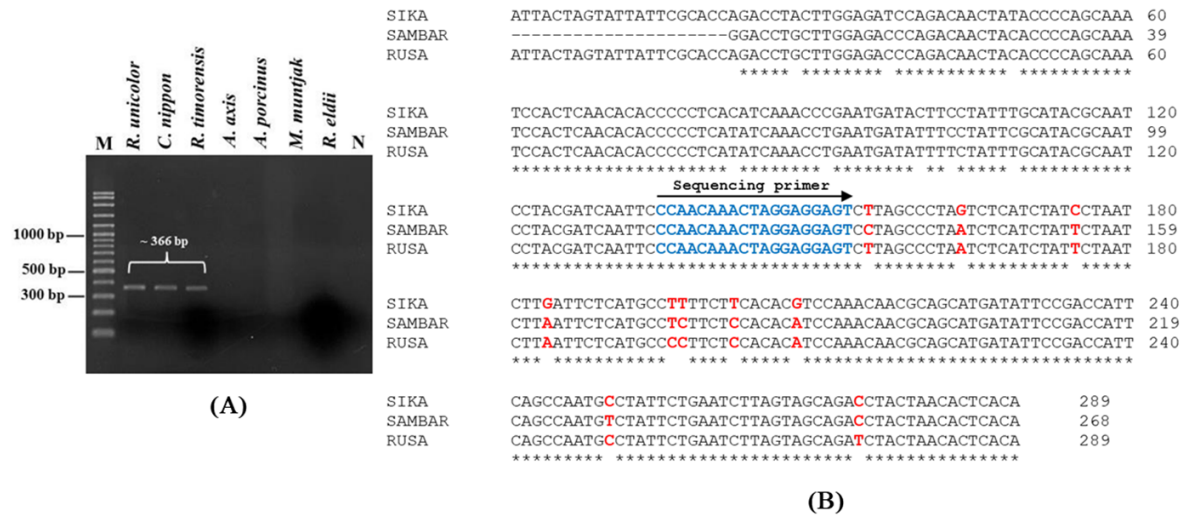
deer species reported in the NCBI database. No SNPs variations were observed except for those of sambar deer, where SNP2 and SNP3 showed major/minor alleles which were G/A and T/C, respectively.

**Pyrosequencing**

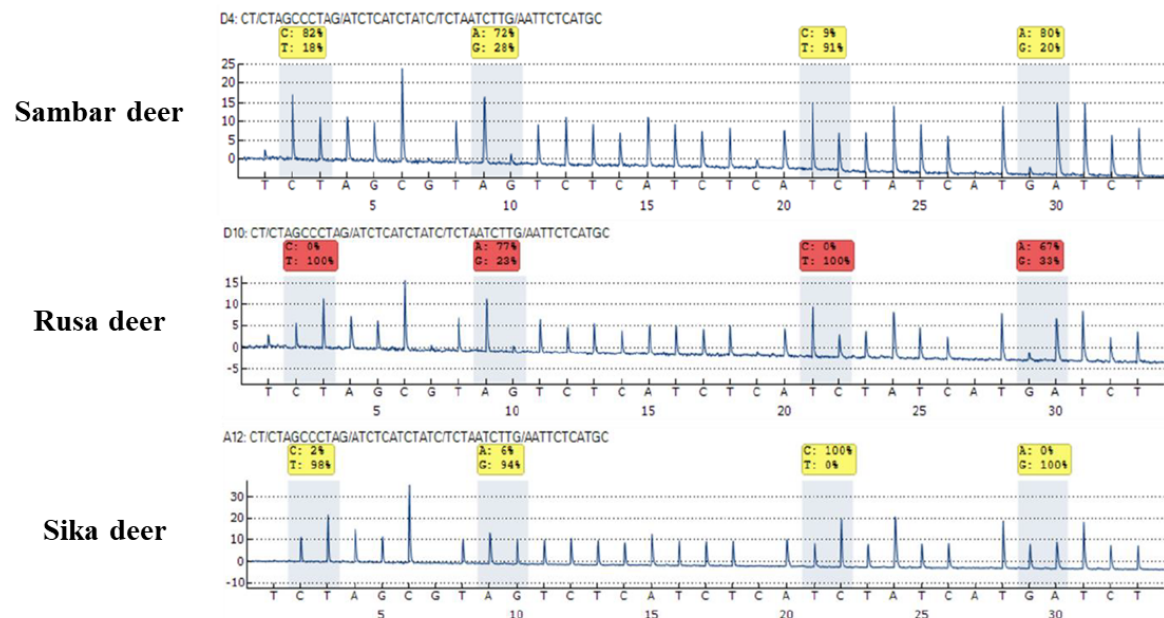
The amplicons of *Cyt b* gene from the screening assay of three deer species were analyzed by pyrosequencing based on four targeted SNPs (SNP1–SNP4) detection using designed sequencing primer. The representing pyrogram from three deer species were shown in Fig. 2. The results showed that the examined targeted SNPs were concordant with their species as indicated in Table 1. From the 5 samples in each species, no variations of SNP1–SNP4 were detected in sika and rusa deer. However, in sambar deer, the variation of SNP3 was detected in which T (60%) was higher than C (40%). Despite the variations of SNP2 shown in Table 1, only a major allele G was detected in sambar deer. These results indicated that pyrosequencing analysis based on these four targeted SNPs enabled identification of the species origin of DNA samples.

**Reproducibility and robustness**

The reproducibility of pyrosequencing assay on species identification of sambar deer was investigated by testing with ten individual sambar deer meats. The expected SNPs of sambar deer species were detected in all samples, resulting in 100% accurate identification (Table 2). This means the pyrosequencing assay is reproducible and would be able to correctly identify sambar deer species. For robustness testing, the developed pyrosequencing assay was used to test sambar deer meats in various conditions including uncooked (frozen meats at  $-4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ ), cooked (boiled, grilled, fried, steamed, autoclaved) and mixed with the mixture of other meats at different percentages (0.5–10% w/w). All meat samples showed expected SNPs for sambar deer species (Table 3), indicating that the developed pyrosequencing assay for sambar deer identification is robust.



**Fig. 1** A screening assay based on PCR amplification of *Cyt b* gene in seven cervids species (A) which are sambar deer (*R. unicolor*), sika deer (*C. Nippon*), rusa deer (*R. timorensis*), chital deer (*Axis axis*), hog deer (*Axis porcinus*), barking deer (*Muntiacus muntjak*) and eld's deer (*Rucervus eldii*). The nucleotide sequences of these amplicons are compared (B). Ten SNPs and designed sequencing primers for pyrosequencing analysis are represented in red and blue, respectively.



**Fig. 2** Representing pyrograms of partial *Cyt b* gene from three deer species. Four targeted SNPs examined by pyrosequencing are shown in colored boxes.

**Species identification of unknown meats**

The species origins of five unknown meat samples were identified by the developed pyrosequencing assay. The results are shown in Table 4. The species origin of unknown meat samples could be revealed by four targeted SNPs. From the SNP markers, sambar deer was the species origin of Unknown 3 and 4. The origin species of Unknown 1 and 2 meat samples were rusa deer. For Unknown 5, as the SNP markers could not be

identified, its species origin was concealed.

**DISCUSSION**

This study aims to identify sambar deer species based on SNP variations on a partial *Cyt b* gene amplified from screening assay [9]. Previous study had shown that three deer species (sambar, rusa, sika) could not be differentiated by amplified products; this is possibly a result of the close genetic relationship

**Table 3** Four targeted SNPs on *Cyt b* gene determined by pyrosequencing from sambar deer meats in various conditions.

Sambar deer meats		Targeted SNPs			
		SNP1	SNP2	SNP3	SNP4
Uncooked	Frozen at -4 °C	C	A	T	A
	Frozen at -20 °C	C	A	T	A
Cooked	Boiled	C	A	T	A
	Grilled	C	A	T	A
	Fried	C	A	T	A
	Steamed	C	A	T	A
	Autoclaved	C	A	T	A
Mixed within the mixture of other meats	0.5%	C	G	C	A
	1%	C	G	C	A
	2%	C	G	C	A
	4%	C	G	C	A
	6%	C	G	C	A
	8%	C	G	C	A
	10%	C	G	C	A

**Table 4** Species origin of unknown meat samples (Unknown 1–5) identified by four targeted SNPs on *Cyt b* gene using pyrosequencing assay.

Unknown meat samples	Targeted SNPs				Identified species
	SNP1	SNP2	SNP3	SNP4	
1	T	A	T	A	Rusa deer
2	T	A	T	A	Rusa deer
3	C	G	C	A	Sambar deer
4	C	G	T	A	Sambar deer
5	N/A	N/A	N/A	N/A	N/A

they share [17]. In addition, supported evidence of evolutionary relationships between sambar and rusa deer [18] or sambar and sika deer [19] have been demonstrated. To fulfill the previous study, the identification of sambar deer species needs to be confirmed. SNP markers on amplified *Cyt b* gene were focused. The amplified *Cyt b* gene (366 bp) from the screening assay of three deer species were sequenced. These nucleotide sequences were blasted, and it showed that all PCR products were from their own species origin, confirming reliability of the screening assay. Next, the nucleotide sequences of sambar deer (268 bp), rusa deer (289 bp), and sika deer (289 bp) were compared. Of ten detected SNPs, four SNPs were targeted considering from the species-specific SNPs and the SNP site close to the conserved region designed for sequencing primer.

The targeted SNPs specific to each deer species were compared with the *Cyt b* sequences in the NCBI to determine the variations of each SNP within their species. No variations of those SNPs were found within rusa and sika deer species. However, variations of SNP2 (G/A) and SNP3 (T/C) were observed within the sambar species. Despite these SNP variations, SNP1

and SNP4 had no variation and was able to differentiate sambar deer from others. Previous study had shown turkey could be differentiated from duck by only single SNP [20]. Additionally, it has been reported that SNP analysis of 12 bases could identify up to 17 animal species [14]. Therefore, pyrosequencing analysis for deer species identification was then developed based on species-specific targeted SNPs of three deer species as follows; sambar deer (C...G/A...T/C...A), rusa deer (T...A...T...A) and sika deer (T...G...C...G).

The developed pyrosequencing assay was introduced to examine four targeted SNPs from the PCR products of three deer species retrieved from the screening assay. The four targeted SNPs of all tested samples were concordant with their own species-specific SNPs, indicating that the developed pyrosequencing assay could identify these deer species based on their SNP markers. However, prior to application for wildlife forensic science, the reproducibility and robustness of the developed pyrosequencing assay need to be studied. Noticeably, the developed pyrosequencing assay was accurate as all sambar deer samples showed four targeted SNP markers concordant to sambar deer species. For robustness, the developed pyrosequencing assay could identify sambar deer origin from a variety of meat samples including uncooked meats (frozen at -4 °C or -20 °C) and cooked meats (boiled, grilled, fried, steamed, autoclaved). The short length of DNA target (33 bp) may possibly explain the success of pyrosequencing assay especially on degraded DNA samples of cooked meats [8]. Pyrosequencing assay was able to identify the species origin of sambar deer in the mixture of meat, indicating the efficacy of this assay.

Five unknown origin of meat samples (Unknown 1–5) from screening assay were subsequently investigated by our developed pyrosequencing assay. Based on the detected SNP markers, the species origin of Unknown 3 and 4 were notably sambar deer while rusa deer was identified as the species origin of Unknown 1 and 2. Interestingly, the pyrogram could not be detected in Unknown 5 sample. This may have resulted from a screening assay in which Unknown 5 had no PCR product, meaning there was no template DNA for pyrosequencing analysis. From these results, the sambar deer species is clearly identified by pyrosequencing assay based on four SNP markers on amplified *Cyt b* gene retrieved from screening assay. Hence, it is feasible to introduce the pyrosequencing assay as a confirmatory method for sambar deer species identification in wildlife forensic science as described by the schematic diagram in Fig. 3.

The pyrosequencing assay developed in this study demonstrated several advantages for application in wildlife forensic science. The method targets short DNA fragments (33 bp), enabling reliable analysis of degraded or processed samples, as evidenced by the successful species identification of cooked meat sam-

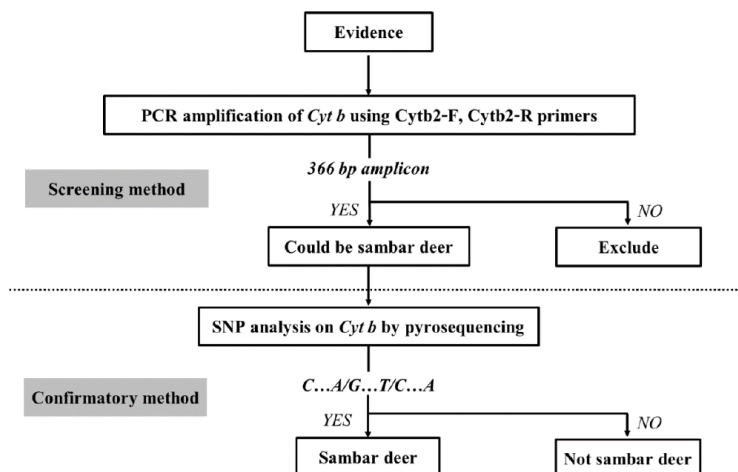


Fig. 3 Schematic diagram of the proposed method for sambar deer identification in wildlife forensic science.

ples in the present study. Nevertheless, this method is restricted to short read length (20–60 bp) and requires prior sequence knowledge for primer design. Compared to automated Sanger sequencing, the gold standard for sequence-based species identification in forensic science [21], pyrosequencing offers practical advantages for routine targeted SNP analysis, including shorter turnaround time and lower cost per sample [22]. Therefore, pyrosequencing is best applied as a rapid and reliable confirmatory assay complementary to existing PCR-based screening methods.

## CONCLUSION

Pyrosequencing assay for determining sambar deer origin was investigated in this study. The *Cyt b* gene from screening assay for species identification of some Cervids (sambar, sika and rusa deer) was targeted. Four bases were identified as species-specific SNPs for sambar deer (C...G/A...T/C...A), rusa deer (T...A...T...A) and sika deer (T...G...C...G). The pyrosequencing method was developed to examine these four bases in samples, successfully determining the sambar origin of the samples. In addition, the developed pyrosequencing assay is reproducible and robust for use with mixed meat, and both cooked and uncooked meat samples. Hence, it is feasible to introduce this method as a confirmatory assay for sambar deer species identification in wildlife forensic science.

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