Original Article

Classification of venomous snakes in Thailand based on the melting temperature specificity of target genes using SYBR Green real-time PCR

Sunutcha Suntrarachun[1*] Tanapong Tawan[1] Lawan Chanhome[1]

Abstract

The SYBR Green real-time PCR was developed for the rapid and accurate classification of major venomous snakes in Thailand. The method relies on melting temperature (Tm) discrimination of target genes from mitochondrial ribosomes. Short single-stranded primers were designed to specifically amplify synthetic DNA of venomous snakes in a single reaction (singleplex), producing distinct melting peaks. Neurotoxic snakes such as cobras (*Naja* spp.), king cobra (*Ophiophagus hannah*) and kraits (*Bungarus* spp.) exhibited melting peaks ranging from 81.00 to 86.00°C, whereas hematotoxic snakes, including the Siamese Russell's viper (*Daboia siamensis*), Malayan pit viper (*Calloselasma rhodostoma*), and green pit vipers (*Trimeresurus* spp.), generated peaks between 77.00 and 80.00°C. The optimal melting rate for singleplex was 0.5°C/sec, with a sensitivity of approximately 10 copies/µL, which is 10-100 times higher than that of agarose gel-based PCR. Primer specificity was confirmed using DNA from snakebite sites on parafilm sheets, demonstrating reliable species-level identification. Multiplex analysis of several snake groups yielded less consistent results due to high genetic similarity among species; only *Naja* spp. could be reliably differentiated from *Bungarus* spp. by applying a slower melting rate of 0.1°C/sec, which improved the resolution of the melting temperature. The coefficient of variation (CV) was less than 5% for both intra- and inter-specific assays, ensuring reproducible outcomes. Overall, SYBR Green real-time PCR provides a sensitive, rapid, and practical tool for distinguishing venomous from non-venomous snakes, supporting accurate identification of snake species from bite wound samples and facilitating appropriate clinical management of snakebite victims.

Keywords: melting temperature, SYBR Green real-time PCR, target gene, venomous snake

[1]*Research and Development, Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand*
****Correspondence**: sunutcha@yahoo.com* (S. Suntrarachun)
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Introduction

Snakebite envenomation remains a neglected tropical disease of global importance, with the World Health Organization (WHO) estimating that 5.4 million people are bitten by snakes each year, resulting in over 140,000 deaths worldwide. Most cases occur in Asia, Africa, and South America, where snakebite is considered a significant public health problem. In Thailand, snakebite incidences are particularly common during seasonal changes and periods of increased agricultural activity, when human encounters with snakes become more frequent. The burden of snakebite is further complicated by the need for accurate and rapid identification of the biting snake species, which directly determines the selection of appropriate antivenom therapy. Failure to promptly recognize the snake species can lead to ineffective treatment, severe complications, or mortality (Vasaruchapong, 2013). The medically significant venomous snakes in Thailand are mainly classified into two families: Elapidae and Viperidae. The family Elapidae includes cobras (*Naja* spp.), the king cobra (*Ophiophagus hannah*), and kraits (*Bungarus* spp.), whose venoms primarily cause neurotoxic effects. The family Viperidae includes the Siamese Russell's viper (*Daboia siamensis*), the Malayan pit viper (*Calloselasma rhodostoma*), and green pit vipers (*Trimeresurus* spp.), which predominantly cause hematotoxic effects by disrupting blood vessel integrity and damaging vital organs. Since clinical symptoms often overlap and bite marks are not always clearly visible, relying solely on clinical diagnosis or patient description is unreliable. This creates a pressing need for a laboratory-based diagnostic method that is both rapid and accurate in identifying the snake species (Pook and McEwing, 2005; Cox *et al*., 2012; Theakston and Laing, 2014).

Traditional diagnostic approaches such as enzyme-linked immunosorbent assays (ELISA) have been employed but suffer from limitations including low sensitivity, lack of reproducibility, and slow turnaround times. In recent years, molecular techniques have offered promising alternatives. In particular, mitochondrial DNA (mtDNA) has been widely used in species identification due to its small genome size, high mutation rate, and abundance in cells. Variations in mitochondrial gene sequences can be exploited to differentiate between closely related snake species (Feng *et al*., 2006; Rastogi *et al*., 2007; Ferri *et al*., 2009; Dubey *et al*., 2011; Singh *et al*., 2012; Ajaz *et al*., 2015).

Real-time polymerase chain reaction (real-time PCR or qPCR) represents a significant advancement over conventional agarose gel-based PCR, enabling simultaneous amplification and quantification of DNA during each cycle. By continuously monitoring fluorescence emitted throughout the reaction, qPCR provides rapid detection as well as precise measurement of target DNA. SYBR Green, an intercalating dye that binds to double-stranded DNA, also enables melting temperature (Tm) analysis, allowing species differentiation based on specific DNA melting characteristics. During amplification, the reaction generates an S-shaped curve (Sigmoidal curve) as the amount of PCR product increases exponentially, and the point at which fluorescence reaches the detection threshold is defined as the cycle threshold (Ct), which reflects the number of DNA amplification cycles required for detectable signal generation. This method is cost-effective, sensitive, and practical for both clinical and laboratory applications (Tobe and Linacre, 2010; Linacre and Tobe, 2011; Fang and Zhang, 2016; Khedkar *et al*., 2016).

In this study, species-specific primers targeting mitochondrial ribosomal RNA genes (12S and 16S) were designed and evaluated using SYBR Green real-time PCR. The objective was to classify major venomous snakes in Thailand by analyzing melting curve profiles to distinguish between neurotoxic and hematotoxic species. The technique was evaluated for sensitivity, specificity, and reproducibility and compared with conventional agarose gel-based PCR. This reliable molecular assay enables rapid and accurate identification of the snake species responsible for envenomation, supporting appropriate antivenom use and improving patient outcomes.

Materials and Methods

Target genes for primer design: To design specific primers for venomous snake identification, the mitochondrial ribosomal RNA genes 12S rRNA and 16S rRNA were selected due to their combination of conserved and variable regions. The primer sequences used in this study were newly designed based on multiple sequence alignment of the target gene regions, using the NCBI accession numbers listed in Table 1, and the final primer sets were generated using Primer3 to ensure optimal specificity and appropriate thermodynamic properties. The specificity of the amplified products was confirmed by the presence of single bands of the expected size in agarose gel-based PCR, together with single melting peaks observed in the SYBR Green real-time PCR melting curve analysis, both of which are indicative of specific and accurate target amplification.

The primer sequences and expected amplicon sizes for neurotoxic and hematotoxic snakes are provided in Table 1. For certain neurotoxic snakes, primer pair SpNeu1216-F and SpNeu1216-R were designed. Additional primer pairs were used to identify specific species: SpNaja-F and SpNeu1216-R for *Naja* spp., including the monocled cobra (*Naja kaouthia*; NK), the Indo-Chinese spitting cobra (*Naja siamensis*; SC), and the golden spitting cobra (*Naja sumatrana*; NSU). SpOph-F and SpNeu1216-R were used to identify the king cobra (*Ophiophagus hannah*; OH), whereas SpBun-F and SpNeu1216-R were applied to detect *Bungarus* spp., including the banded krait (*Bungarus fasciatus*; BF) and the Malayan krait (*Bungarus candidus*; BC). For hematotoxic snakes, SpecHemato1216 primers S1R-F and S2R-R were used. Additional primer sets were designed for specific species: CR-F and S2R-R for the Malayan pit viper (*Calloselasma rhodostoma*; CR), DRS-F and S2R-R for the Siamese Russell's viper (*Daboia siamensis*; DRS), and TATM-F and S2R-R for green pit vipers, including the white-lipped pit viper (*Trimeresurus albolabris*; TA) and the big-eyed pit viper (*Trimeresurus macrops*; TM).

Synthetic DNA fragments: To develop SYBR Green real-time PCR and agarose gel-based PCR, synthetic DNA fragments containing partial sequences of 12S rRNA and 16S rRNA from both neurotoxic and hematotoxic snakes were generated. For neurotoxic snakes, DNA fragments from certain neurotoxic snakes, *Naja* spp., *Ophiophagus hannah* (OH), and *Bungarus* spp., were combined into a single 1,689 bp fragment. For hematotoxic snakes, a synthetic fragment of 1,113 bp was generated, including certain hematotoxic snakes, *Calloselasma rhodostoma* (CR), *Daboia siamensis* (DRS), and *Trimeresurus* spp. (TATM). To ensure reliability, all DNA fragments were synthesized by Integrated DNA Technologies, USA, as standard DNA templates. These synthetic controls were used to verify assay accuracy, confirm purity, and determine DNA copy number based on fragment size (bp) and DNA concentration (ng).

Table 1 Primer sequences and characteristics of 12S rRNA and 16S rRNA genes from neurotoxic and hematotoxic snakes analyzed by agarose gel-based PCR and SYBR Green real-time PCR.

Primer	Sequences (5′ – 3′)	Amplicon size (bp)	Average Tm (°C)	NCBI Accession Number
SpNeu1216 - F	5′ ACATGCAAGCCTCAM(A/C) CACAACAGTG 3′	531	60	NC010225
SpNeu1216 - R	5′ TTGGGTK(G/T) AATCGTGGATTATCG 3′			NC011389
				NC011394
				NC011392
				NC011393
SpNaja - F	5′ TAAAGCACGACTAAAATCAAAY(C/T) AGTC 3′	300	60	LC431744
SpOph - F	5′ GCTCGACCCAGTAAGAGAGTTACCA 3′	385	55	EU921899
SpBun - F	5′ GCATCAGGCTAY(C/T) CGCCCACA 3′	473	55	EU579523
S1R - F	5′ CAGGAGTAATTAACATTAGGCCAT 3′	390	55	NC022820
S2R - R	5′ CGGTTATTR(A/G) GACAGGCTCCTCTA 3′			
CR - F	5′ GACACAATAACCACCACACCTAAC 3′	202	55	AF057190
DRS - F	5′ CAACCCTTAGGATAGAAACCAACC 3′	247	55	EU913478
TATM - F	5′ CGGCGTAAAAATGACTAAAACTAAGTTA 3′	274	55	KF311102

The standard curve: The number of copies of the synthetic DNA template (1,689 bp and 1,113 bp) was calculated from a 500-ng preparation. Standard DNA samples were then serially diluted ten-fold, from 10^8 to 10^0 copies, to generate a dilution series. These samples were analyzed using SYBR Green real-time PCR to establish a standard curve by plotting the Ct values against the known DNA concentrations (Fig. 1). This curve was used to evaluate amplification efficiency and assay accuracy. The coefficient of determination (R^2) was then calculated to confirm the reliability of DNA quantification for each group of venomous snakes.

Agarose gel-based PCR: The singleplex PCR reaction contained synthetic DNA templates (10^8-10^0 copies), 1,000 nM of each primer, 5 mM $MgCl_2$, and 2X Taq Master mix (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia). Reactions were performed on a thermocycler (MWG Biotech, USA) with the following program: 94°C for 3 mins; 94°C for 1 min, 50°C for 1 min, and 72°C for 3 mins for 35 cycles; followed by a final extension at 72°C for 7 mins. The same PCR conditions were applied for multiplex, duplex, and triplex PCR reactions to allow simultaneous analysis. Primer concentrations (100-1000 nM) and $MgCl_2$ concentrations (3-5 mM) were optimized to improve amplification. Annealing temperatures (50-60°C) and times (1-4 mins) were also tested for specific bands. Final products were analyzed using 2% agarose gel electrophoresis in 1XTAE buffer with ethidium bromide staining and appropriate molecular size markers.

SYBR Green real-time PCR: Target genes from different groups of venomous snakes were analyzed using SYBR Green real-time PCR with a CFX 96 Touch™ Real-Time PCR with iTaq Universal SYBR Green Supermix (Biorad, CA, USA). The target genes of each snake group were evaluated concurrently in a single reaction using the same primer set, as in agarose gel-based PCR testing, although the primer concentrations could vary. Each 20 µL reaction contained 2X iTaq universal SYBR Green supermix, 600 nM of each primer, the synthetic DNA template, and nuclease-free water. The reaction conditions were as follows: an initial denaturation at 95°C for 3 mins, followed by 40 cycles of denaturation at 95°C for 5 secs and annealing/extension at 62°C for 30 secs. Gradient PCR was performed with annealing/extension temperatures ranging from 60°C to 65°C to determine the optimal primer temperature. After amplification, a melting curve analysis was carried out to verify PCR specificity. The melting curve was generated by gradually increasing the temperature from 65°C to 95°C at 0.5°C increments every 5 secs. A distinct melting peak indicated a specific PCR product. The same PCR settings were used for multiplex, duplex and triplex, SYBR Green real-time PCR. Primer concentrations ranging from 100 to 1000 nM and increments from 0.1 to 0.5°C were used to optimize the conditions.

Sensitivity, specificity, intra-assay (repeatability), and inter-assay (reproducibility): The sensitivity of the SYBR Green real-time PCR and agarose gel-based PCR was assessed by comparing known concentrations of serially ten-fold diluted synthetic DNA, ranging from 10^8-10^0 copies/µL. Each procedure was performed in triplicate, and the limit of detection was determined. A variety of venomous and non-venomous snakes were used to evaluate specificity.

To verify the precision and repeatability of the SYBR Green real-time PCR assays, the intra-assay precision (repeatability) was evaluated in three runs. Inter-assay variation (reproducibility) was examined

across three independent experimental runs conducted over five days.

Assessment of the efficacy of the SYBR Green real-time PCR: At QSMI, Thai Red Cross Society, venom collection was performed by the voluntary injection of venom into a container covered with a parafilm sheet. According to the manufacturer's recommendations, the bite-marked parafilm sheet was then rinsed with sterile water, and DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). DNA concentration and purity were determined by measuring absorbance at 260 and 280 nm. The acceptable purity ratio (A260/A280) ranged from 1.8 to 2.0. DNA concentration in ng/µL was calculated using the formula OD260 × dilution factor × 50. As described previously, a SYBR Green real-time PCR experiment was also conducted.

Results

Agarose gel-based PCR: For singleplex PCR, the optimal conditions included an annealing temperature of 50°C, 5 mM $MgCl_2$, and 1,000 nM of each primer. For multiplex PCR, the best parameters were 4 mM $MgCl_2$, an annealing temperature of 50°C for 1 min, 300 or 600 nM for the forward primers, and 900 nM for the reverse primers. The detection range of PCR products was from 10^8-10^0 copies/µL, as visualized on a 2% agarose gel electrophoresis. The minimal detection limits were as follows: 10^3 copies/µL for certain neurotoxic snakes, 10^3 copies/µL for *Naja* spp., 10^4 copies/µL for *Ophiophagus hannah* (OH), 10^4 copies/µL for *Bungarus* spp., 10^3 copies/µL for certain hematotoxic snakes, 10^2 copies/µL for *Calloselasma rhodostoma* (CR), 10^4 copies/µL for *Daboia siamensis* (DRS), and 10^3 copies/µL for *Trimeresurus* spp. (TATM), respectively (Fig. 2). However, due to the similarity in amplicon sizes, distinguishing between multiplex, duplex, and triplex PCR products was challenging (Fig. 3).

SYBR Green real-time PCR: When primers were tested with synthetic DNA and agarose gel-based PCR, they were found to be species-specific for each venomous snake and capable of detecting DNA of the expected size. To further determine the specificity of the Tm for the target gene, the same primer sets were evaluated using the SYBR Green real-time PCR. During each reaction cycle, the relative fluorescence intensity (Relative Fluorescence Unit: RFU), which reflected the accumulation of amplified DNA, was measured. The amplification profiles appeared as S-shaped curves, characteristic of exponential DNA amplification. Results were expressed as Ct values, indicating the cycle at which the target DNA was detected, along with Tm values specific to each target gene. The melting temperatures of the synthesized DNA for different snake groups were as follows: *Naja* spp. (NK, SC, and NSU) showed a Tm of 81.50 ± 0.5°C; OH, 84.50 ± 0.5°C; and *Bungarus* spp. (BF and BC), 85.50 ± 1.0°C. The Siamese Russell's viper (DRS) had a Tm of 77.00 ± 0.5°C, while the Malayan pit viper (CR) and *Trimeresurus* spp. (TATM) showed Tm values of 78.00 ± 0.5°C and 79.00 ± 0.5°C, respectively (Figs. 4A to 4F). A duplex SYBR Green real-time PCR revealed melting peaks at 80.00 ± 1.0°C and 85.00 ± 0.5°C, indicating that only *Naja* spp. and *Bungarus* spp. could be clearly separated and identified (Fig. 5).

Sensitivity, specificity, intra-assay (repeatability), and inter-assay (reproducibility) of SYBR Green real-time PCR: Serial ten-fold dilutions of synthetic DNA templates, ranging from 10^8 to 10^1 copies, were used to evaluate the sensitivity, specificity, intra-assay (repeatability), and inter-assay (reproducibility) of SYBR Green real-time PCR. At a concentration of 10^1 copies/µL, 100% of hematotoxic and neurotoxic snake samples were successfully detected (Fig. 6). The coefficient of variation (CV) of the Ct values in both intra- and inter-assay experiments was calculated using three distinct concentrations of synthetic DNA templates. For intra-assay repeatability, each concentration was tested in triplicate within a single run, while inter-assay reproducibility was assessed across three independent runs over five days. The results are summarized in Table 2. Based on the Tm specificity of the target genes, SYBR Green real-time PCR proved highly reliable for classifying and identifying venomous snakes in Thailand. The mean CV of Ct values was less than 5% for both intra-assay repeatability and inter-assay reproducibility.

Assessment of the efficacy of the SYBR Green real-time PCR: As a model for snakebite wounds on human skin, DNA samples obtained from venomous snakebite marks on parafilm sheets were used in the venom extraction process. All primer sets successfully detected DNA from the parafilm sheets. This may be attributed to the relatively high initial DNA concentration from the bite wounds, measured at 10–50 ng/µL, which was sufficient for classifying groups of venomous snakes using group-specific primers, such as those designed for *Naja* spp. (NK and SC) (Fig. 7A). For *Trimeresurus* spp., only the white-lipped viper (TA) and big-eyed pit viper (TM) were identified (Fig. 7B). The SYBR Green real-time PCR demonstrated high specificity when primers were designed for each venomous snake group. However, when applied to other venomous snakes or non-venomous snake samples, the specificity decreased.

Table 2 Intra-specificity and inter-specificity assays of the SYBR Green real-time PCR. The assays were used to evaluate repeatability within runs (intra-specific assay) and reproducibility between runs (inter-specific assay).

Primer	Sequences (5′ – 3′)			Species	Inter-specific assay (Reproducibility)		
	Tm values of amplicon	Mean	CV%		Tm values of amplicon	Mean	CV%
Naja spp.	78.50, 78.66, 78.11	78.42	0.32	*Naja* spp,	78.50, 78.66, 78.11, 78.20, 78.05	78.30	0.32
Ophiophagus hannah (OH)	81.10, 81.53, 82.00	81.54	0.49	*Ophiophagus hannah* (OH)	81.10, 81.53, 82.05, 81.95, 81.67	81.66	0.43
Bungarus spp.	82.35, 82.79, 82.00	82.38	0.42	*Bungarus* spp.	82.35, 82.79, 82.00, 81.98, 82.65	82.35	0.43
Daboia siamensis (DRS)	77.15, 77.68, 77.95	77.59	0.47	*Daboia siamensis* (DRS)	77.15, 77.68, 77.95, 78.06, 77.28	77.62	0.49
Calloselasma rhodostoma (CR)	78.59, 78.24, 79.03	78.62	0.45	*Calloselasma rhodostoma* (CR)	78.59, 78.24, 79.03, 78.68, 78.26	78.56	0.39
Trimeresurus spp. (TATM)	79.38, 79.10, 78.98	79.15	0.23	*Trimeresurus* spp. (TATM)	79.38, 79.10, 78.98, 78.68, 78.29	78.89	0.50

CV% = (Standard deviation/mean) × 100; Tm: Melting temperature.

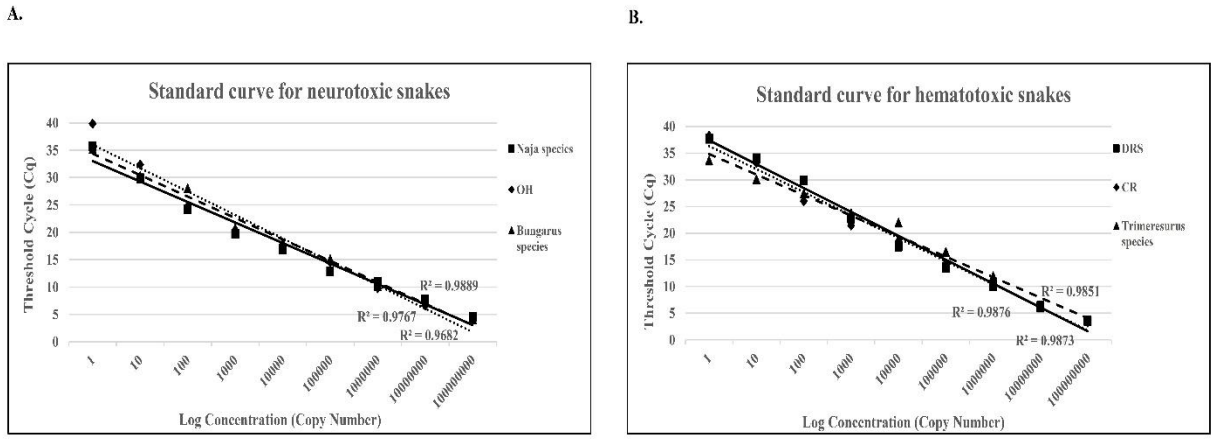

Figure 1 Standard curves for neurotoxic (A) and hematotoxic (B) snakes. A linear relationship was observed between the threshold cycle (Ct) and serially diluted in vitro DNA containing primer-targeting sites (100 to 108 copies/µL). The SYBR Green real-time PCR amplification efficiencies were as follows: *Naja* spp.: 98.89%, OH: 97.67%, *Bungarus* spp.: 96.82%, DRS: 98.51%, CR: 98.76% and *Trimeresurus* spp.: 98.73%.

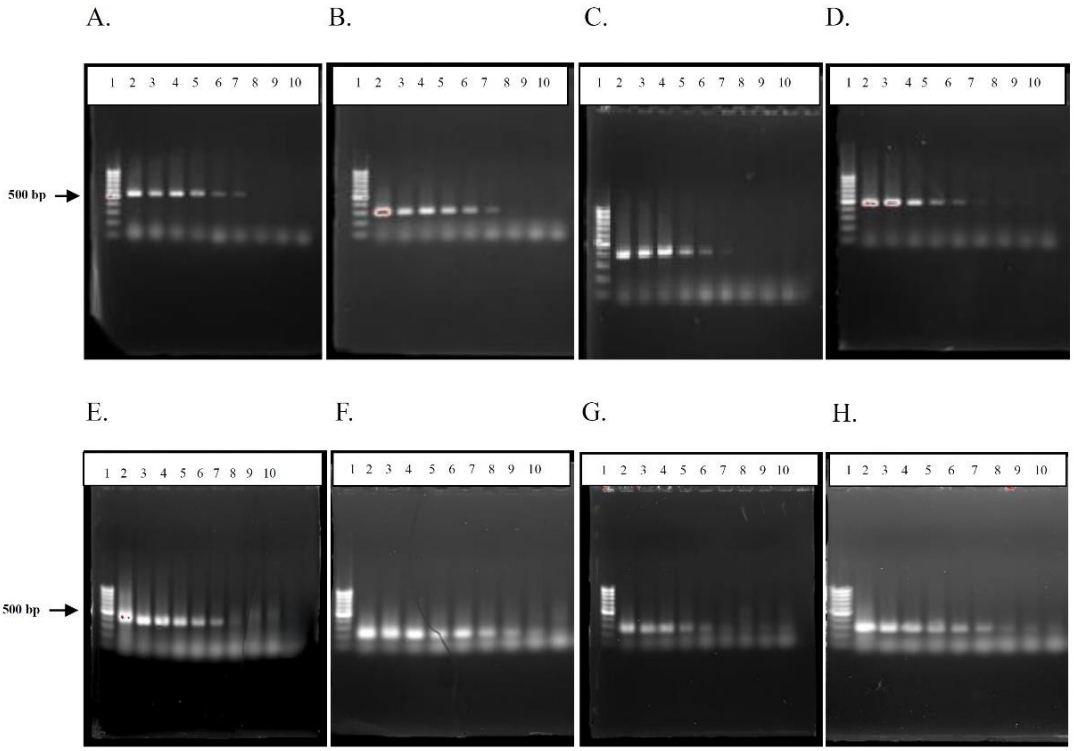

Figure 2 PCR products of neurotoxic and hematotoxic snakes obtained from 10-fold serial dilutions of the DNA fragment, visualized on 2% agarose gel electrophoresis. A: Neurotoxic snakes; 531 bp, B: *Naja* spp.; 300 bp, C: OH; 385 bp, D: *Bungarus* spp.; 473 bp, E: Hematotoxic snakes; 390 bp, F: CR species; 202 bp, G: DRS; 247 bp, and H: TATM; 274 bp. Lane 1: GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific, Sweden); Lane 2-10: Minimal detection limits for neurotoxic and hematotoxic snakes ranging from 10^8 to 10^0 copies/µL.

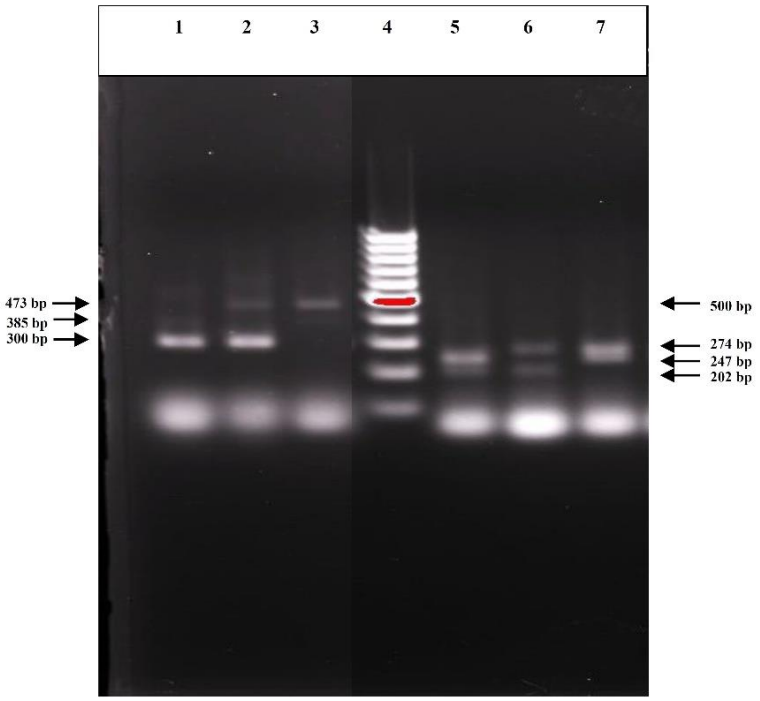

Figure 3 Multiplex PCR of neurotoxic and hematotoxic snake species analyzed on 2% agarose gel electrophoresis. Lane1: *Naja* spp.; 300 bp and OH; 385 bp, lane 2: *Naja* spp.; 300 bp and *Bungarus* spp.; 473 bp, lane 3: OH; 385 bp and *Bungarus* spp.; 475 bp, lane 4: 100 bp MW marker, lane 5: CR; 202 bp and DRS; 247 bp, lane 6: CR; 202 bp and TATM; 274 bp, lane 7: DRS; 247 bp and TATM; 274 bp.

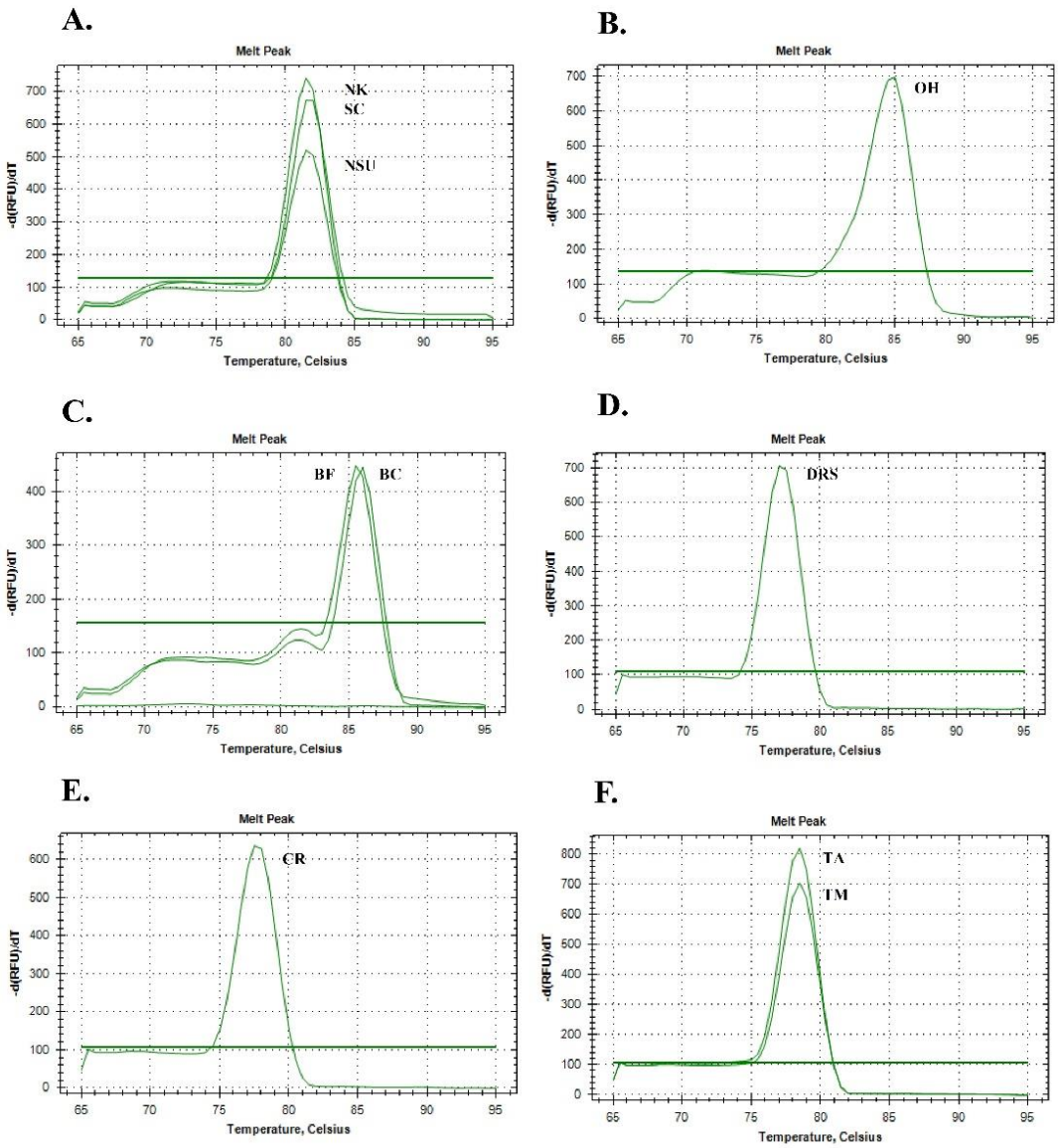

Figure 4 Specific melting temperatures of target genes from different snake group determined by singleplex SYBR Green real-time PCR. The melting temperatures (Tm) were as follows: *Naja* spp. (NK, SC, and NSU): 81.50 ± 0.5°C (A.), *Ophiophagus hannah* (OH): 84.50 ± 0.5°C (B.), *Bungarus* spp. (BF and BC): 85.50 ± 1.0°C (C.), *Daboia siamensis* (DRS): 77.00 ± 0.5°C (D.), *Calloselasma rhodostoma* (CR): 78.00 ± 0.5°C (E.), and *Trimeresurus* spp. (TATM): 79.00 ± 0.5°C (F.).

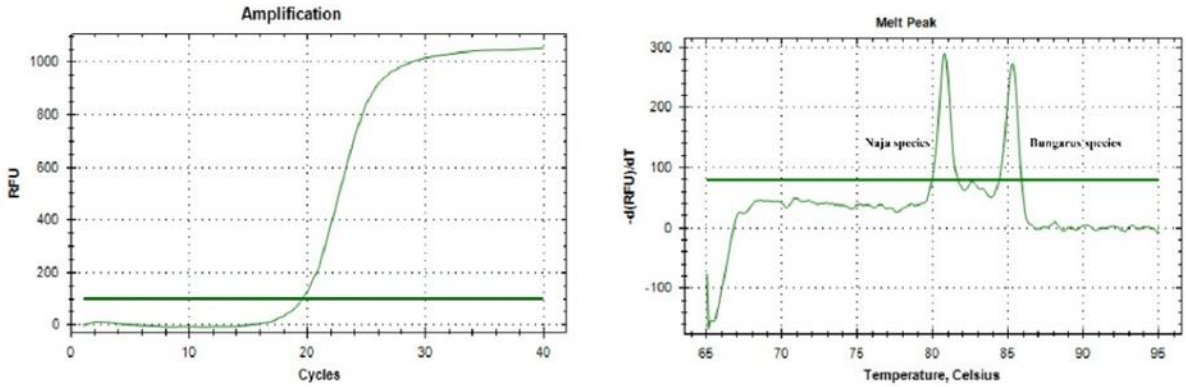

Figure 5 Multiplex (duplex) SYBR Green real-time PCR for the *Naja* spp. (NK, SC, and NSU) and the *Bungarus* spp. (BF and BC), showing distinct melting temperatures of 80.00 ± 1.0°C and 85.00±0.5°C, respectively.

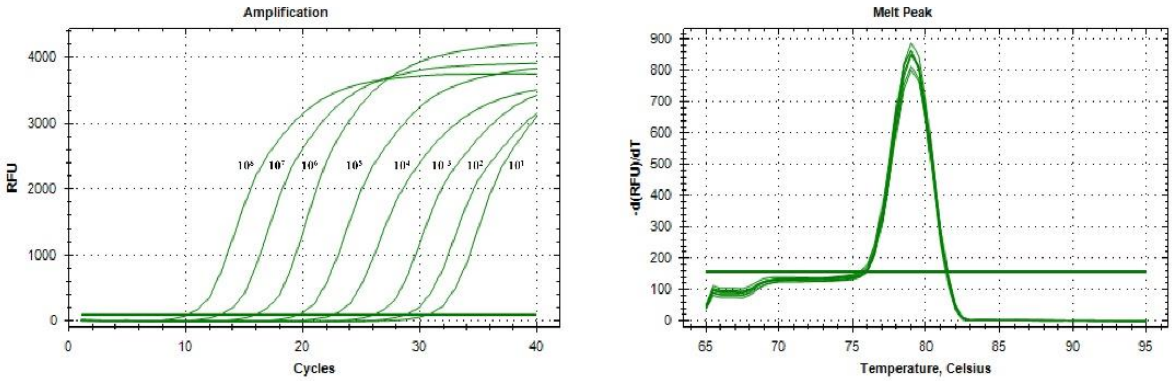

Figure 6 Amplification curves and specific melting temperature obtained from serial ten-fold dilutions (10^8 to 10^1 copies/µL) of synthetic DNA targeting the gene of *Trimeresurus* spp.

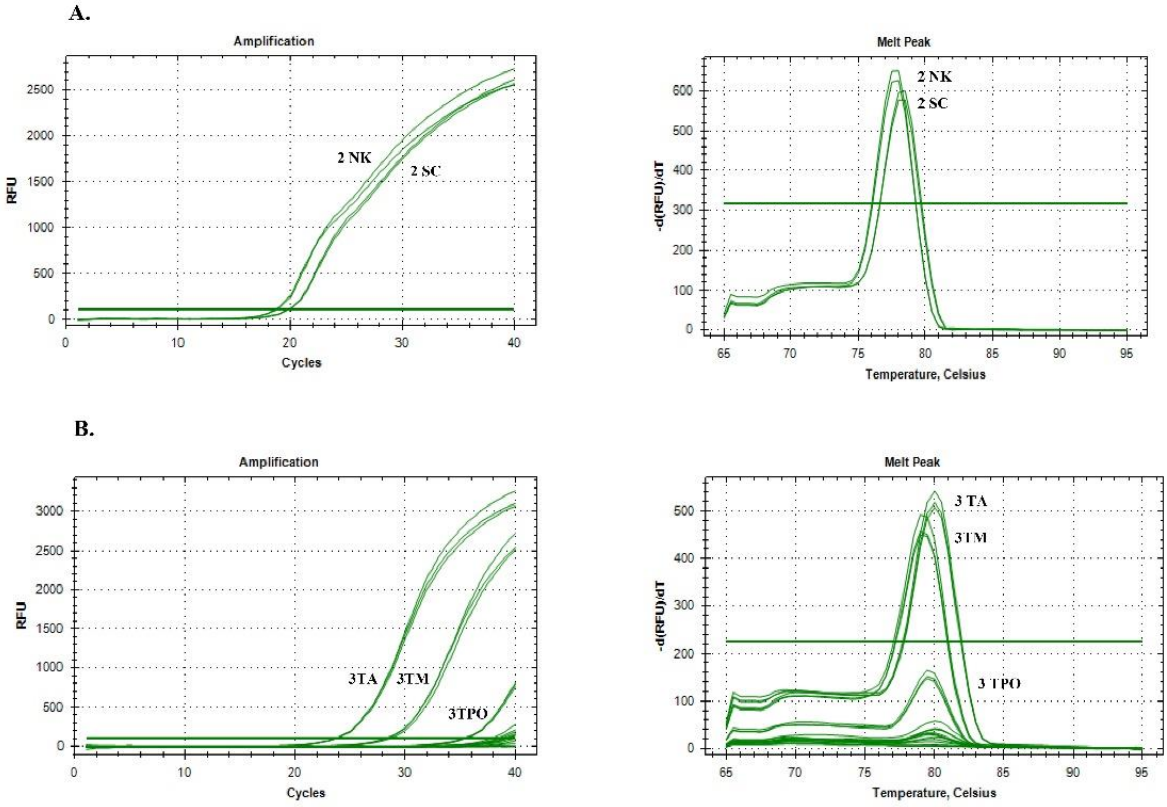

Figure 7 Parafilm sheets used as a model for snakebite wounds on human skin, analyzed by SYBR Green real-time PCR for detecting different groups of venomous snakes. Parafilm samples included *Naja* spp. (NK: 2 samples; SC: 2 samples) (A), and *Trimeresurus* spp. (3 samples each), including *Trimeresurus albolabris* (TA), *Trimeresurus macrops* (TM), *Trimeresurus popeiorum* (TPO), other *Trimeresurus* spp. such as the *Trimeresurus albolabris* complex (TAX), *Trimeresurus gumprechti* (TGU), *Ovaphis monticola* (OM), and *Protobothrops kelomohy* (PKE) (B).

Discussion

This study developed SYBR Green real-time PCR and agarose gel-based PCR assays targeting the mitochondrial 12S rRNA and 16S rRNA genes for identifying medically important venomous snakes in Thailand. These mitochondrial regions contain both conserved and variable domains, which have been widely recognized as effective markers for phylogenetic studies and species discrimination in reptiles. Consistent with previous reports (Powell *et al*., 2006; Rastogi *et al*., 2007; Pyron *et al*., 2013), the present findings demonstrate that mitochondrial rRNA genes provide sufficient interspecific variability for designing genus- or species-specific primers. Moreover, the melting curve profiles generated in this study further confirm that these regions remain highly informative for distinguishing neurotoxic and hematotoxic snakes.

Methodologically, this study builds upon earlier agarose gel-based PCR approaches for snake identification, which, although effective, require post-PCR electrophoresis, increase contamination risk, and prolong turnaround time (Suntrarachun *et al*., 2001).

The incorporation of SYBR Green real-time PCR overcomes these limitations by enabling closed-tube detection with improved sensitivity. Compared with DNA barcoding methods that utilize mitochondrial sequences and rely on sequencing and database comparison (Supikamolseni *et al*., 2015), the present approach allows more rapid species discrimination without post-PCR processing, making it more suitable for clinical application. Consistent with previous findings that melting curve analysis can differentiate closely related species when sufficient sequence divergence exists (Sharma *et al*., 2016), distinct and reproducible melting peaks were observed for Naja and Bungarus species in this study. However, species with high genetic similarity, such as Malayan pit viper and green pit vipers, exhibited closely aligned melting temperatures, highlighting the limitations of SYBR Green assays when multiple targets are analyzed simultaneously.

From a clinical perspective, the ability to rapidly distinguish between neurotoxic and hematotoxic snakes has important implications for snakebite management in Thailand. Neurotoxic envenomation requires immediate respiratory monitoring and administration of neurotoxic antivenom, whereas hematotoxic envenomation necessitates early assessment of coagulation abnormalities and timely hematotoxic antivenom administration. Although early species identification is critical for reducing morbidity and mortality, many diagnostic tools remain impractical in rural healthcare settings. The SYBR Green real-time PCR assay developed in this study provides a rapid, accurate, and accessible molecular tool that can support clinical decision-making in regional hospitals, where snakebite cases are most prevalent.

Despite its advantages, several limitations should be acknowledged. First, multiplexing capacity remains constrained by the genetic similarity among some species, limiting simultaneous multi-target detection within a single reaction. Second, while synthetic DNA fragments were used for assay development, further validation using clinical specimens, venom samples, or field-derived DNA is necessary to confirm performance under real-world conditions. Lastly, melting curve analysis alone, although effective for several species, may not provide sufficient resolution for species with minimal sequence divergence, suggesting that additional markers or probe-based assays may be required for comprehensive identification.

In summary, this study demonstrates that SYBR Green real-time PCR is a practical and effective approach for identifying venomous snake species in Thailand. The assay exhibited high sensitivity, specificity, and reproducibility, enabling clear distinction between neurotoxic and hematotoxic snakes based on characteristic melting temperature profiles. Although duplex SYBR Green real-time PCR was feasible for certain species such as Naja and Bungarus, limitations remained for species with closely related genetic sequences. Nevertheless, the method provides rapid and reliable results, supporting its potential application in diagnostic and epidemiological settings and its contribution to improved snakebite management and clinical outcomes.

Conflicts of interest: The authors have no conflicts of interest to declare.

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