Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (1):** 133-138 (2014)

Research Article

International Journal of Pure & Applied Bioscience

Molecular identification of venomous snakes in Thailand using PCR-RFLP

Sunutcha Suntrarachun[1]*, Lawan Chanhome[1], Wichit Thaweekarn[1], and Montri Sumontha[2]
[1]Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok 10330, Thailand
[2]Ranong Marine Fisheries Station, Ranong 85000, Thailand
*Corresponding Author E-mail: sunutcha@yahoo.com

ABSTRACT

A method for species identification of venomous snakes has been developed on the basis of amplification of a specific part of the mitochondrial cytochrome b gene using PCR. To distinguish among venomous species, the obtained 1,144 bp PCR products were cut with different restriction endonuclease, Alu I and Hinf I, resulting in species-specific restriction fragment length polymorphism (RFLP). Each enzyme generated different-sized fragments which specific to the six neurotoxic and six hematotoxic snake species in Thailand. However, identical patterns were found between Daboia siamensis and Trimeresurus popeorum among hematotoxic snakes when Alu I has been used. These results could be resolved by using additional enzyme such as Hinf I due to no cross reaction between the species was detected in their restriction patterns. The PCR-RFLP developed here is simple, rapid, reliable and reproducible; hence it can be routinely applied for snake species identification, essential for conservation and management of endangered snake species in Thailand.

Keywords: *PCR-RFLP, cytochrome b, neurotoxic, and hematotoxic snakes.*

INTRODUCTION

Snakes are a group of reptiles with many derived morphological characters related to their peculiar behavior and life style. The traditional classification of snakes based on morphological and histological characteristics divided snakes into three major groups: The Scolecophidia (blindsnakes), the Henophidia (primitive snakes), and the Caenophidia (advanced snakes). The majority of snakes are Caenophidians which include many harmless forms and all known venomous species. These advanced snakes include three major groups: (1) the colubrids, typical harmless species eg. rat snakes and racers, (2) the elapids, front-fanged species with neurotoxic venom eg. cobras and coral snakes, and (3) the viperids, species with movable front fangs and hemotoxic venom eg. vipers and rattlesnakes[1]. However, snakes possess adaptational characters in various respects of their morphology, which may mislead taxonomic classification to reconstruct incorrect or unresolved phylogeny. Illegal trade in snake products, especially skin and venom, has increased manifold in recent years. A large number of snakes, such as Indian cobra, are killed in India for skin and venom. The enforcement agencies fail to implement the law because it is difficult to identify the species from the specimen[2]. Therefore, reliable labeling of products offered for sale is important in order to assure the consumers about the identity and quality of the food products they consume for a variety of reasons including adulteration and socio-religious factors. Due to an increase in the consumption demand and high cost, other cheaper animals are being used as fraudulent substitutes or mislabeled[3,4].

The need for accurate and reliable techniques for animal species identification has been increased dramatically during the past few years[5,6]. Species identification based on protein analysis had been widely used and regarded as a reliable method. However, it is difficult to identify the species origin in processed products since the proteins are denatured and morphological features are no longer detectable after

processing[7]. As an alternative to protein analysis, DNA-based techniques for species identification have recently started to be applied towards a wide variety of animals[8,9]. Mitochondrial DNA (mtDNA) is regarded as an important tool in studying evolutionary relationships, species identification and authentication of animal origin[10]. Due to mtDNA contains the conserved protein-coding region, high variability in non-coding sequences, lack of recombination, and easy to isolate and sequence. The sequence divergence accumulates more rapidly in mtDNA than nuclear DNA due to faster mutation rate and lack of repair system in mtDNA which contain high levels of informative variation. However, the rate of evolution for each region in mtDNA is different and has been used to examine various phylogenetic relationships and species identification[11,12]. The mitochondrial cytochrome b (cyt b) gene showed a relatively high mutation rate and sufficient point mutations to enable discrimination of a wide variety of animals, even closely related species belonging to the same family and genus[13,14].

Species recognition by DNA analysis can be carried out by methods based on PCR, sequencing and restriction enzyme analysis. The high cost and arduous nature of DNA sequencing limits its use in small laboratories with restricted facilities. Therefore, this study is aimed at developing a simple and effective molecular technique for identification of endangered snake species of Thailand. Restriction fragment length polymorphism (RFLP) technique has been chosen, because (i) it is less time-consuming, (ii) it is more cost-effective than DNA sequencing, (iii) it requires equipment readily available in most molecular laboratories, and (iv) it has proved its utility in species identification[15].

In this study, for easy and effective identification of the venomous snake species in Thailand, we have used novel primers to amplify the cyt b gene to generate species-specific restriction patterns. The results presented here suggest that this technique may be effective for discriminating endangered snakes among neurotoxic and hematotoxic snake species in Thailand. The technique has proved effective even in the case of degraded DNA recovered from shed snake skins.

MATERIAL AND METHODS

Sample collection

Shed snake skins from Elapinae, Viperinae, and Crotalinae in Thailand were obtained from the following species: Neurotoxic snake species; *Naja kaouthia* (NK), *Naja sumatrana* (NSU), *Naja siamensis* (SC), *Ophiophagus hannah* (OH), *Bungarus fasciatus* (BF), and *Bungarus candidus* (BC). Hematotoxic snake species; *Daboia siamensis* (DRS), *Calloselasma rhodostoma* (CR), *Trimeresurus albolabris* (CA), *Trimeresurus macrops* (CM), *Trimeresurus popeorum* (PP), and *Trimeresurus purpureomaculatus* (CP).

DNA Extraction

Shed snake skins were washed with sterile distilled water, dried in air and cut into small pieces. DNA extraction was performed using Genomic DNA extraction mini kit (Tissue) (RBC Bioscience, USA). DNA from bloods was extracted using Genomic DNA extraction kit (Blood/Bacteria/Cultured cells) (RBC Bioscience, USA).

Oligonucleotide Primers and Amplification

Oligonucleotide primers of cyt b were designed based on NCBI GenBank database (Table 1.). DNA amplification using PCR was carried out with 50 µl reaction buffer containing 10xbuffer, 100mM of each dNTP, 25 mM $MgCl_2$, 50 pmol/µl of sense and antisense primers, Taq DNA polymerase and 10 µl DNA template. The amplification was preceded on a thermocycle (MWG Biotech, USA) at 94°c, 3 minutes, followed by 40 cyclers of 94°c/56°c/72°c one minute each with final extension of 72°c for 7 minutes. The final products were electrophoresed on a 1.5% agarose gel containing ethidium bromide in 1xTAE buffer along with appropriate molecular size markers. The gel fragment containing the amplified product was excised and extracted using Gel/PCR DNA fragments extraction kit (RBC Bioscience, USA).

Nucleotide Sequencing

DNA sequencing was carried out using the same primers used in the PCRs by 1st BASE sequencing (Malaysia-http:// www.base-asia.com). DNA sequences of cyt b from Elapinae, Viperinae, and Crotalinae snakes were aligned using Clustal X program.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Cyt b gene sequences obtained in this study were subjected to electronic RFLP using Webcutter 2.0 software. Based on these preliminary results, two enzymes (Alu I and Hinf I) were selected for further experimental analysis. Fifteen microliters of PCR product (approximately 1 µg) were separately digested with 1 U of Alu I and Hinf I (Vivantis, USA), in a final volume of 20 µl for 4 hr at 37◦C. The final products were electrophoresed on a 2 % agarose gel containing ethidium bromide in 1xTAE buffer along with appropriate molecular size markers.

Table 1.

Primers	Nucleotides (5' ⟶ 3')	Product size (bp)
tRNA-F	5' GCCTGAAAAACCACCGTTGT 3'	1,144
tRNA-R	5' CCGTCTTTGGTTTACAAGAAC 3'	

Oligonucleotide primers of tRNA (Cytochrome b) for DNA sequencing was designed based on NCBI GenBank database: mitochondrion, complete genome of *N. naja* (NC_010225.1), *N. atra* (NC_011389.1), *O. hannah* (NC_011394.1), *B. fasciatus* (NC_011393.1), *B. multicinctus* (NC_011392.1), and *D. siamensis* (NC_011391.1).

RESULTS AND DISCUSSION

Cyt b gene amplification of 6 neurotoxic and 6 hematotoxic venomous snakes in Thailand resulted in a single fragment of 1,144 bp (Table 1.). Four species, *T. albolabris, T. macrops, T. popeorum* and *T. purpureomaculatus* represented the genus *Trimeresurus* since frequently found throughout Thailand. The sequences of 12 venomous snakes were assembled and subjected to electronic RFLP. Following the electronic RFLP, two enzymes AluI and Hinf I were then selected to obtain different restriction digestion patterns among the venomous snakes (Table 2.). These two enzymes generated reproducible species-specific restriction patterns and allowed to discriminating 12 snake species on an agarose gel (Fig 1.). Electrophoresis analysis of Alu I (Fig 2.) and Hinf I (Fig 3.) digestion gave the expected fragments as shown except fragments that smaller than 100 bp could not be seen in the gel. However, *D. siamensis* and *T. popeorum* generated identical patterns among hematotoxic snakes when Alu I has been used. These results could be resolved by using additional enzyme such as Hinf I due to no cross reaction between the species was detected in their restriction patterns. Although digestion of these two snakes with Hinf I produced distinct RFLP patterns, the apparent conservation of a number of enzyme recognition sites suggests that these two snakes are closely related. It is probably that nuclear genomic sequence would provide additional information as to the relationships among the various species of snakes[7]. Color variation and geographic distribution of these snakes could be possible considered as additional information for species identification. *D. siamensis* is light brown with dark brown spots and mostly distributes in central Thailand. *T. popeorum*, on the other hand, is predominantly green and commonly found in northern Thailand[16]. For some species, nevertheless, additional fragments shown up in the gels. Possibly these results could be attributable to a co-amplification of nuclear mitochondrial pseudo genes. These genes were sequences inserted in the nuclear genome that diverge from their ancestral mitochondrial genes with a mutational spectra related to its loss of function. The presence of these mt pseudogenes integrated in nuclear genomes has been largely demonstrated for several taxa[17].

There are many reasons of using cyt b for phylogenetic analysis in animals. The high discriminatory power of the cyt b marker is based on the existence of discrete character classes that exhibit mutation rates reliable for phylogenetic analysis, plus the fact that the gene is maternally inherited and free of recombination. Moreover, the gene evolves slowly in terms of non-synonymous substitutions, but the rate of silent mutations is relatively high. The slow mutation rate and absence of recombination of the cyt b marker seems to make it better suited than microsatellites for differentiating closely related populations and distinguishing populations in close geographical proximity[18].

While DNA sequencing is the most common assay for species identification, the process requires several steps and is relatively expensive. Sequencing analysis involves the manual inspection and interpretation of base called data, sequence alignment and comparison against database samples, as well as phylogenetic

and statistical analysis. Problems with DNA sequencing techniques reflect the vital need of an easy, rapid and cost effective method, which could enable the authentication of snake products[2,10]. In this experiment, a brief PCR-RFLP protocol with a set selected enzyme provides a simple and effective PCR-RFLP technique for rapid identification of common venomous snakes in Thailand. Direct digestion of the PCR products with restriction enzymes generated reproducible species-specific restriction patterns. This method could be performed in the small laboratory with minimal equipments, cost and time. The results also support the worthiness of the method in cases of degraded noninvasive of shed snake skins. Hence, this technique could be recommended for quick identification of venomous snakes in Thailand. It is hopefully that this technique is not only improve snake species identification under "real case condition" but also strengthen the law enforcement for illegal trade of snake conservation[15].

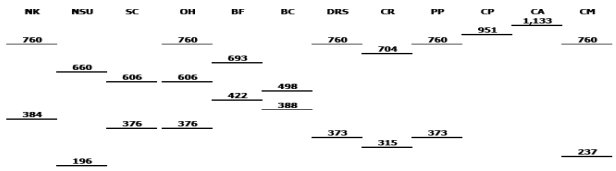
Table 2.

Species	Alu I	Hinf I
Naja kaouthia (NK)	376, 760	87,128,929
Naja sumatrana (NSU)	45,49,86,100,196,660	128, 129,159,728
Naja siamensis (SC)	54,100,376,606	60,72,87,128,129,168,500
Ophiophagus hannah(OH)	154,376,606	128,1,016
Bungarus fasciatus (BF)	130,138,154,693	50,79,215,258,530
Bungarus candidus (BC)	33,154,195,234,498	72,129,307,636
Daboia siamensis (DRS)	373,760	53,154,182,270,485
Calloselasma rhodostoma (CR)	56,57,315,704	203,258,671
Trimeresurus albolabris (CA)	1,133	36,287,821
Trimeresurus macrops (CM)	136,237,760	151,162,185,323
Trimeresurus popeorum (PP)	373, 760	215,322,499
Trimeresurus purpureomaculatus (CP)	182, 951	128,195,821

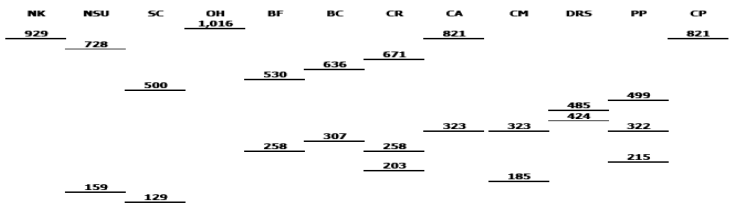
Predicted size of DNA segments (bp) based on electronic restriction fragment length polymorphism analysis with Webcutter 2.0 program.

Fig 1.

A: Alu I

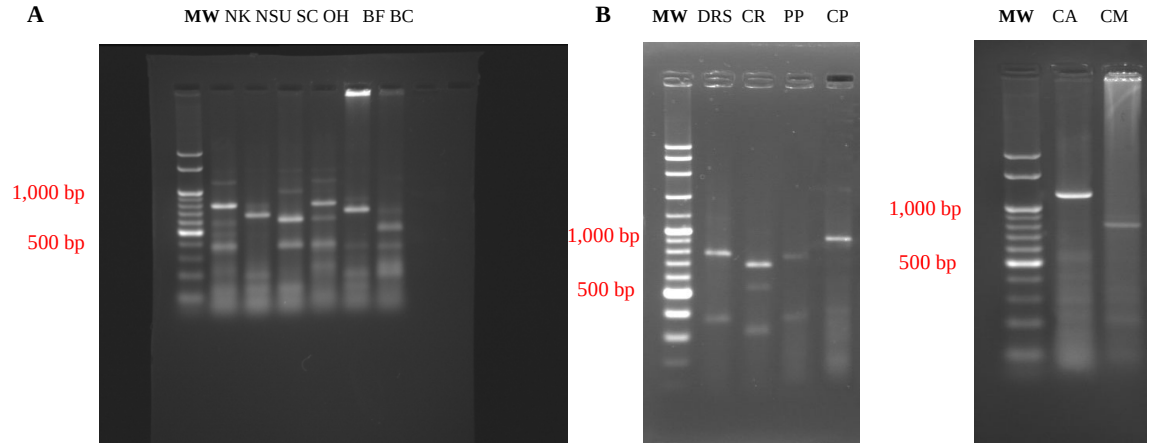


B: Hinf I



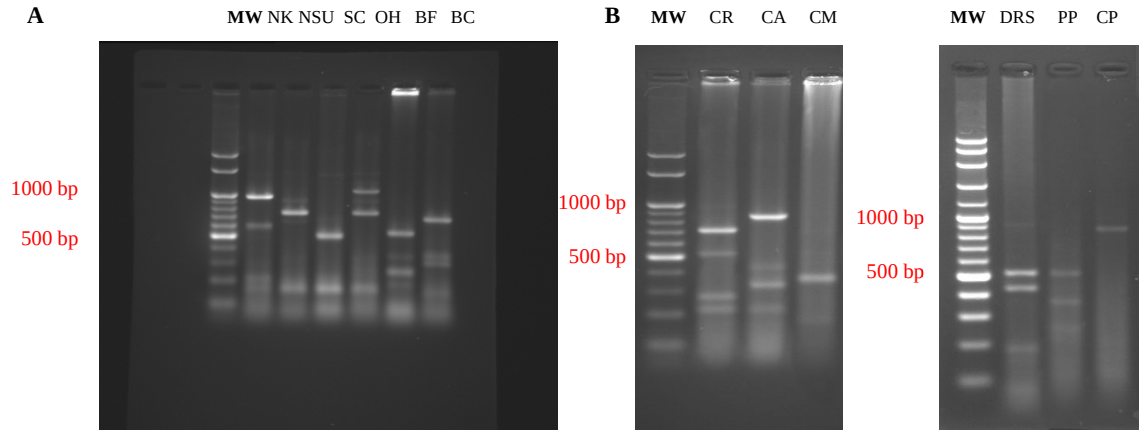
Alu I (A) and Hinf I (B) restriction patterns of cytochrome b based on the analysis of the GenBank DNA sequences. The full length of the PCR product is 1,144 bp. Sizes (bp) for restriction fragments are indicated above the bands.

Fig 2. PCR-RFLP patterns of neurotoxic(A) and hematotoxic (B) snakes for cytochrome b gene obtained by digestion with enzyme Alu I



A; lane 1: 100 bp molecular weight marker; lane 2-7: neurotoxic snakes. **B**; lane 1 and 6: 100 bp molecular weight marker; lane 2-5 and 7-8: hematotoxic snakes.

Fig 3. PCR-RFLP patterns of neurotoxic and hematotoxic snakes for cytochrome b gene obtained by digestion with enzyme Hinf I



A; lane 1: 100 bp molecular weight marker; lane 2-7: neurotoxic snakes. **B**; lane 1 and 5: 100 bp molecular weight marker; lane 2-4 and 6-8: hematotoxic snakes.

CONCLUSION

Cyt b is a highly conserved region and consequently a good molecular marker for diagnostic studies. The PCR-RFLP technique using Alu I and Hinf I restriction enzymes can easily and effectively identify the venomous snakes without any need of sequencing or phylogenetic analysis. The protocol developed in this study proved to be simple, reliable, rapid, and cost-effective method, requiring minimum experimental set up and enabling accurate identification of closely related venomous snake species in Thailand. Therefore, PCR-RFLP technique could be used successfully as a routine method in species identification of venomous snakes in Thailand.

Acknowledgements

The authors would like to thank the staffs of snake farm, Queen Saovabha Memorial Institute for collecting shed snake skins. This work was supported by a grant from Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand.

The authors have no conflict of interest to declare in this study.

REFERENCES

1. Heise, P. J., Maxson, L. R., Dowling, H. G., and Hedges, S. B. Higher-level snake phylogeny inferred from mitochondrial DNA sequences of 12S rRNA and 16S rRNA genes. *Mol. Biol. Evol.* **12(2):** 259-265 (1995)
2. Singh, C. S., Gaur, A., Sreenivas, A., and Singh, L. Species identification from dried snake venom. *J. Forensic. Sci.* **57(3):** 826-828 (2012)
3. Meganathan, P., R., Dubey, B., and Haque, I. Molecular identification of Indian crocodile species: PCR-RFLP method for forensic authentication. *J. Forensic Sci.* **54(5):** 1042-1045 (2009)
4. Pascoal, A., Barros-Velazquez, J., Cepeda, A., Gallardo, J. M., and Calo-Mata, P. Identification of shrimp species in raw and processed food products by means of a polymerase chain reaction-restriction fragment length polymorphism method targeted to cytochrome b mitochondrial sequences. *Electrophoresis* **29(15):** 3220-3228 (2008)
5. Gupta, A. R., Patra, R. C., Das, D.K. Gupta, P. K., Swarup, D., Saini, M. Sequence characyerization and polymerase chain reaction-restriction fragment length polymorphism of the mitochondrial DNA 12S rRNA gene provides a method for species identification of Indian deer. *Mitochondrial DNA.* **19(4):** 394-400 (2008)
6. Wong, K- L., Wang, J., But, P. P-H., and Shaw, P-C. Application of cytochrome b DNA sequences for the authentication of endangered snake species. *Forensic Sci. Int.* **139:** 49-55 (2004)
7. Aranishi, F., Okimoto, T., and Izumi, S. Identification of gadoid species (Pisces, Gadidae) by PCR-RFLP analysis. *J. Appl. Genet.* **46(1):** 69-73 (2005)
8. Hryncewicz-Gwozdz, A., Jagielski, T., Dobrowolska, A., Szepietowski, C., and Baran, E. Identification and differentiation of Trichophyton rubrum clinical isolates using PCR-RFLP and RAPD methods. *Eur. J. Clin. Microbiolo. Infect. Dis.* **30:** 727-731 (2011)
9. Kent, R. J., Deus, S., Williams, M., and Savage, H. M. Development of a multiplexed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay to identify common members of the subgenera *Culex* (*Culex*) and *Culex* (*Phenacomyia*) in Guatemala. *Am. J. Trop. Med. Hyg.* **83(2):** 285-291 (2010)
10. Pook, C.E. and McEwing, R. Mitochondrial DNA sequences from dried snake venom: a DNA barcoding approach to the identification of venom samples. *Toxicon.* **46:** 711-715 (2005)
11. Chakraborty, A., Aranishi, F., and Iwatsuki, Y. Molecular identification of hairtail species (Pisces: Trichiuridae) based on PCR-RFLP analysis of the mitochondrial 16S rRNA gene. *J. Appl. Genet.* **46(4):** 381-385 (2005)
12. Quinta, R., Gomes, L., and dos Santos, A. T. A mitochondrial DNA PCR-RFLP marker for population studies of the black scabbardfish (*Aphanopus carbo*). *J. Marine Sci.* **61:** 864-867 (2004)
13. Slowinski, J. B., and Keogh, J. S. Phylogenetic relationships of elapid snakes based on cytochrome b mtDNA sequences. *Mol. Phylogenet. Evol.* **15(1):** 157-164 (2000)
14. Rohilla, M. S., and Tiwari, P. K. Restriction fragment length polymorphism of mitochondrial DNA and phylogenetic relationships among five species of Indian freshwater turtles. *J. Appl Genet.* **49(2):** 167-182 (2008)
15. Dubey, B., Meganathan, P. R., and Haque, I. Molecular identification of three Indian snake species using simple PCR-RFLP method. *J. Forensic Sci.* **55(4):** 1065-1067 (2010)
16. Cox, M. J., Hoover, M. F., Chanhome, L., and Thirakhupt, K. The snakes of Thailand. 1st Ed. Sirabutr Printing. Bangkok (2012)
17. Bravi, C. M., Liron, J. P., Mirol, P. M., Ripoli, M. V., Peral-Garcia, P., and Giovambattista, G. A simple method for domestic animal identification in Argentina using PCR-RFLP analysis of cytochrome b gene. *Legal Med.* **6:** 246-251 (2004)
18. Hamarsheh, O., Presber, W., Al-Jawabreh, A., Abdeen, Z., Amro, A., and Schonian, G. Molecular markers for Phlebotomus papatasi (Diptera: Psychodidae) and their usefulness for population genetic analysis. *Trans. R. Soc. Trop. Med. Hyg.* **103:** 1085-1086 (2009)