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Molecular genetic assessment of formalin-fixed ethanol-preserved King Cobra: A comparative study on pretreatment protocols and DNA extraction methods

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ABSTRACT

Four different pretreatment protocols for formalin-fixed ethanol-preserved (FFEP) King Cobra (*Ophiophagus hannah*) specimens were tested to remove excess formalin before DNA extraction. Either TE buffer or TE buffer combined with absolute ethanol seemed to be the most efficient protocol to pretreat FFEP specimens. Moreover, commercial DNA extraction kit appeared to be the most successful method for DNA extraction from FFEP King Cobra compared with modified phenol-chloroform isoamyl alcohol and Chelex DNA extraction methods, respectively. Due to the degraded nature of DNA found in FFEP tissues, amplification by overlapping segments, between 150 and 250 bp, within 737 bp of NADH dehydrogenase subunit2 gene was recommended to increase a success rate of amplification. DNA sequence of the FFEP King Cobra, from Phetchabun province, was closely related to the sequences of King Cobra shed skins from northern Thailand, Myanmar, and southern China. Meanwhile, DNA sequences of the other FFEP samples, with no locality information, were identified as King Cobra from southern Thailand. Thus, archival FFEP specimens could be a useful source for studying molecular genetics, biodiversity and conservation of the most dangerous snake, King Cobra, when properly prepared fresh shed skins were not available.

Key words: archival FFEP King Cobra, pretreatment protocols, DNA extraction methods, DNA sequencing

INTRODUCTION

The fundamental purpose of most natural museums is to collect and preserve biological materials for scientific research. During the past decades, specimens in museums have often become the last and only sources available to compare present day and historical genetic structure and to assess genetic diversity and population structure of animals (Joshi *et al*., 2013). Formalin has become the most commonly used for preserving biological tissues and archival museum specimens (Chatigny, 2000). However, tissues and museum specimens preserved in formalin have been difficult in DNA extraction for molecular studies, which arose from various adverse effects of formalin, both direct and indirect, on the structure on DNA. Bibi *et al*., (2015) reported that formalin-fixation of samples damaged DNA in many ways including irreversible denaturation, fragmentation, base modification and cross-linkage within the DNA itself or between DNA and proteins. The correlation has been found between concentration and purity of DNA isolated from tissues fixed for 8 and 48 hr but not for 96 hr. Short-term fixation has shown to produce high molecular weight DNA, while long-term fixation has only produced smaller DNA fragments. It seems likely that long-term fixation has decreased the amount of DNA. Moreover, the concentration of formalin might have an effect to the purity of DNA. For zoological purposes, 1-10% formalin has been commonly used for tissues fixation. Many studies (Fang *et al*., 2002; Schander and Halanych, 2003) have been pointed out that ethanol is a good medium for removing formalin after fixation. Most archival samples have been fixed in 10% formalin and stored in 70-80% ethanol including reptiles. Thus, removing formalin has become the most critical point to DNA extraction (Diaz-Viloria *et al*., 2005; Kuch *et al*., 1999).

Suntrarachun *et al*., (2014) have studied phylogenetic analysis of the most dangerous venomous snake King Cobra, *Ophiophagus hannah*, in Thailand based on mitochondrial DNA. King Cobra is the world's largest venomous snake and distribute across Thailand. Mitochondrial DNA has been extracted from fresh shed skins of 12 individuals King Cobra from different localities across Thailand. The mitochondrial NADH dehydrogenase subunit2 (NADH2) and control region of *O. hannah* from different localities across Thailand have been compared to the published sequences of *O. hannah* deposited in NCBI GenBank database from other countries. However, fresh samples of King Cobra from some localities across Thailand, including eastern and northeastern Thailand, have not been available. Only FFEP King Cobra specimens have been kept in Snake Farm, Queen Saovabha Memorial

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Institute, The Thai Red Cross Society as archival museum specimens. DNA extraction methods, from FFEP samples, might be different in performance with different species of animals. Finding an appropriate pretreatment protocol and DNA extraction method would be very important for representing an invaluable source of information for conservation, phylogenetic and population genetic studies.

This study was carried out on four different pretreatment methods combined with dehydration for removing formalin from FFEP King Cobra specimens before DNA extraction. Standard methods for DNA extraction might not be successful for all archival animal tissues. The modification should be used for further investigation to optimize the extraction and purification of DNA from archival snakes. Three methods for DNA extraction were also compared in order to determine amplification success rate of mitochondrial DNA gene from archival FFEP King Cobra. DNA sequences of archival FFEP King Cobra were identified and compared with the sequences of King Cobra shed skins from different parts of Thailand and published sequences deposited in NCBI GenBank database. The most suitable protocol would be generated in FFEP venomous and non venomous snakes for forensic and conservation studies when fresh shed skins from some localities were not available.

METHODS

Specimens

Ten formalin-fixed ethanol-preserved (FFEP) muscle tissues from King Cobra (*Ophiophagus hannah*) were obtained from Snake Farm, Queen Saovabha Memorial Institute (QSMI), The Thai Red Cross Society. Only one FFEP snake came from Phetchabun province, Thailand. The other nine samples were not given the exact locality information. The samples had been previously fixed in the 10% formalin overnight. The samples were then rinsed using distilled water and preserved in 70% ethanol. Archival samples of King Cobra were stored in 70% ethanol for 2 to 10 years. Approximately 1-2 cm section of preserved muscle tissues were excised from each of the intact specimens.

Pretreatment samples

Each sample was excised into approximately 3-5 mm portion. The samples were pretreated in various methods include: (1) Treatment with phosphate buffer saline (PBS) pH7.2 by soaking for 3 days and the buffer has been changed every 8 hours. The sample were completely chopped and dehydrated overnight. (2) Treatment with PBS pH7.2 by soaking for 3 days and the buffer has been changed every 8 hours. The sample was completely chopped and hydrated with absolute ethanol. Then, the samples were dehydrated overnight. (3) Treatment with TE buffer (containing Tris 10mM and EDTA 1 mM, pH 7.6) by soaking for 3 days and the buffer has been changed every 8 hours. The sample were completely chopped and dehydrated overnight. (4) Treatment with TE buffer by soaking for 3 days and the buffer has been changed every 8 hours. The sample was completely chopped and hydrated with absolute ethanol. Then, the samples were dehydrated overnight.

DNA extraction

Three different techniques were used for DNA extraction method from pretreated muscle tissues from FFEP King Cobra.

1. Modified phenol-chloroform isoamyl alcohol DNA extraction method:

Tissue muscles were resuspended in lysis buffer pH8.0 and proteinase K. The samples were incubated 60°C for 3 hr. An equal volume of 25:24:1 of phenol-chloroform isoamyl alcohol was added to the DNA solution. The samples were mixed and centrifuged for 5 min at high speed. An aqueous phase was removed to a new tube (If a white precipitate was present at the aqueous interface, reextracted the organic phage and pooled the aqueous phase). The DNA was precipitated with 1/10 volume 3M sodium acetate pH5.5. Two volumes of absolute ethanol were added and mixed inversion slowly. The samples were centrifuged for 15 min at high speed and supernatant was removed. The pellet was washed with 70% ethanol and centrifuged for 5 min at high speed. The supernatant was removed and the pellet was air dried briefly but did not overdried (Ausubel *et al*., 1995; De Moraes-Barros and Morgante, 2007).

2. Commercial DNA isolation kit:

The extraction steps were performed according to the user guide of commercial DNA isolation kit (RBC Bioscience, Taiwan). The tissue was digested with 180 ml of GT buffer and homogenized sample with graining. Next, 20 ml of proteinase K (10mg/ml) was added and incubated at 60°C for 30 min. During the incubation, the tube was inverted every 5 min. Then, 200 ml of GB buffer was added, incubated 70°C for 20 min, followed by mixing with 200 ml of absolute ethanol. The solution was transferred into a spin column, centrifuged for 2 min at high speed and washed with 400 ml of W1 buffer and 600 ml of Wash buffer. DNA was eluted with 80 ml of Elution buffer preheated to 70°C and further incubated at 70°C for 5 min before collection by centrifugation.

3. Chelex DNA extraction method:

Tissue muscles were resuspended in 100 ml of 5% Chelex 100 resin. Then the sample was allowed to boil at the heat block of 100 °C for 20 min. The solution was centrifuged at high speed for 5 min. The supernatant was collected as DNA template for PCR amplification.

Oligonucleotide primers and PCR amplification

The Oligonucleotide primer of NADH dehydrogenase subunit2 (NADH2) was designed based on NCBI GenBank database. The primers of 737 bp of NADH2 gene were also designed overlapping 150-250 bp segments for four fragments (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

Table 1. Oligonucleotide primers of NADH2 were designed based on NCBI GenBank nucleotide sequence database. GenBank Accession No. for NADH2: AY059008.1, AY058997.1 and AY059002.1.

Primers	Nucleotides (5' ⟶ 3')	Product size (bp)
NADH2-F	5' CGGGCAACAGAAGCTGCTAC3'	160
NADH2.1-R	5' TTATTAGGGCCATGGTAATG 3'	
NADH2.2-F	5'CATTACCATGGCCCTAATAA 3'	182
NADH2.2-R	5'ATTGCAGATGATAATAAAAATG 3'	
NADH2.3-F	5'CATCTGCAATCCTATCTGTT 3'	205
NADH2.3-R	5' GTTAGTGTGTGGTTAATAGA 3'	
NADH2.4-F	5' TCTATTAACCACACACTAAC 3'	222
NADH2-R	5' GTCGTAGATAGACGTATAGGCT3'	

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 min each with final extension of 72°c for 7 min. The final products were electrophoresed on a 1.5% agarose gel containing ethidium bromide in 1xTAE buffer along with appropriate molecular size markers.

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 NADH2 were blasted and compared with other King Cobra in different parts across Thailand (Suntrarachun *et al*., 2014) and from deposited in NCBI GenBank database.

RESULTS

The results obtained from Table 2. indicate that the most effective method for DNA amplification of NADH2 gene from King Cobra successfully occurred, 71-93%, when using Commercial DNA isolation kit after 3 days pretreatment with either TE buffer or TE buffer combined with absolute ethanol. As the same pretreatment, DNA extraction using Phenol-chloroform Isoamyl Alcohol present 64-79% which was lower success rates than Commercial DNA isolation kit. However, Chelex DNA extraction method seemed to be the less effective method of DNA extraction after pretreatment using both TE buffer (21-29%) and TE buffer combined with absolute ethanol (14-29%). Moreover, comparison of pretreatments before DNA extractions, between TE buffer and PBS including combination with absolute ethanol, has been conducted. DNA extraction after pretreatments with PBS and PBS combined with absolute ethanol, using these three methods, produced lower success rates than pretreatments with TE buffer and TE buffer combined with absolute ethanol. Only 50-71% of DNA amplification for four fragments NADH2 gene occurred when using Commercial DNA isolation kit after pretreatment with PBS and PBS combined with absolute ethanol. Meanwhile, DNA extraction using Phenol-chloroform Isoamyl Alcohol after pretreatments with PBS (43-64%) and PBS combined with absolute ethanol (50-64%) were lower success rates than Commercial DNA isolation kit. Furthermore, the lowest success rate of DNA amplification came from the extraction of DNA using Chelex after pretreatments with PBS (7-14%) and PBS combined with absolute ethanol (14-29%), respectively (Table 2).

Due to degrade nature of the DNA found in FFEP tissue of King Cobra, sets of primers were designed bracketing overlapping 150-250 bp segments within 737 bp section of NADH2 gene. No significant difference in the success rate of amplification has occurred among DNA fragment sizes 160, 182, 205, and 222 bp (Figure 1). Pretreatments and extraction methods could be the crucial points for DNA amplification of FFEP specimens. The amplification of short sequences, less than 250 bp, should be recommended for situation in which DNA degradation is likely such as FFEP tissues or any archival specimens.

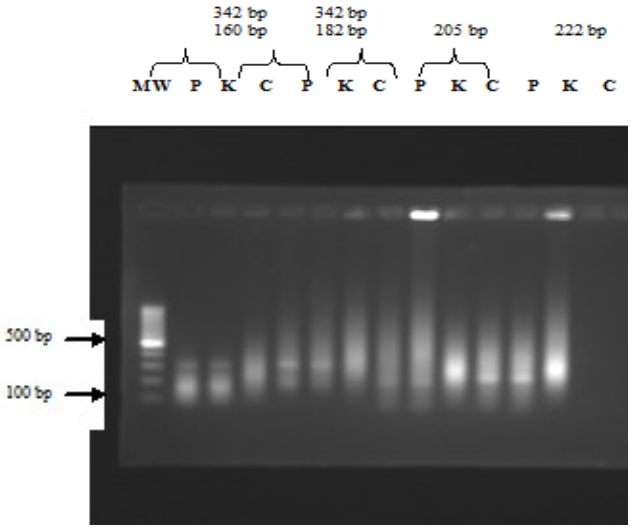

Figure 1. Electrophoresis patterns of DNA amplification obtained by each extraction method. These muscle tissues from archival King Cobra were pretreated with PBS combined with absolute ethanol. Amplified DNA fragments: lane 1: 100 bp molecular weight marker, lane 2-4: 160 and 342 bp, lane 5-7: 182 and 342 bp, lane 8-10: 205 bp, lane 11-13: 222 bp with DNA extracted from Phenol-chloroform Isoamyl Alcohol (P), Commercial DNA isolation kit (K), and Chelex DNA extraction (C) methods.

Table 2. Polymerase chain reaction amplification of 14 formalin-fixed ethanol-preserved tissues from King Cobra (*Ophiophagus hannah*) at Snake Farm, QSMI. Numbers of positive amplified fragments from NADH2 gene were shown with percentage. Summary of successful PCR amplification was present in the percentages.

DNA extraction methods With DNA fragment (bp)	PBS (Number of positive in percentage)	PBS with ethanol (Number of positive in percentage)	TE (Number of positive in percentage)	TE with ethanol (Number of positive in percentage)
Phenol-Chloroform Isoamyl alcohol				
- NADH2.1; 160 bp	8/14 (57%)	8/14 (57%)	11/14 (79%)	9/14 (64%)
- NADH2.2; 182 bp	6/14 (43%)	7/14 (50%)	11/14 (79%)	9/14 (64%)
- NADH2.3; 205 bp	8/14 (57%)	9/14 (64%)	9/14 (64%)	11/14 (79%)
- NADH2.4; 222 bp	9/14 (64%)	8/14 (57%)	9/14 (64%)	10/14 (71%)
Commercial DNA isolation kit				
- NADH2.1; 160 bp	7/14 (50%)	9/14 (64%)	11/14 (79%)	11/14 (79%)
- NADH2.2; 182 bp	9/14 (64%)	7/14 (50%)	10/14 (71%)	10/14 (71%)
- NADH2.3; 205 bp	10/14 (71%)	10/14 (71%)	13/14 ((93%)	13/14 (93%)
- NADH2.4; 222 bp	9/14 (64%)	10/14 (71%)	13/14 (93%)	11/14 (79%)
Chelex DNA extraction method				
- NADH2.1; 160 bp	1/14 (7%)	2/14 (14%)	3/14 (21%)	4/14 (29%)
- NADH2.2; 182 bp	1/14 (7%)	2/14 (14%)	4/14 (29%)	3/14 (21%)
- NADH2.3; 205 bp	2/14 (14%)	4/14 (29%)	3/14 (21%)	2/14 (14%)
- NADH2.4; 222 bp	2/14 (14%)	2/14 (14%)	3/14 (21%)	3/14 (21%)

DNA sequencing of NADH2 gene from archival FFEP specimens were compared with the sequences from fresh shed skins of King Cobra in different localities across Thailand (Fig 2.). NADH2 sequence of FFEP specimen, from Phetchabun province, was similar to the sequence of King Cobra from northern Thailand. Meanwhile, archival FFEP specimens, with no detailed locality information, had the similar sequences as King Cobra collected from southern Thailand. In addition, the sequences of FFEP specimens from Phetchabun province and fresh shed skins King Cobra from northern Thailand were closely related to King Cobra in Myanmar and southern China which already deposited in NCBI GenBank database.

DISCUSSION

When live tissues are fixed with formalin, it penetrates into the tissues and causes cross-linking of DNA with DNA, DNA with proteins, and proteins with proteins in the chromosomes. It is believed that formalin impede PCR reaction and can either cause failure in PCR amplification or reduce the available sequence length of amplified products. Thus, formalin must be eliminated when molecular biology and phylogenetics are studied (Bucklin and Allen, 2004). Various techniques or special processes are required to resolve the problems from formalin. The wide variation in preserving methods adopted for storage of specimens. Many studies (Diaz-Viloria *et al*., 2005; Schander and Halanych, 2003) successfully amplified mtDNA fragments because the tissue was normally fixed in 1-10% formalin for 8-48 hr and then preserved in 70-80% alcohol for various time periods. On the other hand, after a long time in formalin preservation such as 96 hr, it is unlikely that mtDNA fragments can be recovered by amplification. Mitochondrial DNA could be amplified on formalin-fixed ethanol-preserved samples over short time period, but not on longer periods of preservation in formalin. Since the archival museum King Cobra specimens were fixed overnight by using 10% formalin and long term 70% ethanol preservation, the differences in the results attributed to the differences in pretreatment method to remove formalin and the DNA extraction procedure. To extract good quality DNA for PCR amplification and carry out studies about genetic diversity, we strongly recommend that fixation in formalin should be less than 48 hr and followed by preservation in 70% ethanol. Formalin is not a

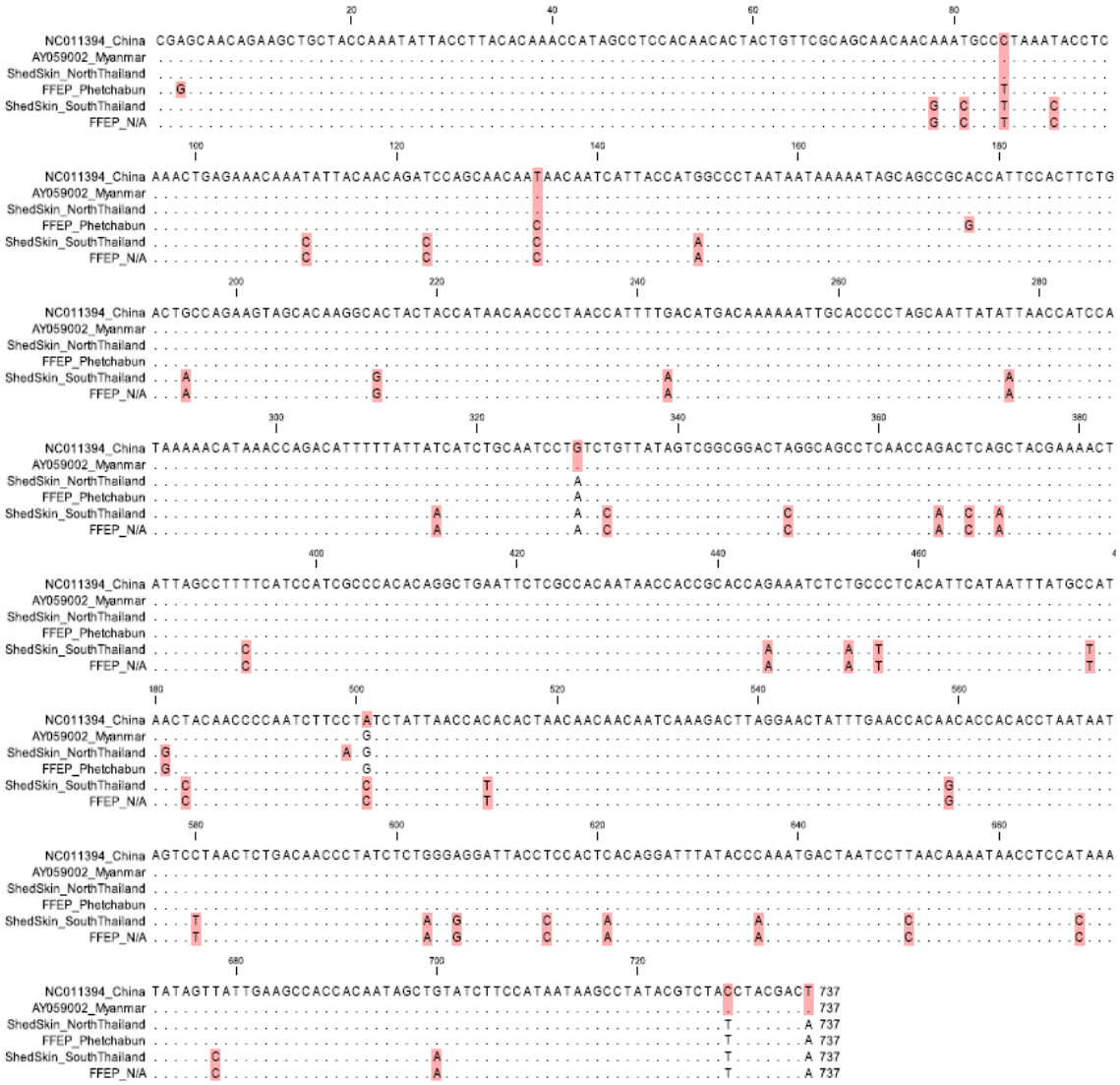

Figure 2. Multiple sequence alignment, for 737 bp of NADH2 gene, from FFEP tissues and fresh shed skins of King Cobra were compared with published sequences deposited in NCBI GenBank database including King Cobra from Myanmar and southern China. Identity sequences were denoted by dots. FFEP N/A: No locality information; FFEP Phetchabun: Phetchabun Province, Thailand.

good medium for preserving DNA, and today tissue for molecular studies is either frozen or formalin-fixed and preserved in alcohol (Pelero *et al*., 2010).

Many studies have been carried out on pretreatment of formalin preserved and FFEP samples to increase the quality and yield of the DNA (Chakraborty *et al*., 2006). Pre-treatment methods often used including treatment with a hot alkali and additional NaOH to the digestion buffer, adding glycine to the digestion buffer as a binding agent to release the formalin, and drying using graded ethanol washes. Furthermore, NaOH and PBS have been used as a washing buffer for removing formalin from preserved samples by enhancing the pH value prior to the extraction (Bibi *et al*., 2015). Most of these studies have been limited to samples preserved for a short duration in formalin. Joshi *et al* (2013) revealed that PBS treatment was the best method for DNA extraction from formalin-preserved samples of longer period (2 to 7 years) because of higher success rate in amplifying mitochondrial gene than NaOH treatment. TE buffer is also a common used buffer solution in molecular biology, especially in procedures involving DNA and RNA. The purpose of using TE buffer is to protecting DNA and RNA from degradation. Pretreatment 70% ethanol-preserved samples using TE buffer and TE buffer with 100% ethanol seemed to be a gradient of ethanol washing system before DNA extraction. Pretreatment with a gradient of ethanol washing system seemed to be a useful tool to study of genetic structure and phylogenesis of endangered animal including reptile, using specimens in museum and herbarium (Yuan *et al*., 2014). The recovery of long DNA sequences after extraction may depend on several variables such as how the sample is prepared, storage and time since specimen collection and any treatment previously applied to the museum specimens. However, the success rate of amplification has been consistent and could be varied between 12-74% for 100-400 bp for both mitochondrial and nuclear genes (Joshi *et al*., 2013). It was reported that such cross-linking can inhibit primer annealing and suppresses the replication process during

the PCR reaction. The condition of a museum specimen and its quality in terms of using mtDNA and nuclear DNA are important in molecular studies and conservation genetics. DNA extraction from FFEP samples and PCR amplification for mitochondrial and nuclear genes has been attempted from different species eg. fish, mammals etc (Raja *et al*., 2011). Several studies (Friedman and Desalle, 2008; Joshi *et al*., 2013) reported unsuccessful amplification of 400 bp of mitochondrial DNA using published protocols for the samples that had been stored for long periods (3-4 years) in formalin. They concluded that DNA structure is less affected by cross-linking when specimens are stored for short periods, less than 24 hr, in formalin which leads to easy extraction and amplification of mitochondrial DNA up to size 400 bp. It is probably that a reduced exposure of samples to formalin fixation would reduce the degradation of DNA and extraction from these samples is easy. According to high DNA damage rates by formalin, working with small mtDNA fragments less than 250 bp were crucial steps for consistent amplification of FFEP specimens (Chase *et al*., 1998; France and Kocher, 1996).

DNA extraction using Commercial DNA isolation kit is more practical and successful method for PCR amplification but its high cost limits its use. Although, modified phenol-chloroform isoamyl alcohol DNA extraction method is cheaper than Commercial kit, the efficiency of DNA extraction was low and the process generates toxic waste that must be disposed with care and its lengthy procedure reduces its benefits (Mirmomeni *et al*., 2010). Lastly, DNA extraction method using Chelex 100 resin resulted in a low quantity and poor quality DNA which could be due to degradation of DNA from boiling the samples. In addition, DNA sequencing should be performed from these products to be determined whether the sequences were modified as a result of exposure to formalin (Shedlock *et al*, 1996; Shokralla *et al*., 2010). Finally, the disparity of amplification obtained from each sample could be due to the inherent differences in tissue makeup of musculature of the archival museum specimens. For example, the intercostal muscle tissue from the venomous snake, *M. fulvius*, was noticeably more diffuse than the denser musculature apparent in the constrictor snake, *L. triangulum*. The implication is that the denser muscle tissue of the constricting colubrid may contain a greater number of mitochondrial than the more diffuse tissue in the intercostal region of the venomous elapid. The amount of mitochondrial DNA could vary between cell types and within cell types at varying metabolic levels. Thus, careful consideration should be given when the type of tissue from formalin-fixed sample is used (Friedman and Desalle, 2008).

It seems likely that PCR amplification and DNA sequencing from archival FFEP King Cobra specimens have a correlation with the sequences of samples from fresh tissues such as fresh shed skins. However, small fragments especially less than 250 bp would be recommended for both amplification and sequencing. The sequence divergence among King Cobra in Thailand could be useful for inferring intraspecific relationships of King Cobra through Southeast Asia. There are two lineages of King Cobra across Thailand. The first lineage of King Cobra is commonly found in northern Thailand, western Thailand, Myanmar and southern China. The second lineage of King Cobra distributes only in southern Thailand and is different from other parts across Thailand. It is possible that this lineage might be similar to King Cobra in the Malay Peninsula, Indonesia, and the Philippine Islands. Determination of long term FFEP specimens in museum could inform new information for understanding the evolutionary, genetic relationships, and species identification.

This study reveals that DNA extraction, from FFEP King Cobra for 2 to 10 years, should remove formalin by pretreatment 3 days with either TE buffer or TE combined with absolute ethanol before drying overnight. The great potential method of DNA extraction after pretreatment was Commercial DNA extraction kit but Phenol-Chloroform Isoamyl alcohol was optional. The amplification of NADH2 gene containing short segments, 150-250 bp, by overlapping within gene is recommended. DNA amplification and sequencing from FFEP King Cobra specimens could be used to identify the lineage of King Cobra distributes in Thailand.

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