THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Veterinary Pathology)
DEGREE

Veterinary Pathology
FIELD

Pathology
DEPARTMENT

TITLE: Differentiation of *Varanus* spp. in Thailand by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

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THESIS

DIFFERENTIATION OF *VARANUS* SPP. IN THAILAND BY POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

WALLAYA PHONGPHAEW

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Veterinary Pathology)
Graduate School, Kasetsart University
2010

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Wallaya Phongphaew 2010: Differentiation of *Varanus* spp. in Thailand by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Master of Science (Veterinary Pathology), Major Field: Veterinary Pathology, Department of Pathology. Thesis Advisor: Associate Professor Theerapol Sirinarumitr, Ph.D. 70 pages.

The twenty two samples of monitor from Thailand were differentiated by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique, this study worked based on cytochrome b gene and 12S rRNA gene. The PCR products were approximate 376 and 450 bp respectively. The cytochrome b gene products were treated with BstXI, BanI, HpaII and EcoRI , the 12S rRNA gene products were treated with ApaI, BamHI and HaeIII to observe the different patterns, which specific to each monitor. The results from both genes analysis showed the different patterns among species, but the variations between subspecies were observed in cytochrome b gene only. These results suggested that the PCR-RFLP based on the cytochrome b gene could be used to differentiate the species and subspecies of monitors in Thailand, while the PCR-RFLP based on the 12S rRNA gene could not be differentiate at subspecies levels.

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Student's signature Thesis Advisor's signature

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ACKNOWLEDGMENTS

I would like to specially thank my advisor, Associate Professor Dr. Theerapol Sirinarumitr and Associate Professor Dr. Chaleow Salakij, for their guidance and support on this research work.

I would like to thank Ms. Rungtiwa Sinsiri, a molecular technician from Molecular Diagnostic Laboratory, Veterinary Diagnosis Laboratory, The Faculty of Veterinary Medicine, Kasetsart University, for her support and guidance.

I would like to thank the Faculty of Veterinary Medicine, Kasetsart University, Bang-khen campus, Bangkok, for providing facilities to work on this research.

Specially thank to my colleagues who assisted me and shared their comment during doing research.

Last but not least, I would like to appreciate and deeply thank to my parents. They have always understood and encouraged me to complete my graduate study.

Wallaya Phongphaew

April 2010

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS	i
LIST OF TABLES	ii
LIST OF FIGURES	iii
LIST OF ABBREVIATIONS	v
INTRODUCTION	1
OBJECTIVES	3
LITERATURE REVIEW	4
MATERIALS AND METHODS	25
RESULTS	30
DISCUSSIONS	49
CONCLUSSION	52
LITERATURE CITED	53
APPENDICES	62
Appendix A Chemical reagents and substances	63
Appendix B The standard methods	65
CURRICULUM VITAE	70

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LIST OF TABLES

Table		Page
1	Numbers of samples	25
2	Sequence sets of primers	27
3	Numbers and sizes of restriction fragments of 12S rRNA gene	37
4	Numbers and sizes of restriction fragments of cytochrome b gene	41
5	Monitor species and GenBank accession number information, partial 12S rRNA gene sequences.	45
6	Monitor species and GenBank accession number information, partial cytochrome b gene sequences	47

Appendix Table

B1	Reaction component of pGEM®-T Easy system	68

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LIST OF FIGURES

Figure		Page
1	A phylogeny of the *Varanus* spp. based on chromosomal morphology	5
2	A phylogeny of monitor lizards based on microcomplement fixation (MCF)	6
3	A phylogeny of *Varanus* spp.	
	A: Based on hemipeneal morphology	7
	B: Based on lung morphology	7
4	Phylogenetic tree based on 12S rRNA sequence analysis	8
5	A cladogram of phylogenetic relationship for Varanoidae based on long PCR product sequences from mitochondrial DNA	9
6	Juvenile Asian water monitor	11
7	Adult Asian water monitor	11
8	Black jungle monitor (*V. salvator komaini*)	12
9	A red-headed monitor (*V. dumerilii*)	13
10	The rough-necked monitor (*V. rudicollis*)	14
11	The clouded monitor (*V. bengalensis nebulosus*)	15
12	Distribution map of *Varanus* spp.	15
13	The vertebrate mitochondrial genome	18
14	1% Agarose gel electrophoresis of the PCR products of partial mitochondrial 12S rRNA gene	30
15	1% Agarose gel electrophoresis of the PCR products of partial cytochrome b gene	31
16	1% Agarose gel electrophoresis of the PCR products of the partial 12S rRNA gene using T7 and SP6 specific primers	32
17	1% Agarose gel electrophoresis of the PCR products of the partial cytochrome b gene using T7 and SP6 specific primers	33
18	Restriction patterns of the partial 12S rRNA fragments of Asian water monitor and black jungle monitor	34

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LIST OF FIGURES (Continued)

Figure		Page
19	Restriction patterns of the partial 12S rRNA fragments of red-headed monitor and rough-necked monitor	35
20	Restriction patterns of the partial 12S rRNA fragments of clouded monitor	36
21	Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of Asian water monitor	38
22	Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR products of black jungle monitor	39
23	Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of red-headed monitor	39
24	Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of rough-necked monitor	40
25	Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of clouded monitor	40
26	Nucleotide alignment of 12S rRNA gene sequences	42
27	Nucleotide alignment of cytochrome b gene	44
28	The phylogenetic tree of monitor lizards in Thailand based on partial 12S rRNA gene sequences	46
29	The phylogenetic tree of monitor lizards in Thailand based on partial cytochrome b gene sequences	48

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LIST OF ABBREVIATIONS

tRNA	=	Transfer ribonucleic acid
12S rRNA	=	12 small ribosomal ribonucleic acid
CITES	=	The Convention on International Trade in Endangered Species of Wild Fauna and Flora
cm	=	centimeter
µl	=	microliter
ml	=	milliliter
µg	=	microgram
mg	=	milligram
bp	=	base pair
mM	=	milimolar
M	=	molar
ATP	=	Adenosine triphosphate
DNA	=	Deoxyribonucleic acid
mtDNA	=	mitochondrial Deoxyribonucleic acid
RNA	=	Ribonucleic acid
PCR	=	Polymerase Chain Reaction
RFLP	=	Restriction fragments length polymorphism
rpm	=	round per minute
°C	=	Degree Celsius
OD	=	Optical Density
V. salvator	=	*Varanus salvator*
V. dumerilii	=	*Varanus dumerilii*
V. rudicollis	=	*Varanus rudicollis*
V. bengalensis	=	*Varanus bengalensis*
ND1	=	NADH dehydrogenase subunit 1
ND2	=	NADH dehydrogenase subunit 2
TAE	=	Tris-Acetate-EDTA
TE	=	Tris-EDTA
sec	=	second
min	=	minute

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LIST OF ABBREVIATIONS (Continued)

IPTG	=	Isopropyl β-D-1-thiogalactopyranoside
X-gal	=	Bromo-chloro-indolyl-galactopyranoside
U	=	unit
E. coli	=	*Escherichia coli*
VSS	=	*Varanus salvator salvator*
VSK	=	*Varanus salvator komaini*
VD	=	*Varanus dumerilii*
VR	=	*Varanus rudicollis*
VB	=	*Varanus bengalensis*
Vnilo	=	*Varanus niloticus*
Th	=	Thailand
Jap	=	Japan
AUS	=	Australia
Chi	=	China
USA	=	United State of America

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DIFFERENTIATION OF *VARANUS* SPP. IN THAILAND BY POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)

INTRODUCTION

Monitor lizard is a common name of about 60 species *Varanus* spp. It is classified in Family *Varanidae*, Class *Reptilia*, Order *Squamata* and Suborder *Sauria*. In the past, their systematic studied using external morphology, chromosomal characters (King and Max, 1975), microcomplement fixation technique (Baverstock *et al*., 1993), morphology of neurocranial skull and molecular techniques, based on tRNA and 12S rRNA gene (Fuller *et al*., 1998; Ast, 2001). They are worldwide distribution such as Africa, Asia, especially Southeast Asia and Australia. Although all *Varanus* spp. are on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) but their population have been decreased, they are hunted by human for their skins and meat, Chinese medicinal purpose and indirectly from the communal growth and human's agriculture. Nowadays in Thailand, there are 6 taxa of monitors including of 5 species and 1 subspecies such as common water monitor (*Varanus salvator salvator*), red-headed monitor (*Varanus dumerlii*), rough-necked monitor (*Varanus rudicollis*), clouded monitor (*Varanus bengalensis nebulosus*), yellow monitor (*Varanus flavescens*) and black jungle monitors or black water monitor (*Varanus salvator komaini*). These monitors are listed on Act of Animals Protection and Conservation since 1992. At the present time in Thailand, the red-headed monitor and rough-necked monitors are classified as the endangered species, whereas the black water monitor is the critical endangered species. Due to the difficulty to determine their species from morphology (sometimes), meat, blood or skin, therefore molecular techniques are needed to identify the species of monitor. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) technique is generally used to identify species for several organisms. The principle of this technique is to amplify the specific fragment of deoxyribonucleic acid (DNA) and follows by restriction endonuclease digestion at specific sequences. Each individual or species has the restriction patterns specific for each individual or species. In this

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study, PCR-RFLP technique was used to analyze partial cytochrome b gene and partial 12S rRNA gene. Because these regions are on mitochondrial DNA that is maternal inheritance, low occurrence of DNA recombination and had enough polymorphism between species and some subspecies.

Aims of this study were to differentiate *Varanus* species in Thailand by using PCR-RFLP and study the phylogenetic relationship of these *Varanus* by using partial 12S rRNA and partial cytochrome b sequences.

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OBJECTIVES

1. To apply PCR-RFLPs technique for identify species in genus *Varanus*, observe restriction pattern or restriction profile of partial mitochondrial cytochrome b gene and 12S rRNA gene of *V. salvator, V. bengalensis, V. dumerilii, V. rudicollis* and *V. salvator komaini*.

2. To study for phylogenetic tree of these *Varanus* by DNA sequencing of partial cytochrome b gene and partial 12S rRNA**.**

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LITERATURE REVIEW

1. Overview of *Varanus* spp. (monitors)

1.1 Taxonomy

Monitor lizard is the common name of *Varanus* spp. It is classified in kingdom Animalia, phylum Chordata, class Reptilia, order Squamata, superfamily Varanoidea including family of Varanidae, Helodermatidae and Lanthoanotidae. The genus *Varanus* is classified in family Varanidae and first appeared in the fossil deposits of the Miocene (15-20 million years BP) in the Eastern Europe, Africa and Australia (King and Green, 1993). It is likely that the genus originated in Asia and radiated outward into Africa and Australia. The close relative animals of the varanoid are the snakes (King and Green, 1993). The Nile monitor was the first varanid named by Linneaus in 1758, as Lacerta monitor. In 1942, Mertens attempted to classify the Varanidae by using osteology and external morphology (King and Green, 1993, King and Green, 1999). In 1975, King and Max divided the *Varanus* into six groups such as A: gouldii group, B: varius group, C: salvator group, D: odatria group, E: grigeus group and F: niloticus group based on karyotypes (morphology and size of chromosomes). They found that this lizard had 40 chromosomes and it divided to eight pairs of large chromosomes and twelve pairs of micro chromosomes. They constructed a phylogeny by using chromosomal morphology, fossil evidence and the current distribution (Figure 1). The conclusion of this study indicated that there were three major radiation including African, Indo-Asian and Australian radiations.

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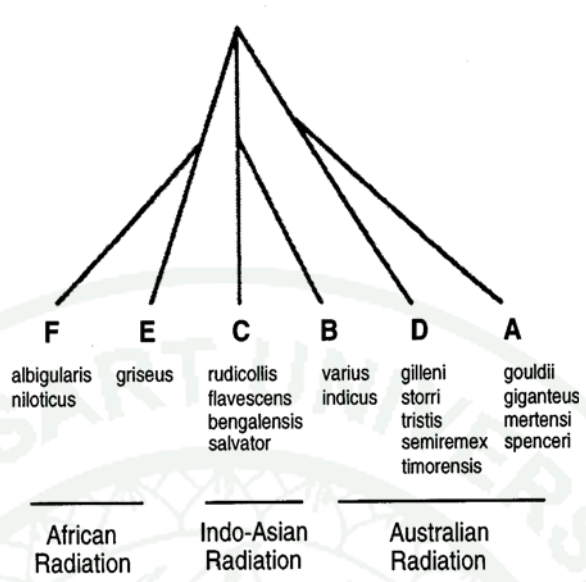

Figure 1 A phylogeny of the *Varanus* based on chromosomal morphology

Source: Fuller *et al*. (1998)

Baverstock *et al*. (1993) used the micro complement fixation technique to generate phylogenetic tree by study of the cross reactivity of antisera albumin-albumin protein microcomplement fixation among varanids (Figure 2) (Fuller *et al*., 1998). According to this study, the *Varanus* was divided into four radiations; Australian, African, Asian and Indo-Asian radiation. This result agrees with previous phylogeny based on chromosomal morphology.

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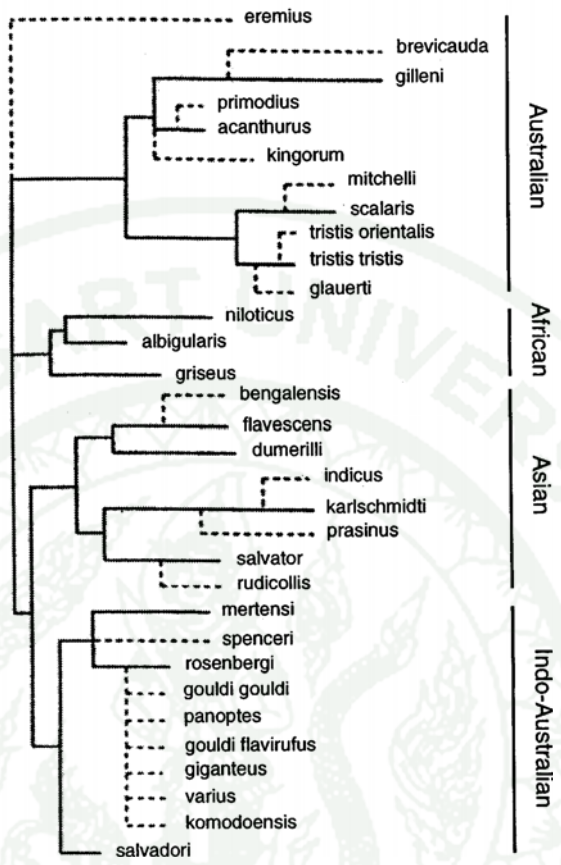

Figure 2 A phylogeny of monitor lizards based on microcomplement fixation (MCF).

Source: Fuller *et al*. (1998)

After Baverstock (1993), Branch (1982) and Böhme (1988) attempted to classify this genus based on hemipeneal morphology. The result of this method showed agreement with chromosomal and immunological information. However, the phylogeny based on lung morphology (Becker *et al*., 1989) did not agree with the other studies, they had difference in the position of the Asia species-cluster relatively to the African cluster and the two Australian clusters (Figure 3).

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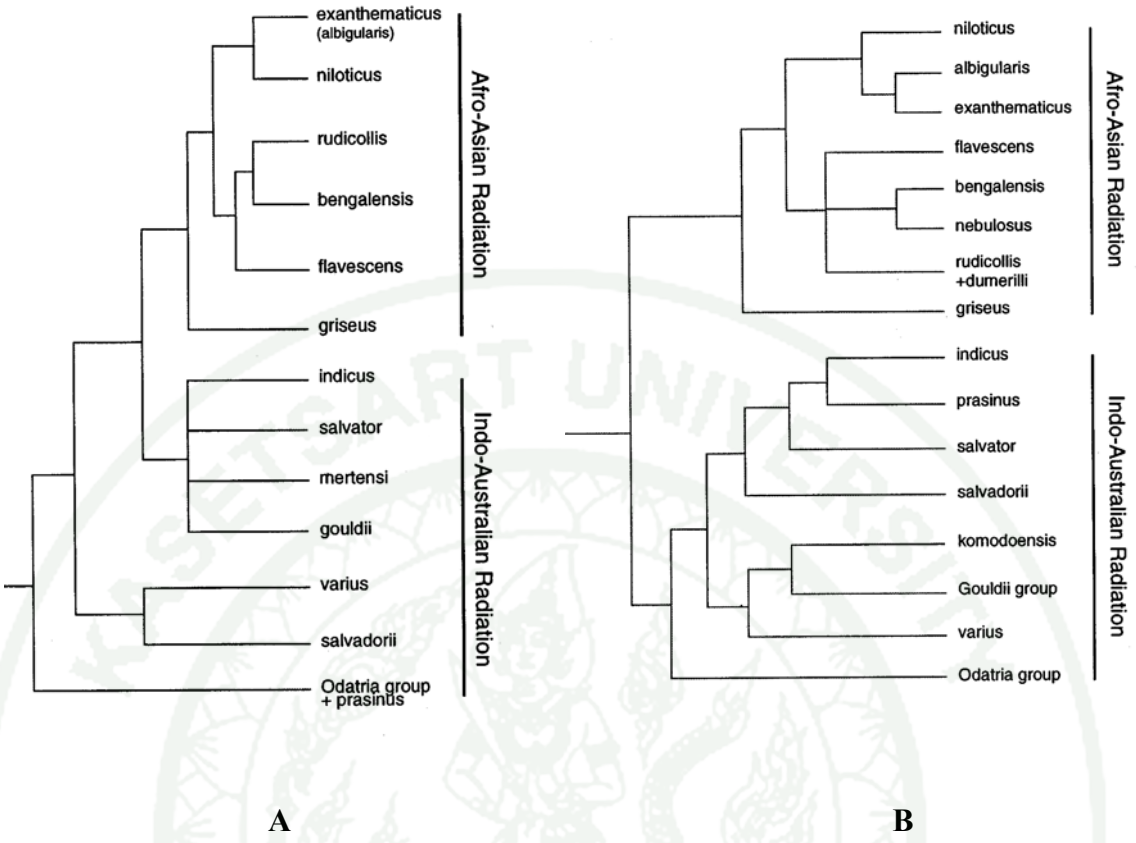

Figure 3 A phylogeny of *Varanus* spp. based on hemipeneal morphology (A) and a tree from lung morphology studied (B).

Source: Fuller *et al*. (1998)

In 1998, Fuller *et al*. analyzed the genetic variation of 12S rRNA sequence and constructed the phylogenetic tree for 23 varanoid based on maximum parsimony method (Figure 4). The phylogenetic tree from this study showed four clades of varanoids, the Asian species was paraphyletic with the Australian species, while the African species were most divergent from Australian taxa. They concluded that the Australian subgenera *Odatria* and *Varanus* had Asia origin. The phylogeny based on 12S rRNA sequences was supported by previous phylogeny based on microcomplement fixation test data, although these trees had major difference because the Australian species using 12S rRNA phylogeny was a single form.

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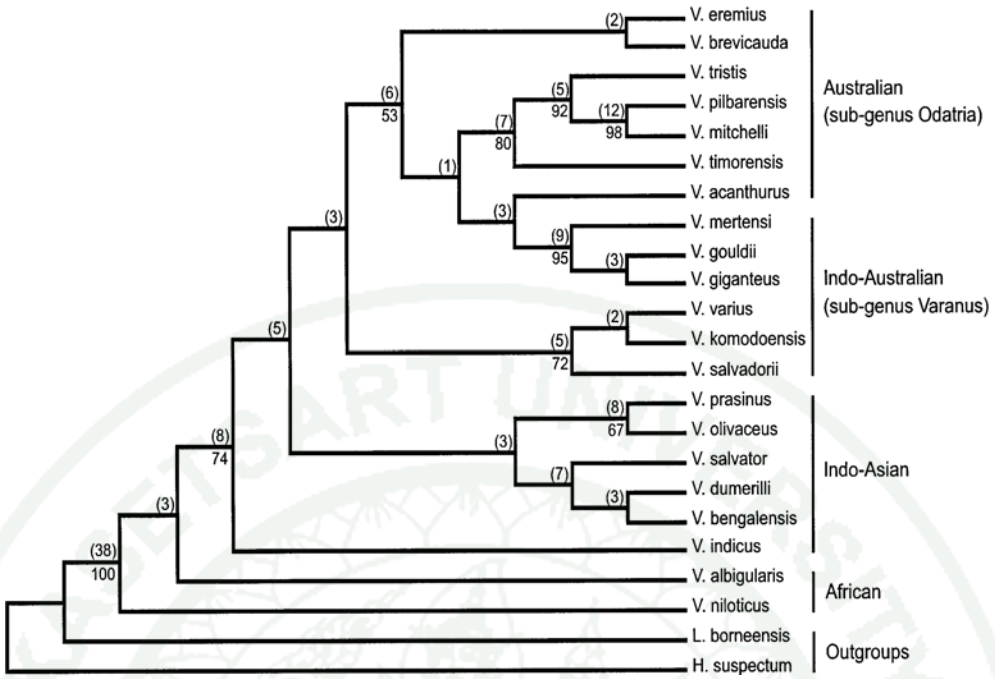

Figure 4 Phylogenetic tree based on 12S rRNA sequence analysis

Source: Fuller *et al*. (1998)

Ast (2001) attempted to classify *Varanus* spp. using 16S rRNA, ND1, ND2 and nine tRNAs genes to test the relationship among varanids. According to this study, the *Varanus* were divided into three major clades, an African clade, an Indo-Asian clade and Indo-Australian clade. This phylogeny showed more details that covered to subspecies taxa (Figure 5).

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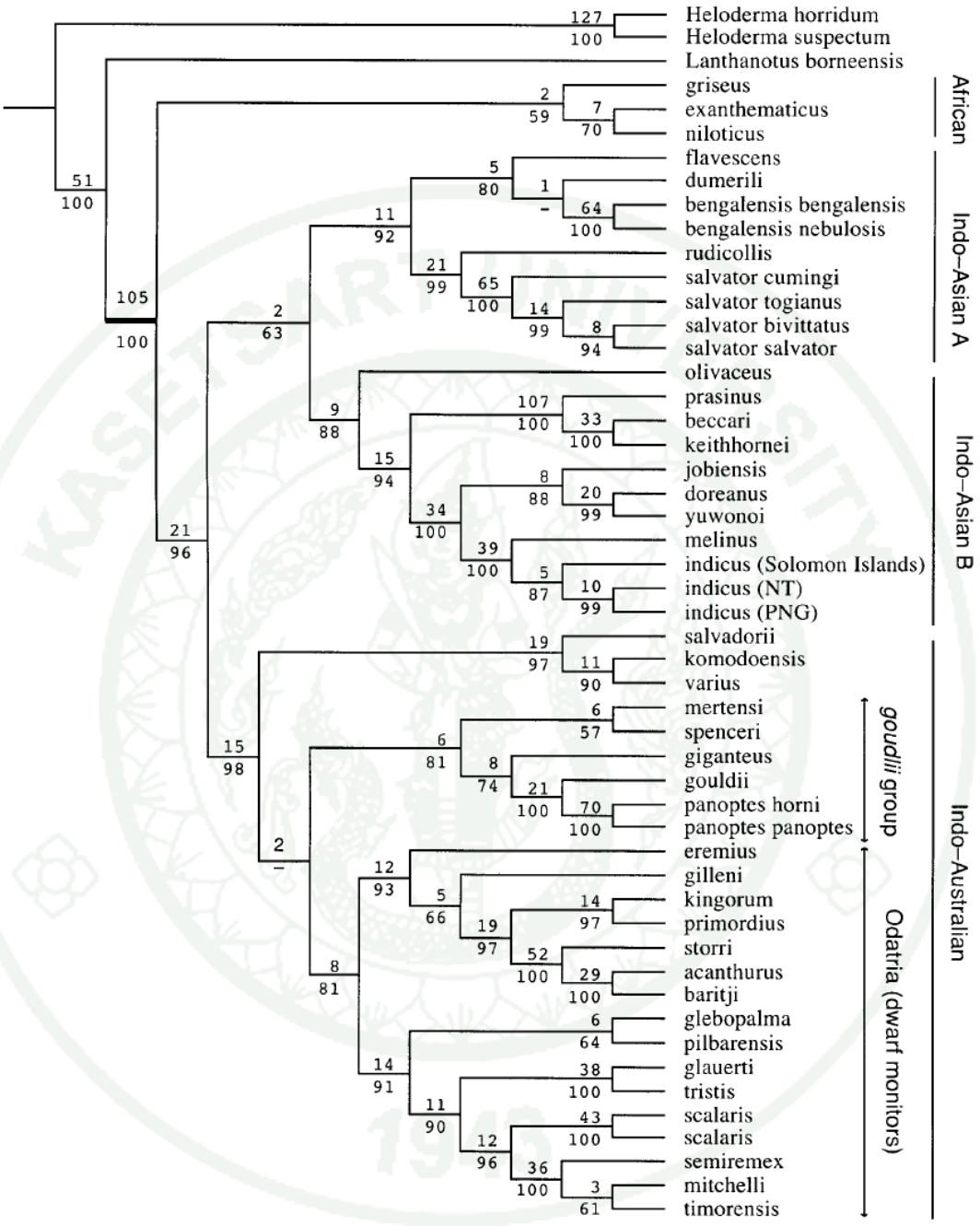

Figure 5 A cladogram of phylogenetic relationship for Varanoidae based on long PCR product sequences from mitochondrial DNA, the fragment recovered many gene such as 16S rRNA, ND1, ND2 and nine tRNAs.

Source: Ast (2001)

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Recently, Schuett *et al*. (2009) used prevalence of bipedal postures for phylogeny in thirty-two *Varanus* spp. This result was broadly supported by the phylogenetic tree using mitochondrial DNA sequences by Ast (2001).

1.2 Morphology

Although monitors have conservative morphology but their body size are varied widely which are approximately 0.2-3.5 m in length or 0.017-70 kg body weight. *Varanus brevicauda* is the smallest monitor while *Varanus komodoensis* is the biggest (King and Green, 1993). The *Varanus* share a small head, long neck, elongated snout, the nares range in position from near the tip of the snout to close to the eyes, the long, slender, bifid smooth and retractable tongue. Body and limbs are sturdy and the tail is long and round or laterally flattened in cross section. Its length in relation to the snout-vent length (SVL) is variable.

Body of these animals is covered with many small juxtaposed scales. The scales on top of head always are small and fragmented, and the scale shapes or scale pattern is different in some species (King and Green, 1993).

1.2.1 The *Varanus salvator salvator* (water monitor)

Water monitor is the second largest monitor and the adult has approximately 220 cm in total length. They have black olive color skin with lines of pale yellow-white spots or ocelli across transversely on the body, and the ventral has yellow markings that form series in diamond shapes, The young and juvenile water monitor has spots which are more brightly and larger than spots in adult. It forms 80-95 transverse rows. Scales at the top of head are larger than nuchal scales and the head's scales are larger than the neck's scale. The scales at the back are larger more than the head's and neck's scales (Lauprasert and Thirakhupt, 1999).

Young water monitor is dark brown or black with small yellow spots and larger round spots in transverse series. The lower part of body is yellow

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with narrow black vertical V-shaped marks. Limbs are black with small whitish spots. The tail of this monitor is striped of black and white color (Lauprasert and Thirakhupt, 1999).

Figure 6 Juvenile Asian water monitor

Figure 7 Adult Asian water monitor

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1.2.2 The *Varanus salvator komaini* (black jungle monitor)

Varanus salvator komaini, synonym is *Varanus salvator macromaculatus* or Black jungle monitor or black water monitor is classified in *Varanus salvator* complex. This subspecies was described by Nutphand *et al*. in 1981 at Satun province in Thailand (Lauprasert and Thirakhupt, 1993). Differently from water monitor (*V. salvator*), the black water monitor is dull blackish skin without any line or dot. It has grey ventral abdomen, grey-bluish tongues and scales are minor difference from water monitor. However, the name of this monitor has been unclear and debatable (Lauprasert and Thirakhupt, 1993).

Figure 8 Black jungle monitor (*V. salvator komaini*)

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1.2.3 The *Varanus dumerilii* (red-headed monitor)

Red-headed monitor is the smallest monitor in Thailand. It is approximately 50-120 cm in total length. The young monitor has red color on the top at head but in adult, it has brown on the top of head. Its dorsolateral side of the head has two black bands that they join at the ventral of neck to form a U-shaped dark band marking. The body is black or black brown, it has three bands of cream dots or cream bands and the ventral is yellow with dark transverse bands. The back has dark transverse bars and the tail is black with yellow transverse bands (Lauprasert and Thirakhupt, 1999).

Figure 9 A red-headed monitor (*V. dumerilii*)

Source: Dusit zoo

1.2.4 The *Varanus rudicollis* (rough-necked monitor)

Rough-necked monitor is the most poorly study of the Asian species. The adult was recorded to reach a maximum size of 146 cm. the name "rudicollis" comes from a word "rudi" that refers to rough and a word "colli" that means to neck. It receives this name because it has very large scales on the neck (nuchal scales), like a dull thorns. Its skin is olive brown with rows of white-yellow

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ocelli at the flank and rear part of body. The tail has transverse board black stripes and narrow pale yellow bands (Lauprasert and Thirakhupt, 1999).

A **B**

Figure 10 The rough-necked monitor (*V. rudicollis*)

1.2.5 The *Varanus bengalensis nebulosus* (clouded monitor)

Clouded monitor in Thailand is about 125 cm in total length. Its skin is dark olive or brown with yellow-white dots or marbles. The young has olive to black color and the chin is strongly barred in bluish and yellow-cream. There are irregular white chevron shaped bars on the neck. Head is black with a series of white spots on the upper lip (Lauprasert and Thirakhupt, 1999).

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Figure 11 The clouded monitor (*V. bengalensis nebulosus*)

1.3 Distribution

Monitors are currently recognized worldwide and all occur in Africa, Asia, especially South-East Asia and Australia, they have greatest diversity in Australia (Pianka, 2004).

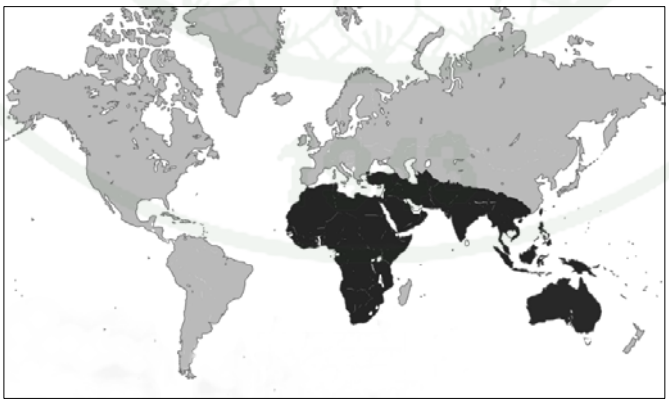

Figure 12 Distribution map of *Varanus* spp. (black area)

Source: Lauprasert and Thirakhupt (1999)

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In Thailand, there are 6 kinds of monitors including common water monitors (*Varanus salvator salvator*), black jungle monitors (*Varanus salvator komaini*), red-headed monitors (*Varanus dumerilii*), rough-necked monitors (*Varanus rudicollis*), clouded monitors (*Varanus bengalensis nebulosus*) and yellow monitors (*Varanus flavescens*) (Lauprasert and Thirakhupt, 1999). Water monitors are found in many countries of Asia such as India, Sri Lang Ka and Thailand. In Thailand, they are common in all parts of Thailand whereas black jungle monitors are found only on beach and some islands in west part of Southern Thailand (Lauprasert and Thirakhupt, 1999). Red-headed monitors live in rainforests in Southern Thailand, Malaysia, Singapore and Indonesia (Lauprasert and Thirakhupt, 1999). Rough-necked monitor are found in the Southern Thailand but also are found in Burma and the Philippines. Clouded monitors inhabit in Asia: India, Thailand and Burma. In the present the yellow monitor is not found. (Lauprasert and Thirakhupt, 1999).

1.4 Feeding and ecology

Almost all monitor lizards are active predator that raid vertebrate nests and eat large vertebrate prey. Some smaller species such as red-headed monitor also feed extensively on invertebrates, including centipedes, large insects, earthworms, crustaceans and snails. Varanids are very active lizards that forage widely, using their forked tongues extensively to locate and discriminate among their prey by scent (Pianka, 2004).

1.5 Economic significance

Many countries in South-East Asia and Africa have been exporting huge numbers of skins of several species such as *V. salvator, V. bengalensis, V. indicus, V. flavescens, V. niloticus* and *V. exanthematicus* for use in the leather goods industry. The major exporters of water monitor lizard skins are Indonesia, the Philippines and Thailand (King and Green, 1993).

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1.6 Conservation status

All varanids are on the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) list. CITES reported that *V. salvator, V. rudicollis and V. dumerilii* are listed in appendix II, while *V. bengalensis nebulosus* is listed in appendix I of the CITES convention (United Nations Environment Programme-World Conservation Monitoring Centre, 2009). In Thailand, all monitor lizards are protected animal under the Wild Animals Reservation and Protections Act of 1992. *V. bengalensis nebulosus, V. dumerilii* and *V. rudicollis* are classified as threatened animal, while *V. salvator komaini* is indicated as critically endangered species (Lauprasert and Thirakhupt, 1999).

2. Overview of mitochondrial DNA (mtDNA)

2.1 Mitochondrial DNA (mtDNA)

Mitochondria are organelles, existed in virtually every cell. Theirs functions associate with energy production to cells, or powerful house of cells. They convert chemical energy to adenosine triphosphate (ATP) by oxidative phosphorylation and electron transport. Some theories suggest that mitochondrion is a bacterium, entered a primordial cell and established a mutually beneficial relationship. Mitochondria compose two distinct membranes, an outer membrane and inner membrane. It contains two compartment including matrix and intermembrane space (Kobilinsky *et al*., 2005). Mitochondria contain their own unique from of DNA, mitochondrial DNA (mtDNA) which replicate automously within the cell. The genome is a closed circle of double-stranded, antiparallel, double-helical DNA. Two strands of mtDNA are called heavy strand (H) and light strand (L), the heavy strand is rich in the purines (adenine and guanine) whereas the light strand is rich in the pyrimidines (cytosine and thymine). Unlike nuclear DNA, mtDNA is passed from mother to offspring without recombination. It is maternally inherited and passes unchanged except spontaneous mutation. In addition, there are several copies of mtDNA whereas contain only one or two copy of nuclear DNA (Kobilinsky *et al*.,

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2005). The mitochondrial genomes consist of approximately 16-18 kb. It encodes for 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs (Bohme *et al*., 2007; Kobilinsky *et al*., 2005; Kumazawa and Endo, 2004; Shen *et al*., 1999). Most coding region is found on heavy strand. The mtDNA has small region known as the control region that controls the transcription of genes within the coding region as well as replication of the genome itself (Kobilinsky *et al*., 2005).

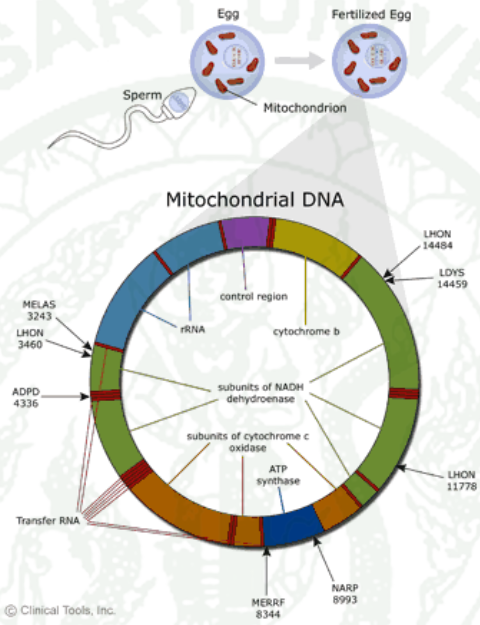

Figure 13 The vertebrate mitochondrial genome

Source: Genetics Education Networking for Innovation and Excellence (GENIE) (2009)

Two reasons of mtDNA are interested for forensic science. The first reason is highly polymorphic on control region; it is useful for person identification. The errors of mtDNA replication and mutations result to the high level of polymorphic. Many studied showed that mtDNA mutates 5-10 times faster than nuclear DNA and the difference in base sequence within this region depending on ethnicity. Another reason, mtDNA has very high copy number within the cell, therefore it is very helpful when analyzing tissues that have little nuclear DNA such as hair and degraded nuclear DNA tissue such as teeth or bone (Kobilinsky *et al*.,

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2005). In addition, mitochondrial DNA can provide a high resolution for distinguishing between closely related organisms (Li and Graur, 1991).

Many studies used mtDNA to generate phylogeny and studied for evolution in several organisms. Many mitochondrial genes are used to study such as 16S rRNA, 12S rRNA, cytochrome b, ND1, ND2 and tRNAs (Amer and Kumazawa, 2008; Bohme *et al*., 2007; Kumazawa and Endo, 2004; Auliya *et al*., 2002; Hsieh *et al*., 2001; Shen *et al*., 1999; Hassinin *et al*., 1998; Fuller *et al*., 1998 Keogh *et al*., 1998). However, cytochrome b gene and 12S rRNA gene are mostly used to study the phylogenetic relationship analysis among species. Cytochrome b is also used to classify or study phylogeny between subspecies (Bradley and Becker 2001; Linacre, 2005).

2.2 The cytochrome b gene

The cytochrome b gene is encoded on heavy strand of mitochondrial genome. It associates with oxidative phosphorylation which is one of the nine proteins that are encoded on mitochondrial genome. It makes up complex III of the mitochondrial oxidative phosphorylation system. There is a wide range of cytochrome b polymorphisms that could exist within different species. Cytochrome b gene sequences analysis is a very sensitive and powerful tool for forensic species identification.

In Taiwan, Hsieh *et al*. (2001) used partial cytochrome b gene to identify species in several conservative animals. They designed a pair of primer from sequence of human mitochondrial DNA. They conclude that the partial sequences of cytochrome b gene adopted in their study proved to be usable for animal identification. Keogh *et al*. (1998) studied the phylogenetic relationship among venomous Australo-Papuan elapid snakes based on sequences of partial cytochrome b gene. The result displayed little intraspecific variation in seven species from twenty-nine species, two species showed larger intraspecific variation. Bradley and Baker (2001) found intraspecific variation in cytochrome b gene sequences of bat population

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which indicated intraspecific variation in cytochrome b gene sequences of some animals. Therefore, it is possible to use the cytochrome b gene to study the population genetic diversity. Lau *et al*. (1998) using cytochrome b gene sequences to study genetic diversity of Asian water buffalo (*Bubalus bubalis*) and found five polymorphic nucleotide positions which can divide Asian water buffalo into 5 haplotype . Chen *et al*. (2006) study genetic variation in 13 indigenous Chinese goat breeds concluded that the cytochrome b gene sequence alone might not be enough for phylogeny analysis among breeds within species because it showed fewer polymorphic sites. Yang *et al*. (2004) also found no intraspecific variation in cytochrome b gene sequences to identify salmon bone from North America; however, the intra- species sequence variation can be found by using D-loop region.

Beside blood or meat specimens, the cytochrome b gene was extracted and amplified from bone or shell specimens. From Hsieh *et al*. (2008) study, they were successful in amplification of partial fragments of cytochrome b gene from turtle shells using universal primers. Furthermore vertebrates, the cytochrome b was used to study for cephalopods identification (Chapela, 2003). This gene fragments were analyzed by using the Forensically Informative Nucleotide Sequencings (FINs) and PCR-RFLP method. They had success in using both techniques to identify species of seventeen food products.

2.2 The 12 small ribosomal RNA (12S rRNA) gene

Like cytochrome b gene, 12S rRNA gene is on heavy strand of mitochondrial DNA. The functional product of this gene is single strand RNA molecule that exhibits extensive secondary structure and binds with ribosomal proteins to form the ribosomal subunits that associates with protein assembly. This gene was preferred to using in molecular phylogeny and identification for several species. Ribosomal RNA is commonly used over a wide range of divergence levels. Sullivan *et al*. (1995) used this gene to constructed phylogeny of Sigmodontine rodents. The conclusion of this study showed that 12S rRNA sequences were high among-site rate variation while the cytochrome b gene sequences were moderate

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level. In contrast the study in by Nobata et al. (2007) attempted to identify Falconifomes in Japan based on PCR-RFLP of partial 12S rRNA gene, they found interspecific variation in most species but they didn't find intraspecific variation among them.

Wang *et al*. (1999) developed the universal primers of 12S rRNA gene based on published 12S rRNA sequences from five animal species including frog, chicken, fish, lizard and green turtle. Their primers succeed to amplify 12S rRNA fragment from different species. Rastogi *et al*. (2004) indicated that the 12S rRNA didn't damaged during extended periods of meat storage or by cooking. This information is useful for forensic study. Korte et al. (2005) used pyrosequencing of partial 12S rRNA gene for species identification. From experimental, they found high degree of variation and they succeed to discriminate between different species. But they did not observed any variation in the same species.

3. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

There are many techniques for genetic diversity study based on DNA base sequences variation include microsatellites or short tandem repeat (STR), variable number tandem repeats (VNTR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), single nucleotide polymorphism (SNP) and DNA sequencing.

Microsatellite loci are tandem repeats of short DNA segments, typically 1-5 bases in length, this is highly variable in different species because slippage during DNA replication. Microsatellites have advantages more than other DNA markers as they combine high variability with nuclear co-dominant inheritance and they can be typed following non-invasive sampling. Although, this method has disadvantages, they must be developed anew for each species, though primers from closely related species (Frankham *et al*., 2002).

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VNTR or minisatellites are DNA sequences that have core repeat sequences with length in the range of 10 to 100 bases. The number of repeats is highly variable between unrelated individuals. Variability in repeat number is generated by unequal crossing-over. Minisatellites DNA fragments are released after restriction enzyme cleaving at the outside the repeat and these fragments are separated on agarose gel electrophoresis (Frankham *et al*., 2002). This method is used to identify species or strain for several bacteria. The disadvantages of this method are that individual loci are not normally identifiable and the inheritance bands is not defined and they cannot be typed following non-invasive sampling as they require considerable amounts of DNA (Frankham *et al*., 2002).

RAPD is random primer sequences using for PCR amplification, usually 10-20 bp in length, these yield a series of DNA fragments, which separated on agarose gel. Typically, several fragments in the 100-200 bp in length are detected for each primer. If there is variation in the priming site in the DNA, then some bands will reveal a presence-absence pattern. RAPDs have been widely used in plants, but less so in animals. However, this method has low repeatability (Frankham *et al*., 2002).

AFLP is method that ligates the short synthetic DNA fragments to restriction genomic DNA fragments which are digested with restriction enzymes and subsequently, these fragments are amplified by PCR. This generated PCR products that widely variable in sizes on agarose gel electrophoresis (Frankham *et al*., 2002; Blears *et al*., 1998).

SNPs are a position in the DNA of a species at which two or more alternative bases occur at appreciable frequency. These are detected by sequencing or using DNA chips. SNPs play important in population genetics and evolutionary studies (Frankham *et al*., 2002).

DNA sequencing is the best measure for genetic diversity studies. It is high reliability but it is expensive and requires many times and labors.

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Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) is a technique for study genetic diversity. It is a classic and inexpensive method of genotyping that is based on restriction endonuclease cleavage (Panneerchelvam and Norazmi, 2003). This method is combination of PCR and RFLP technique.

A restriction enzyme or restriction endonuclease is an enzyme that cut both double-strand and single-strand by breakdown phosphodiester bond. It is a bacterial enzyme that providing the defense mechanism against invading viruses. In bacterial cells, the restriction enzymes cut foreign DNA by the process that associated with methyltransferase and endonuclease enzymes that called "Restriction modification or RM system". The methylation is bacterial process to protect DNA from cutting by self restriction enzyme's activity. The restriction endonuclease is divided into 3 type including type I, type II and type III based on their composition, cofactor requirement, the nature of their target sequences and the position of their DNA cleavage sites (Aggarwal *et al*., 1995).

Type II restriction endonuclease is useful for PCR-RFLP technique because it has only endonuclease activity and cleaves DNA at specific recognition sequence that it is palindromic sequence. Because type II endonucleases digest DNA at specific recognition site, so they generate a number of DNA fragments that vary in length. Polymorphism in the DNA sequences of individuals or species can cause one or more sites to be lost or gain or may otherwise cause variation between individuals in these fragments length. If the DNA fragments are placed on the gel and an electric field is applied, the different size of DNA fragments can move at varying distances across the gel. This evidence generates the restriction patterns that specific to each individuals or species. These patterns like the supermarket bar codes, it is relatively easy to determine that two samples are different.

Many studies analyzed DNA fragments with PCR-RFLP technique. They generated many restriction patterns that specific to each species including both prokaryotes: bacteria (Okhravi *et al*., 2000; Poussier *et al*., 2000; Kawasaki *et al*.,

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2008) and eukaryotes: fungi (Hajek *et al*., 2003), mammals (Alifakiotis *et al*., 2003; Ferreira *et al*., 2005), reptiles (Moore *et al*., 2003; Giovannotti *et al*., 2007), birds (Ballantyne *et al*., 2004; Dybus and Knapik, 2005; Nobata *et al*., 2007), insects (Szalanski and McKern, 2007) and fishes (Aranishi *et al*., 2004; Cheng and Lu, 2005). Zehner *et al*. (1998) used this method for identification the origin of meat using cytochrome b gene. The conclusion of this study is that ii is possible to identify various animals by using PCR-RFLP and this method was useful in cases of mixed samples. However, they found that species with only a short evolutionary distance might not be differentiated by this method, because they shared in RFLP patterns. Wolf *et al*. (1999) also used this method to identify meat species for game meat products. They treated samples with nineteen endonucleases and found different RFLP patterns in various species. In the case of same species, this technique did not show intraspecific variation except in only one species. Thus, PCR-RFLP technique was efficient identification tool. It was simple, reliable and it had repeatability. This method could show variation between different species but in the same species, it has been debated because some species had low level of polymorphism.

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MATERIALS AND METHODS

1. Samples collection

EDTA-blood samples (Table 1) were collected via median tail vein about 1.5-2 ml by aseptic techniques from 22 monitors and were kept at -20 ºC until used.

Table 1 Numbers of samples

Common name	Sciencetific name	Number (n)
Asian water monitor	*Varanus salvator salvator*	15
black water monitor	*Varanus salvator salvator*	2
red-headed monitor	*Varanus dumerilii*	3
rough-necked monitor	*Varanus rudicollis*	1
clouded monitor	*Varanus bengalensis nebulosus*	1

2. Amplification of partial mitochondrial cytochrome b gene and 12S rRNA gene.

2.1 DNA extraction.

DNA was extracted from EDTA-blood by Phenol-Chloroform method adapted from described earlier (Sambrook and Russel, 2001). Briefly, 50 µl of whole blood of monitor were mixed with 500 µl of Denaturing-solution (4 M guanidine thiocyanate, 50 mM TrisCl, 20 mM EDTA) and were incubated at the room temperature for 5 minutes. Subsequently, 200 µl each of phenol and chloroform were added, vortexed and centrifuged at 13,000 rpm for 5 minutes. DNA were precipitated with 700 µl of absolute isopropanol and centrifuged at 13,000 rpm for 10 minutes. DNA pellet was washed with 75% ethanol and were centrifuged at 13,000 rpm for 5 minutes. Finally, the DNA pellet was air-dried and resuspended in 300 µl of TE buffer (pH 8). The concentration and purity of DNA were measured by spectrophotometer (Beckman Coulter, Indianapolis, USA) at A_{260} and A_{280}.

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2.2 Cytochrome b gene and 12S rRNA gene amplification

Partial cytochrome b and 12S rRNA genes were amplified using 2 sets of primer (Table 2) that previous described (Kocher *et al*., 1989; Hassanin and Douzery, 1998; Auliya *et al*., 2002). Amplification reaction mixture were prepared in a volume of 20 µl containing 200 µM of each deoxynucleoside triphosphates (dNTPs) (Fermentus, Vilnius, Lithuania), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.5 µmol of each primer (Forward primer and reverse primer), 2.5 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 2 µl of template DNA (Innis *et al.,* 1990). The reaction was performed using a thermal cycle (PTC 200 DNA engine, MJ Research, Watertown, MA, USA). After an initial denaturation at 95°C for 5 minutes, the PCR profile were set as follows: 1 minute of denaturation at 95°C, 1 minute of primer annealing at 53°C for cytochrome b gene or at 55ºC for 12S rRNA and 1 minute of primer extension at 72°C, for a total of 35 cycles, with a final extension at 72°C for 10 minutes. After the completion of amplification, 9 µl of the reaction mixture were fractionated by electrophoresis (100V for 23 minutes) on a 1.0% (wt/vol) agarose gel containing 1X TAE (40 mM Tris-acetate (pH 8.0), 1mM EDTA) and gel was stained with ethidium bromide (0.5 µg/ml). DNA bands were visualized under UV light.

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Table 2 Sequence sets of primers

Name	Sequences (5′→3′)	Length (bp)
L14841 (cytb forward)	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	35
H15149 (cytb reverse)	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A	34
L1091 (12S rRNA forward)	AAA AAG CTT CAA ACT GGG ATT AGA TAC CCC ACT AT	35
H1478 (12S rRNA reverse)	TGA CTG CAG AGG CTC ACG GGC GGT GTG T	28

3. Transformation

PCR products were extracted and purified from agarose gel by QIA® quick gel extraction kit, (QIAGEN) according to manufacturer recommendation. The purified PCR products were ligated with pGEM-T® Easy vector (Promega) and the ligated plasmids were used to transform into DH5-α *E.coli* (Gibco) competent cells. Briefly, 3 µl of PCR products were transferred into the 1.5 ml microcentrifuge tube and mixed with recombination of 5 µl of 2X ligation buffer, 1 µl of pGEM-T® easy vector and 1 µl of T4 DNA ligase (on ice) respectively. The reaction was incubated at 4ºC for overnight. After ligation, the ligated vector was transformed into DH5-α *E.coli* competent cells. Ten microliters of ligation mixture were mixed with 100 µl of DH5-α competent cells and incubated on ice for 30 min. Then they were shocked by heat at 42ºC for 90 sec. Immediately, the reaction solution were incubated on ice for 5 min. Subsequently, transformed *E. coli* were incubated with 900 µl LB broth at 37ºC for 1 hour and were harvested by centrifugation at 4,000 rpm for 2 min. The pellet was broken by pipetting up and down and were used to spread on LB agar containing 20 µl of 50 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal), 100 µl of 0.1 M isopropyl-ß-D-thiogalactopyranoside (IPTG) and 20 µl of 100 mg/ml ampicillin and incubated at 37ºC for overnight. Only white colony was selected (approximately 10 colonies) and subcultured on LB agar containing with IPTG, X-gal and ampicillin (20

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mg/ml). Single colony of the *E. coli* was selected and grown in 7 ml LB broth containing 100mg/ml ampicillin and incubated overnight with 200 rpm constant rotation. Plasmids were extracted by GeneJET™ plasmid miniprep kit (Fermentus, Vilnius, Lithuania) accordingly the manufacturer protocol. *E. coli* in LB broth was centrifuged at 8,000 rpm for 2 min. The pelleted cells were resuspended in 250 µl of resuspension solution and the cell suspension was transferred to a microcentrifuge tube. Two hundred fifty microlitres of lysis solution were added the cell suspension and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. The lysis reaction were neutralized by 350 µl of the neutralization solution and mixed immediately by inverting for 4-6 times. For cell debris and chromosomal DNA sedimentation, the tube were centrifuged for 5 min at 13,000 rpm, transferred to the supplied GeneJET™ spin column and centrifuged for 1 min at 13,000 rpm. The GeneJET spin column were washed with 500 µl of washing solution and centrifuged for 30-60 seconds. This step was repeated 3 times. The GeneJET spin column were transferred into a fresh 1.5 ml micro centrifuge tube and 50 µl of the Elution buffer were added into the center of GeneJET spin column membrane to elute the plasmid DNA. The spin column were incubated for 2 min at room temperature and centrifuged for 2 min at 13,000 rpm. The purified plasmid DNA was stored at -20ºC until used.

4. DNA sequencing and sequencing analysis

Purified plasmids DNA were analyzed at the 1st base sequencing laboratory (Ward medic, Malaysia). Sequence homology was evaluated using the software BLASTN 2.0 and FASTA 3.0 together with the EMBL-nucleotide sequence database (EMBL, outstation EBI, Hinxton, UK) via internet. DNA sequences were analyzed in online program, NEB cutter v.2.0 for endonucleases selection.

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5. Restriciton analysis

The purified PCR products of 12S rRNA gene were treated with endonucleases; ApaI, BamHI and HaeIII (fast digest®, Fermentus). The PCR products of cytochorme b gene were treated with BstXI, BanI, HpaII and EcoRI (fast digest®, Fermentus). Followed the product recommendation, the restricted reaction was prepared in 30 µl that contained with 17 µl distilled water, 10 µl of 0.2 µg PCR products, 1 U fast digest® endonuclease, 2 µl of 10X fast digest® buffer (Fermentus). The reaction mixture was mixed and incubated at 37˚C for 30 minutes. Finally, the DNA fragments were separated on 2% agarose gel electrophoresis.

6. Phylogenetic analysis

All sequences of cytochrome b gene and 12S rRNA gene were aligned with stand alone version of ClustalW program and analyzed by Mega version 4.0 software to generate the phylogenetic tree. These sequences were computed based on Juke and Cantors model to calculate the genetic distances and constructed the trees with Minimum Evolution (ME) and Maximum Parsimony methods with 1,000 replications of bootstrapping test. All sequences were analyzed to observe numbers and positions of polymorphism by DnaSP version 4.0 software.

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RESULTS

1. Construction of the partial mitochondrial 12S rRNA and cytochorme b gene using PCR

1.1 The partial 12S rRNA gene construction

Total DNA was extracted from EDTA-blood and amplified using 12S rRNA universal primers. The size of PCR products were approximately 450 bp in length (Figure 14).

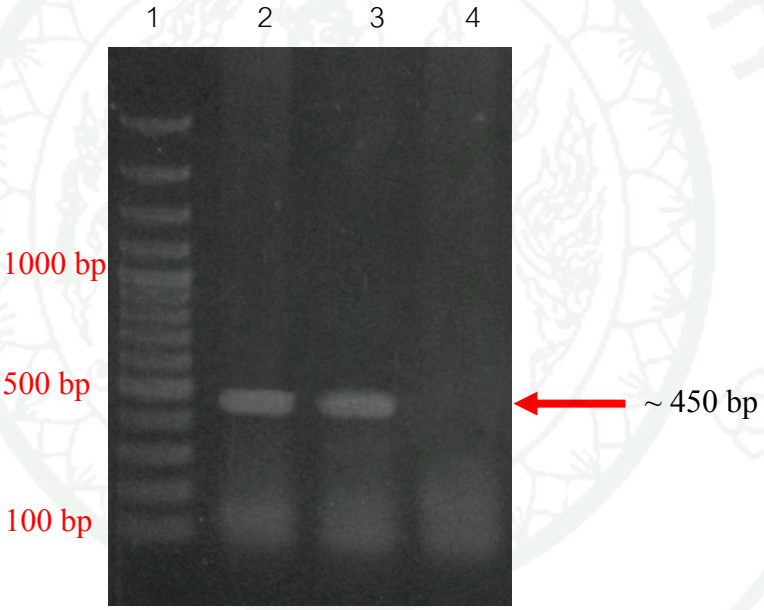

Figure 14 1% Agarose gel electrophoresis of the PCR products of partial mitochondrial 12S rRNA gene. Lane 1: 100 bp DNA marker; lane 2: Monitor's 12S rRNA; lane 3: positive control (Orangutan's 12S rRNA) and 4: negative control (no template control).

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1.2 The mitochondrial cytochrome b gene construction

Similar to 12S rRNA, EDTA-blood samples were extracted and amplified using a pair of universal primers for vertebrate's cytochrome b gene. The PCR products were approximately 376 bp in length (Figure 15).

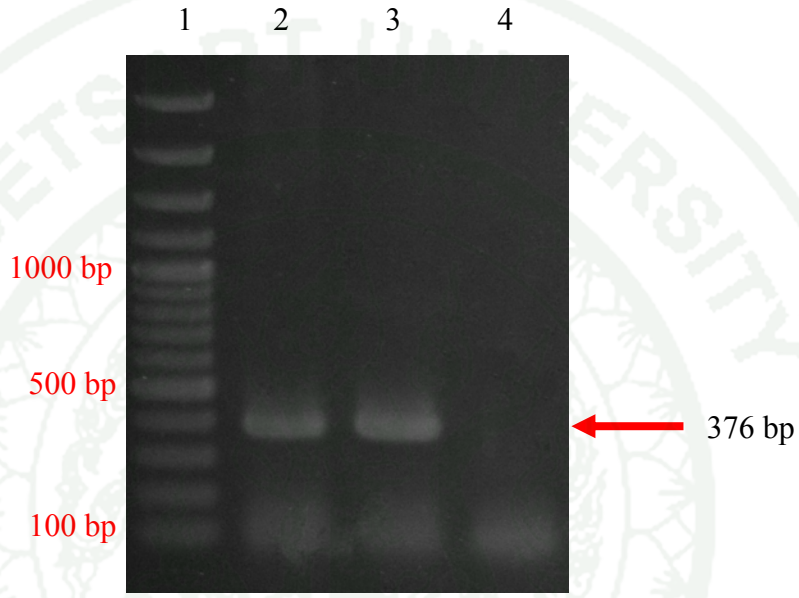

Figure 15 1% Agarose gel electrophoresis of the PCR products of partial cytochrome b gene. Lane 1: 100 bp DNA marker; lane 2: Monitor's cytochrome b; lane 3: positive control (Orangutan's cytochrome b) and 4: negative control.

1.3 Cloning of 12S rRNA PCR products into plasmid pGEM-T® Easy

This step was made to amplify the target DNA for the sequencing. After the purification of PCR product with Gel extraction kit (QIAGEN), the purified product was amplification by DNA cloning. The PCR product of partial 12S rRNA fragment was inserted into plasmid pGEM-T Easy and was used to transform into *E. coli* DH5-α competent cells. The positive clones were confirmed by PCR using T7 and SP6 specific primers flanking between cloning sites. On 1% agarose gel electrophoresis, the PCR products were approximately 600 bp in length (Figure 16).

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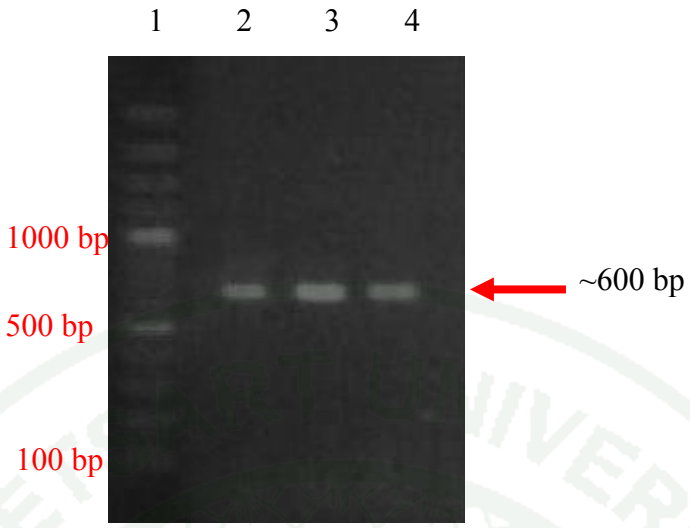

Figure 16 1% Agarose gel electrophoresis of the PCR products of the partial 12S rRNA gene using T7 and SP6 specific primers. Lane 1: 100 bp DNA marker, lane 2-4: PCR product from colonies grown on selective medium.

1.4 Cloning of partial cytochrome b into plasmid pGEM-T® Easy

The purified PCR product of partial cytochrome b gene was ligated with plasmid vector pGEM-T Easy and was used to transform into *E. coli* competent cell for target DNA production. The plasmid which had the target DNA were purified and sent to sequencing laboratory. The positive clones were confirmed by PCR using T7 and SP6 specific primers flanking between cloning sites. On 1% agarose gel electrophoresis, the PCR products were approximately 500 bp in length (Figure 17).

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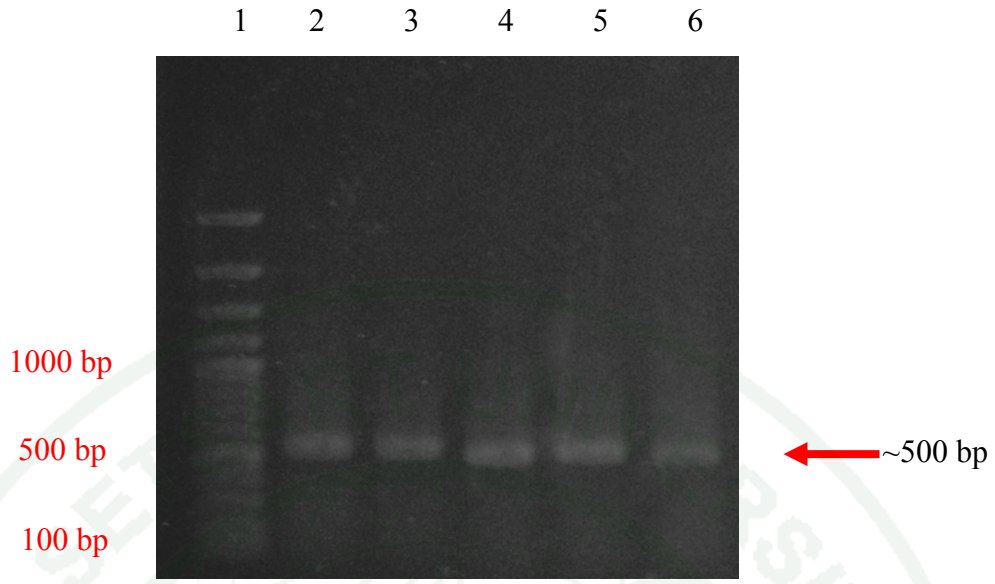

Figure 17 1% Agarose gel electrophoresis of the PCR products of the partial cytochrome b gene using T7 and SP6 specific primers. Lane 1: 100 bp DNA marker, lane 2-4: PCR product from colonies grown on selective medium.

1.5 Restriction patterns

1.5.1 Restriction patterns of partial 12S rRNA for differentiation of *Varanus* spp.

The PCR products of partial 12S rRNA fragments were digested with ApaI, BamHI and HaeIII restriction endonucleases. There were 4 restriction patterns that were specific to each species (Figure 18-20).

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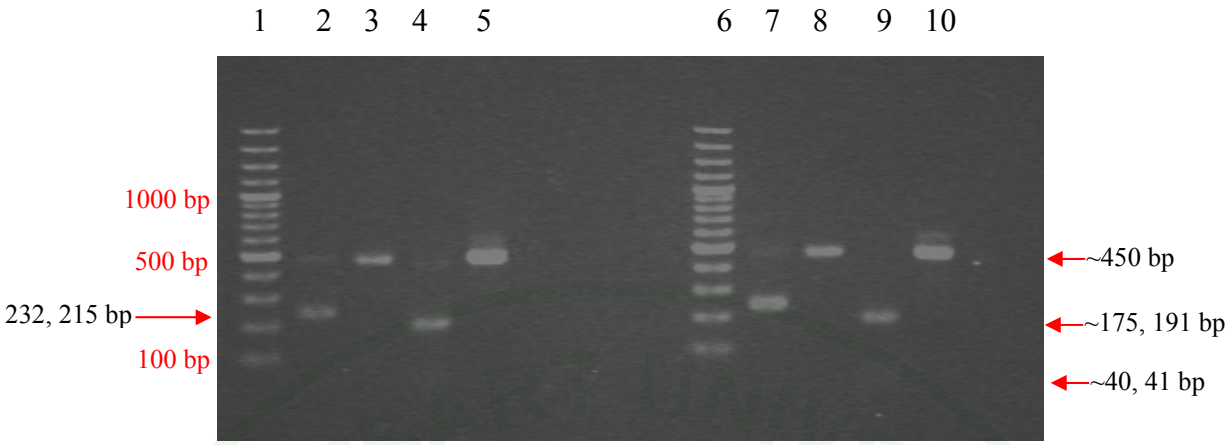

Figure 18 Restriction patterns of the partial 12S rRNA fragments of Asian water monitor (lane2-5) and black jungle monitor (lane 7-10). Lane 1 and 6: 100 bp standard DNA markers, lane 2 and 7: digested with ApaI restriction endonuclease, lane 3 and 8: digested with BamHI restriction endonuclease, lane 4 and 9: digested with HaeIII restriction endonuclease, lane 5 and 6: Uncut DNA (the 12S rRNA of Asian water monitor and black jungle monitor respectively).

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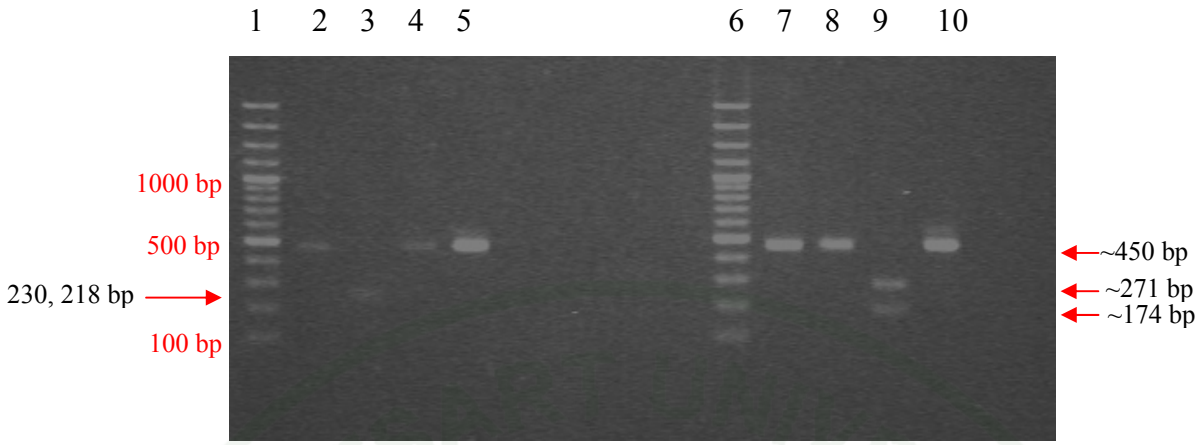

Figure 19 Restriction patterns of the partial 12S rRNA fragments of red-headed monitor (lane2-5) and rough-necked monitor (lane 7-10). Lane 1 and 6: 100 bp standard DNA marker, lane 2 and 7: digested with ApaI restriction endonuclease, lane 3 and 8: digested with BamHI restriction endonuclease, lane 4 and 9: digested with HaeIII restriction endonuclease, lane 5 and 6: Uncut DNA (the 12S rRNA of red-headed monitor and rough-necked monitor respectively).

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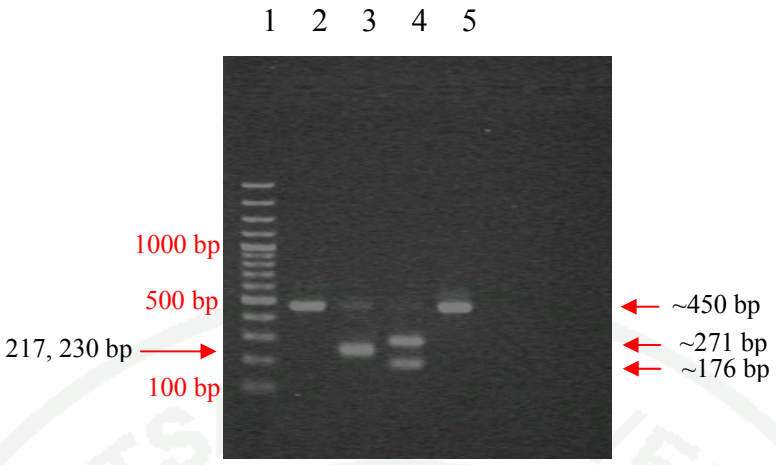

Figure 20 Restriction patterns of the partial 12S rRNA fragments of clouded monitor. Lane 1: 100 bp standard DNA marker, lane 2: digested with ApaI restriction endonuclease, lane 3: digested with BamHI restriction endonuclease, lane 4: digested with HaeIII restriction endonuclease and lane 5: Uncut DNA (the 12S rRNA of clouded monitor).

ApaI restriction endonuclease digested only the partial 12S rRNA of *V. salvator* complex and gave two DNA fragments of approximately 232 and 215 bp in length (Figure 18). BamHI digested the partial 12S rRNA of red-headed monitor and clouded monitor into two fragments that were approximately 218 and 230 bp in length (Figure 19, 20). But it can visualize only one band on 2% agarose gel. HaeIII digested the partial 12S rRNA of water monitor and black jungle monitor into 4 fragments which were approximately 40, 41, 175 and 191 bp in length (Figure 18), whereas the clouded monitor and rough-necked monitor were cut into two fragments which were approximately 271 and 174-176 bp in length (Figure 19, 20).

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Table 3 Numbers and sizes of restriction fragments of 12S rRNA gene

name of monitors	Restriction enzymes		
	ApaI	BamHI	HaeIII
	GGGG↓ CC	G ↓ GATCC	GG ↓ CC
Asian water monitor	215/232	447	40/41/175/191
black jungle monitor	215/232	447	40/41/175/191
red-headed monitor	448	218/230	447
rough-necked monitor	445	445	174/271
clouded monitor	447	217/230	176/271

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1.5.2 Restriction patterns of partial cytochrome b gene for differentiation of *Varanus* spp.

The PCR products of partial cytochrome b were digested with BstXI, BanI, HpaII and EcoRI restriction endonucleases. There were five different restriction patterns of all five species of monitor lizards (Figure 21-25).

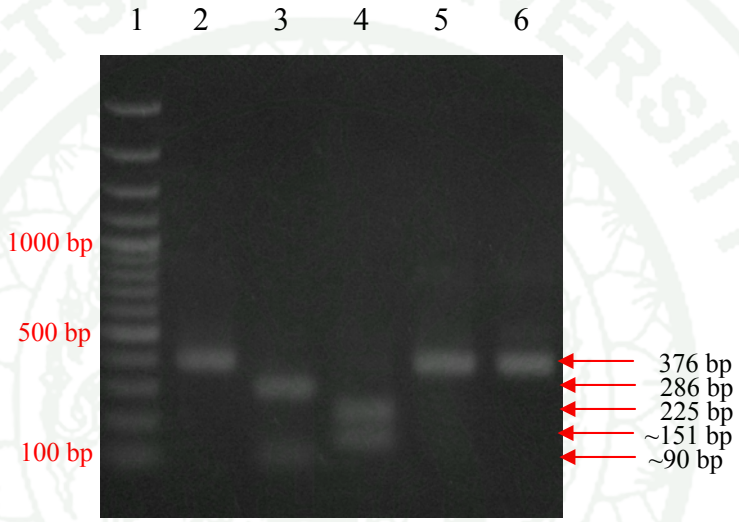

Figure 21 Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of Asian water monitor, lane 1: 100 bp standard DNA marker, lane 2: restriction fragment from BstXI treatment, lane 3: BanI treatment, lane 4: HpaII treatment, lane 5: EcoRI treatment and lane 6: uncut of the partial cytochrome b gene.

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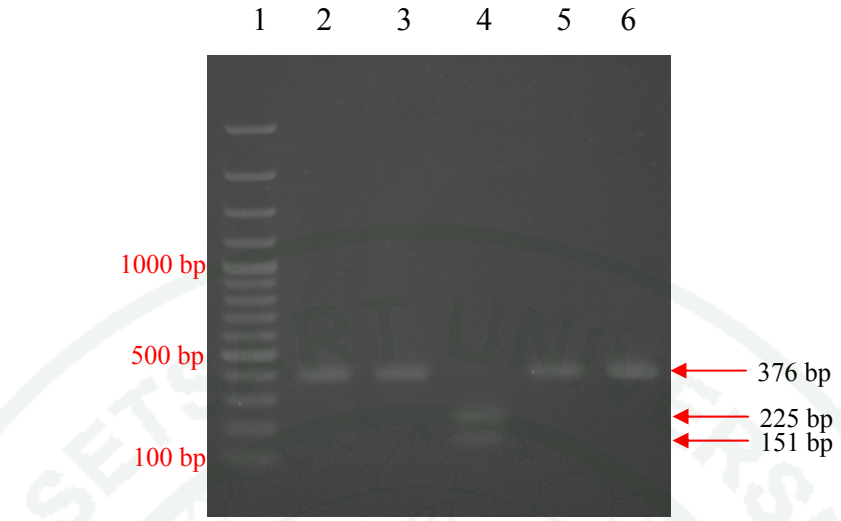

Figure 22 Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR products of black jungle monitor; lane 1: 100 bp standard DNA marker, lane 2: restriction fragment from BstXI treatment, lane 3: BanI treatment, lane 4: HpaII treatment, lane 5: EcoRI treatment and lane 6: uncut of the partial cytochrome b gene.

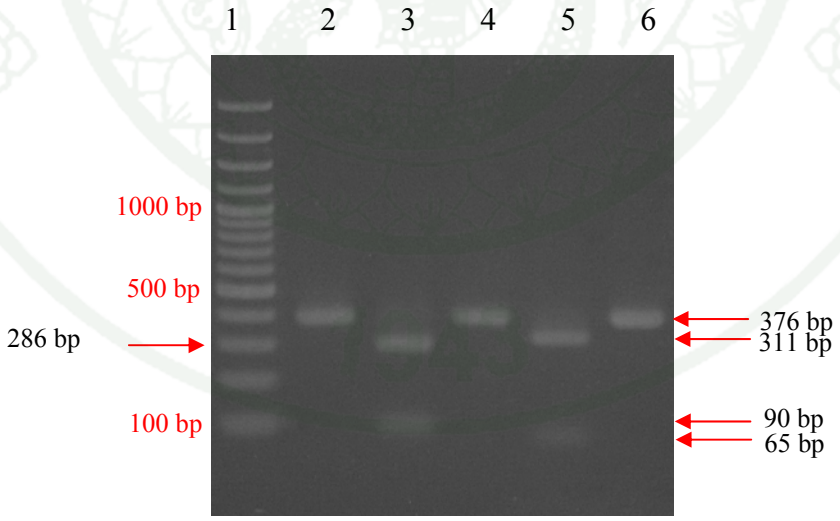

Figure 23 Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of red-headed monitor; lane 1: 100 bp standard DNA marker, lane 2: restriction fragment from BstXI treatment,

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lane 3: BanI treatment, lane 4: HpaII treatment, lane 5: EcoRI treatment and lane 6: uncut of the partial cytochrome b gene.

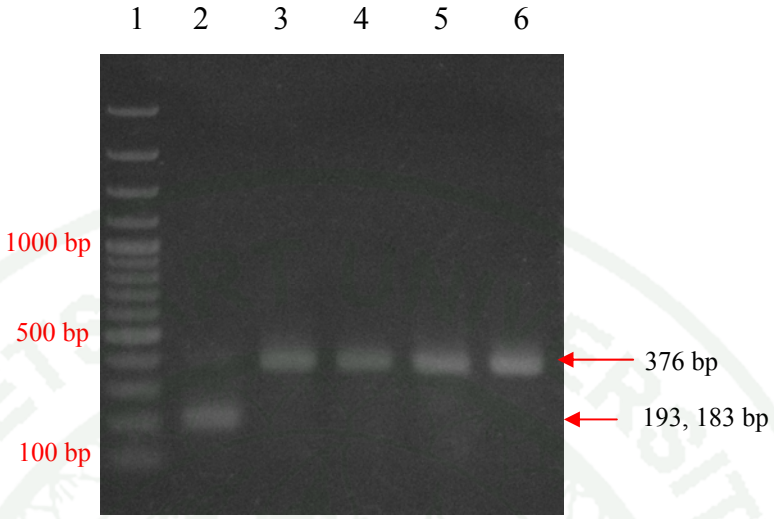

Figure 24 Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of rough-necked monitor; lane 1: 100 bp standard DNA marker, lane 2: restriction fragment from BstXI treatment, lane 3: BanI treatment, lane 4: HpaII treatment, lane 5: EcoRI treatment and lane 6: uncut of the partial cytochrome b gene.

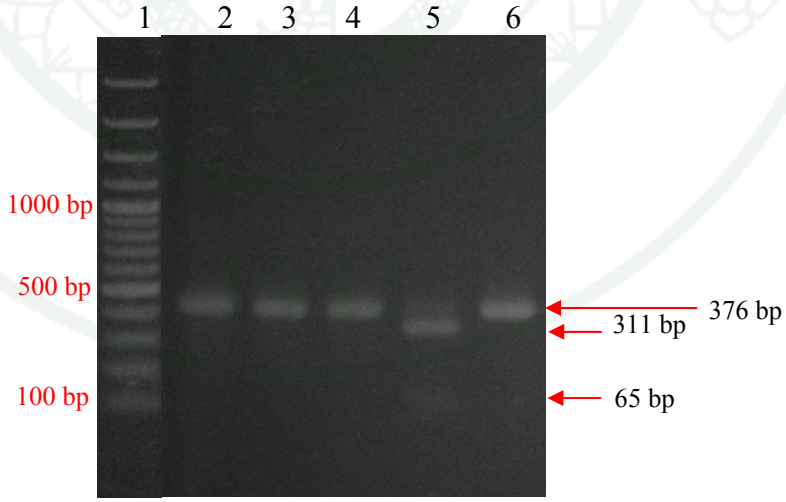

Figure 25 Restriction patterns with BstXI, BanI, HpaII and EcoRI treatment of the cytochrome b gene PCR product of clouded monitor; lane 1: 100 bp standard DNA marker, lane 2: restriction fragment from BstXI treatment,

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lane 3: BanI treatment, lane 4: HpaII treatment, lane 5: EcoRI treatment and lane 6: uncut of the partial cytochrome b gene.

BstXI digestion, only the cytochrome b gene of rough-necked was digested and generated two fragments of approximately 183 and 193 bp in length (Figure 24). BanI digested cytochrome b gene of Asian water monitor and red-headed monitor into two DNA fragments of approximately 286 and 90 bp in length (Figure 21, 23). HpaII digested cytochrome b gene of *Varanus salvator* complex into 2 fragments of approximately 225 and 151 bp in length (Figure 21-22). For EcoRI digestion, only cytochrome b gene of clouded monitor can be cut and gave 2 fragments of approximately 65 and 311 bp (Figure 25). There were five restriction patterns generated by four restriction endonucleases digestion which was specific to each species of monitors.

Table 4 Numbers and sizes of restriction fragments of cytochrome b gene

name of monitors	Restriction enzymes			
	BstXI	BanI	HpaII	EcoRI
	CCANNNNN / NTGG	G / GYRCC	CC / GG	G / AATTC
Asian water monitor	376	90/286	151/225	376
black jungle monitor	376	376	151/225	376
red-headed monitor	376	90/286	376	65/311
rough-necked monitor	183/193	376	376	376
clouded monitor	376	376	376	65/311

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1.6 Numbers of Polymorphism

1.6.1 Polymorphism for 12S rRNA gene sequences

Twenty-two sequences of 12S rRNA gene were analyzed with DnaSP software to observe positions and numbers of polymorphism among sequences. The result displayed 64 polymorphisms from 386 nucleotides (Figure 26).

```
[                10        20        30        40        50        60    ]
[                 *         *         *         *         *         *    ]
VSS1Thai   CGGCTAATCTACCGCACCCGACCAGCCTCCACA-TACCTGCTAAAGC-CTCTCCAGTCACTCCT
VSS2Thai   ................................-...........-.................
VSS3Thai   ................................-...........-.................
VSS4Thai   ................................-...........-.................
VSS5Thai   ................................-...........-.................
VSS6Thai   ................................-...........-.................
VSS7Thai   ................................-...........-.................
VSS8Thai   ................................-...........-.................
VSS9Thai   ................................-...........-.................
VSS10Thai  ................................-...........-.................
VSS11Thai  ................................-...........-.................
VSS12Thai  ................................-...........-.................
VSS13Thai  ................................-...........-.................
VSS14Thai  ........T.....T.................-...........-.................
VSS15Thai  ................................-...........-.................
VSK1Thai   ................................-...........-.................
VSK2Thai   ................................-...........-.................
VD1Thai    AAATC.T..C..AA...T.A..ATAT..T.CG.AC.T..AGC..GA.-....TT.A...AC...
VD2Thai    AAATC.T..C..AA...T.A..ATAT..T.CG.AC.T..AGC..GA.-....TT.A...AC...
VD3Thai    AAATC.T..C..AA...T.A..ATAT..T.CG.AC.T..AGC..GA.-....TT.A...AC...
VRThai     T.A.CC.C.C...A..-.....A.A.A..AC..-CG.ACA.-.G...-.C..TT.........C
VBThai     T.ATCCC..CGA.A.G..TACATTAT.CTTCA--C....AA.G..ATAT.TCTTG.CTT..TT.
```

Figure 26 Nucleotide alignment of 12S rRNA gene sequences. Only the segment exhibiting differences was shown.

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There was no polymorphism in the sequence of black water (*V. salvator komaini*) monitor and Asian water monitor (*V. salvator salvator*), except one Asian water monitors (VSS14) had transition of two cytosine bases to thymine bases.

1.6.2 Polymorphism for cytochrome b gene sequences

Cytochrome b gene sequences of 22 monitors were analyzed with DnaSP software and revealed 79 positions of polymorphism from 307 characters. The polymorphisms can be found in the Asian water monitor group which had transition of thymine base to cytosine base in sequences of VSS4 and VSS8, adenine base to guanine base in VSS6 sequence and guanine to adenine in VSS13 (Figure 27).

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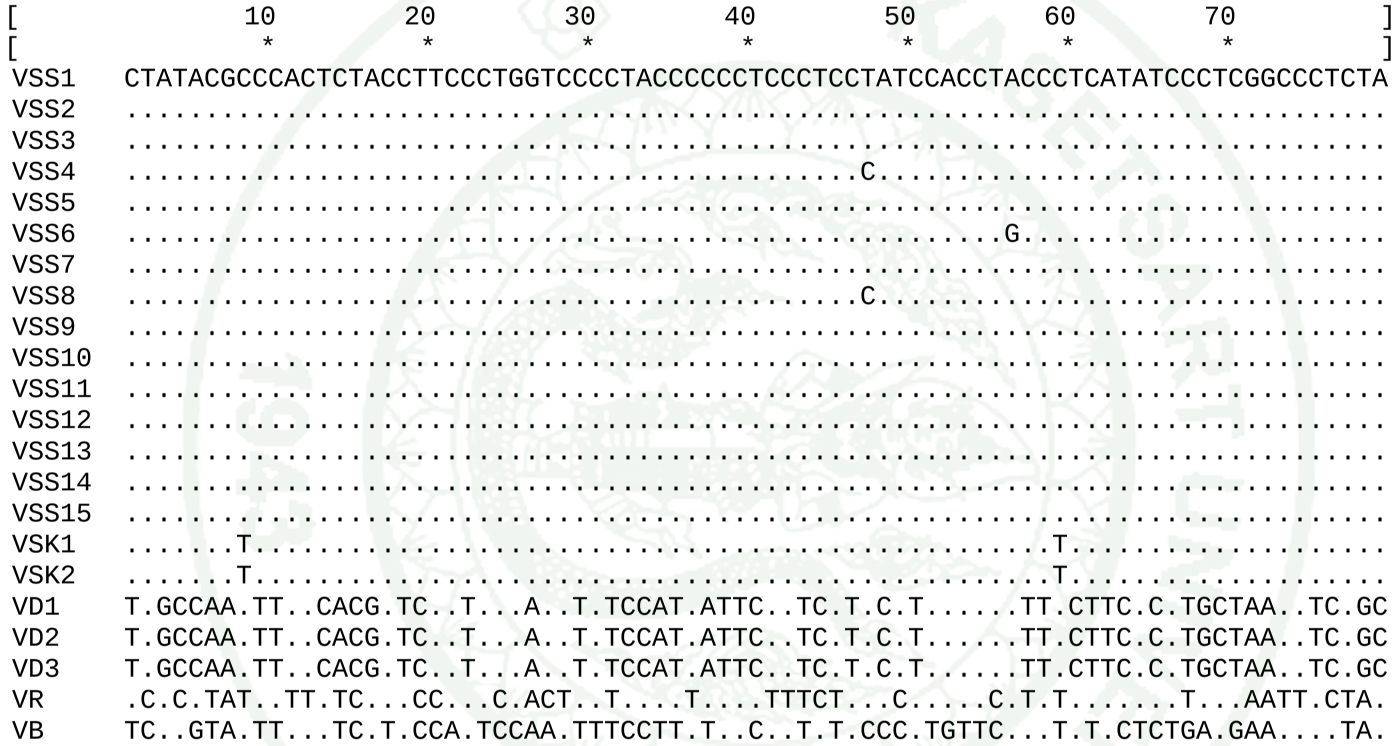

Figure 27 Nucleotide alignment of cytochrome b gene. Only the segment exhibiting differences was shown.

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1.7 Phylogenetic analysis

1.7.1 Phylogenetic analysis of 12S rRNA gene

Twenty-two sequences of monitors' 12S rRNA gene in this study and other sequences from GenBank (Table 3) were analyzed by Mega version 4.0 to construct a phylogenetic tree. These sequences were computed by genetic distance and the tree was built with minimal evolution method based on Jukes and Cantor model. They were tested with 1,000 replication of bootstrapping.

Table 5 Monitors species and GenBank accession number information, partial 12S rRNA gene sequences.

Name	*Common name*	*Species*	*Origin*	*GenBank accession no.*
VS_USA	Water monitors	*Varanus salvator* salvator	USA	NC_010974
VD_Aus	Red-headed monitors	*Varanus dumerilii*	Australia	AH005470
Komodo Jap	Komodo dragons	*Varanus komodoensis*	Japan	AB080275
VB_Chi	Clouded monitors	*Varanus bengalensis*	China	EU621803
VB_Aus	Clouded monitors	*Varanus bengalensis*	Australia	AF004486
Vnilo Jap	Nile monitors	*Varanus niloticus*	Japan	AB185327
Shini Jap	Chinese crocodile lizards	*Shinisaurus crocodilurus*	Japan	NC_00595
VSS1-VSS15_Th	Asian water monitors	*Varanus salvator salvator*	Thailand	GU219865-79
VSK1-VSK2_Th	Black jungle monitors	*Varanus salvator komaini*	Thailand	GU219863-64
VD1-VD3_Th	Red-headed monitors	*Varanus dumerilii*	Thailand	GU219859-61
VR_Th	Rough-necked monitors	*Varanus rudicollis*	Thailand	GU219862
VB_Th	Clouded monitors	*Varanus bangalensis*	Thailand	GU219858

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Based on a cladogram (Figure 28) revealed that Asian water monitor and black jungle monitor had no genetic divergence between them and were monophyletic. Rough-necked monitor was the sister group or paraphyletic group to both *salvator* subspecies and had 0.038 in genetic divergence (the genetic distance table was showed in appendix). Both water monitor were closely related to rough-necked, red-headed and clouded monitor respectively. The clouded monitor had genetic distance close to Komodo dragon and Nile monitor more than other species.

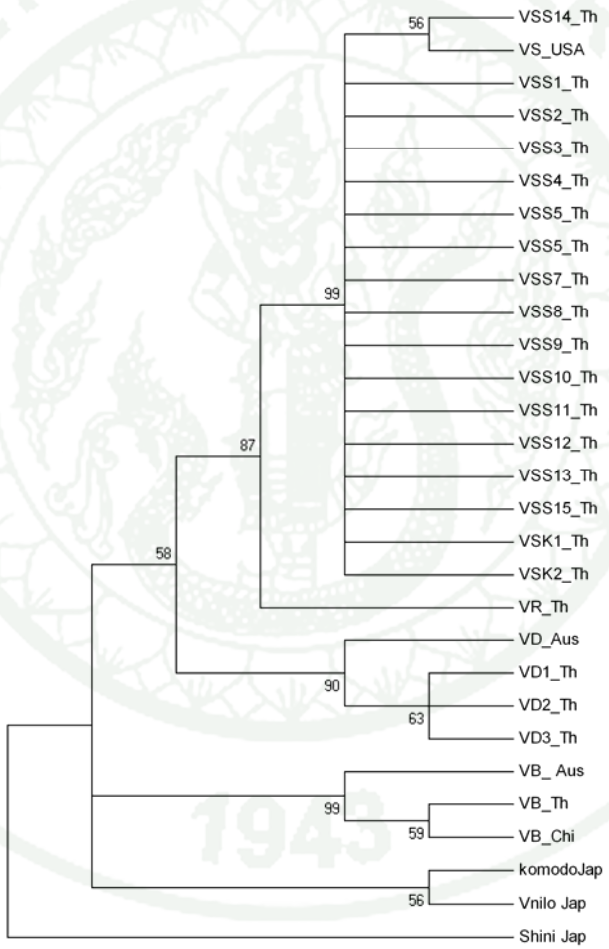

Figure 28 The phylogenetic tree of monitor lizards in Thailand based on partial 12S rRNA gene sequences, ME method with 1,000 replication of bootstrapping test.

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1.6.2 Phylogenetic analysis of cytochrome b gene

Twenty-two sequences of cytochrome b gene in this study and eight sequences from GenBank (Table 4) were used to study the phylogenetic relationship. The tree was built with minimal evolution method based on Jukes and Cantor model using Mega version 4.0 software. In this tree, Chinese crocodile lizard was used as out group taxon.

Table 6 Monitors species and GenBank accession number information, partial cytochrome b gene sequences

Name	*Common name*	*Species*	*Origin*	*GenBank accession no.*
VS1_USA-VS2_USA	Water monitor	*Varanus salvator*	USA	NC_010974, EU747731
Komodo Jap	Komodo dragon	*Varanus komodoensis*	Japan	AB080276
VB_Malay	Clouded monitor	*Varanus bengalensis nebulosis*	Malaysia	EU145735
VN_Jap, VN_USA	Nile monitor	*Varanus niloticus*	Japan, USA	AB185327 NC_008778
Shini _Jap	Chinese crocodile lizards	*Shinisaurus crocodilurus*	Japan	NC_00595
VR_Malay	Rough-necked monitor	*Varanus rudicollis*	Malaysia	EU145739
VSS1_Th-VSS15_Th	Asian water monitor	*Varanus salvator salvator*	Thailand	GU476579-93
VSK1_Th-VSK2_Th	Black jungle monitor	*Varanus salvator komaini*	Thailand	GU476594-95
VD1_Th-VD3_Th	Red-headed monitor	*Varanus dumerilii*	Thailand	GU476596-98
VR_Th	Rough-necked monitor	*Varanus rudicollis*	Thailand	GU476599
VB_Th	Clouded monitor	*Varanus bangalensis*	Thailand	GU476600

From cladogram (Figure 29), black jungle monitor was the sister group of Asian water monitor. According to the cladogram of cytochrome b gene, the *V. salvator* complex was closely to rough-necked monitor more than red-headed monitor, clouded monitor respectively. The genetic divergence between Asian water

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monitor and black jungle monitor was 0.006, whereas the genetic divergence between the Asian water monitor and rough-necked monitor was 0.106.

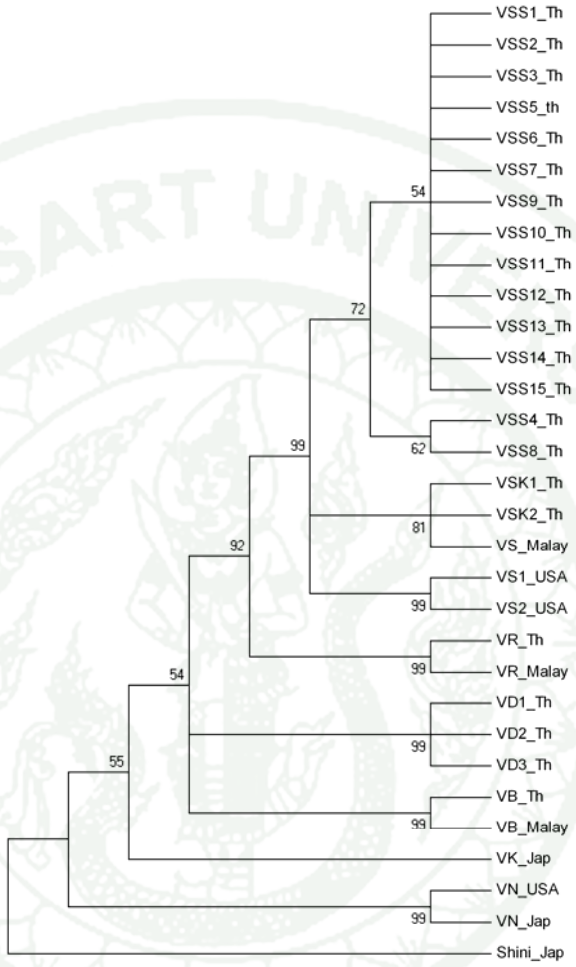

Figure 29 The phylogenetic tree of monitor lizards in Thailand based on partial cytochrome b gene sequences, ME method with bootstrapping test 1,000 replication.

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DISCUSSION

This study was successful to amplify the partial 12S rRNA and cytochrome b gene using universal primers for animals (Kocher *et al*., 1989; Auliya *et al*., 2002; Hassanin and Douzery, 1998). This primer sets were design based on comparative of publish sequences of mammal, frog and fly. In the previous study, these primer sets were used to construct a 307 base-pair segment of cytochrome b gene and a 386 bp segment of 12S rRNA segments for several mammals, birds, amphibian and reptile. In this study, the primer L14841 and H15149 amplified a 307 bp segment of cytochrome b gene. Likewise, the primer L1091 and H1478 amplified a 382-385 bp segment of 12S rRNA gene; 382 bp segment for rough-necked monitor, 384 bp for Asian water monitor, black jungle monitor and clouded monitor and 385 bp for red-headed monitor. The PCR products from both genes were analyzed by RFLP technique with three endonucleases for 12S rRNA gene and four endonucleases for cytochrome b gene.

From PCR-RFLP analysis for 12S rRNA gene, numbers of inter-species variation were observed, but in the same species were not. The 12S rRNA of Asian water monitor and black jungle monitor can be digested with ApaI and HaeIII, but the red-headed monitor was digested with BamHI. While the 12S rRNA of rough-necked monitor was digested with HaeIII and the clouded monitor was digested with both HaeIII and BamHI. However, Asian water and black jungle monitors had the same restriction pattern which was in agreement with the result from DnaSP analysis. In contrast, the different restriction patterns of both Asian water and black jungle monitors were observed in the RFLP analysis of the cytochrome b gene using BanI restriction endonuclease. Because only cytochrome b of Asian water monitor can be digested. Both water monitor were differentiate from others monitor by HpaII. Similar to the Asian water monitor's cytochrome b gene, the red-headed monitor's cytochrome b gene was digested by BanI but it could not be digested by HpaII. The rough-necked monitor was differentiated from others monitor by BstXI and the clouded monitor was differentiated from others monitor by EcoRI. Clearly, numbers

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of inter- and intraspecific variation of RFLP patterns were observed by using the RFLP analysis for the cytochrome b gene with BstXI, BanI, HpaII and EcoRI.

This evidence suggested that the polymorphisms of the 12S rRNA gene were less than the cytochrome b gene. The RFLP analysis for 12S rRNA gene with ApaI, BamHI and HaeIII could differentiate *Varanus* species in Thailand but could not differentiate Asian water from black jungle monitors. The RFLP analysis of the cytochrome b gene using BstXI, BanI, HpaII and EcoRI can differentiate both species and subspecies level of monitors. This conclusion was supported by previous studies. In the other vertebrates studies (Parson *et al*., 2000; Bradley and Baker, 2001; Linacre and Lee, 2005), they indicated that the cytochrome b could be differentiate reach to subspecies level. Whereas the 12S rRNA gene could be differentiate as far as species level, because there was polymorphisms less than the cytochrome b gene.

According to DnaSP analysis, it displayed substitution of pyrimidine and purine bases within the Asian water group. For the 12S rRNA gene sequence of VSS14, there were two transitions of cytosine to thymine. For the cytochrome b gene, transition of thymine to cytosine was found in two Asian water monitors and adenine to guanine was found in one member of Asian water monitor group. These changes indicated that there was variation of nucleotide sequences within population. However, the nucleotide changes did not effect to amino acid sequences. In addition, the transition of cytosine to thymine was found in cytochrome b of black jungle monitor compared with Asian water monitor. This change was an effect on BanI activity, BanI could not digest the PCR product of cytochrome b gene of black jungle monitor because the specific recognition sequences was substituted. Furthermore, this study found nucleotide insertion and deletion. When compared with the sequence of 12S rRNA gene of the Asian water monitor, the adenine base insertion was found in the sequence of the red-headed monitor. The cytosine deletion was found in the sequence of the rough-necked monitor. However this alteration was unconfident because a few number of sample. Moreover, the result of DnaSP analysis showed the great numbers of polymorphisms in the sequence of the cytochrome b gene.

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This study constructed phylogenetic trees based on partial 12S rRNA gene and cytochrome b gene sequences. According to 12S rRNA gene phylogenetic tree, the black jungle monitor was found to be monophyletic with the Asian water monitor. Within the *V.salvator* group, it can be divided into two subclusters and the sample VSS14 was classified in the same group of one sequence from GenBank (accession number = NC_010974). Whereas the black jungle monitors were not divided into subcluster because there was not genetic divergence. The rough-necked monitor was the sister group of both *V. salvator*. According to the cytochrome b phylogenetic tree, the black jungle monitor was in the paraphyletic group for the Asian water monitor and had the genetic divergence of 0.006 which was higher than the genetic divergence within the Asian water monitor group. Similar to the 12S rRNA phylogeny, the *V. salvator* was closer to rough-necked monitor than the red-headed and clouded monitor. In summary, these trees were in accordant to the immunophylogeny (Baverstock *et al*., 1993) and phylogeny based on long PCR product sequences of mitochondrial DNA (Ast, 2001). But it was not in agreement with hemipeneal and lung morphologic phylogeny (Böhme *et al*., 1988; Becker *et al*., 1989), they indicated that the rough-necked monitor was more closely related with clouded monitor than the water monitor.

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CONCLUSION

Five taxa of monitor lizard in Thailand including Asian water monitor, black jungle monitor, red-headed monitor, rough-necked monitor and clouded monitor could be differentiated by PCR-RFLP based on the partial 12S rRNA gene and cytochrome b gene. The purified PCR product of the 12S rRNA gene was treated with ApaI, BamHI and HaeIII. While the purified PCR product of the cytochrome b gene was treated with BstXI, BanI, HpaII and EcoRI. The species differentiation was successful in the PCR-RFLP of both genes. However, the differentiation in subspecies level of these monitors was successful only in the PCR-RFLP of the partial cytochrome b gene. This conclusion supported that the cytochrome b gene had DNA polymorphisms more than the 12S rRNA gene.

According to the results of PCR-RFLP and phylogeny based on the partial sequence of 12S rRNA and cytochrome b gene, they confirmed that the black jungle monitor was classified to subspecies of *Varanus salvator*.

This study demonstrated that the PCR-RFLP technique was simple, less time consumption and inexpensive technique for species determination compared with DNA sequencing. It had advantage for animal forensic and conservation. Furthermore the differentiation of *Varanus*, this technique was applied to identification in other animals, both domestic and wild life animals. Moreover the value for the forensic science, this technique was used to diseases diagnosis, to investigate the cause of diseases. However, this technique had limit in some species that they were less or without polymorphism.

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APPENDICES

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Appendix A

Chemical reagents and substances

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Appendix A

Chemical reagents and substances

1. **Denaturing-solution (D-solution)**
   - 4 M guanidinium thiocyanate
   - 25 mM Sodium citrate
   - 0.5% (W/V) Sodium lauryl sarcosinate

2. **TE buffer (pH 8.0)**
   - 10 mM Tris-a
   - 1 mM EDTA

3. **50X TAE buffer (pH 8.0)**
   - 242 g Tris-Base
   - 100 ml of 0.5 M EDTA
   - 57.1 g glacial acetic acid
   - Add ddH_2O to 1 Liter and adjust ph to 8.0

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Appendix B

The standard methods

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Appendix B

The standard methods

1. Preparation of *E. coli* competent cells for transformation

1. Culture *E. coli* strain DH5α cells on LB agar plate at 37 °C overnight.
2. Pick up a large colony and culture in 1 ml of LB broth at 37°C overnight with vigorous shaking (~ 250 rpm).
3. From 500 µl of overnight culture, subculture to 100 ml of SOB medium containing 25 µg/ml kanamycin, incubate at 37°C until OD_{600} is 0.4 - 0.8 (approximately 3 - 4 hrs).
4. Store the culture on ice for 10 minutes.
5. Centrifuge at 4 °C, for 10 minutes at 3,000 rpm, discard the supernatant.
6. Gently resuspend the pellet in 33 ml of ice-cold TB and store on ice for additional 10 minutes
7. Centrifuge at 4°C, for 10 minutes at 3,000 rpm, discard the supernatant.
8. Gently resuspend the pellet with 2 ml of ice-cold TB, then add 7% DMSO (150 ul)
9. Aliquot the cell to ependorf tube each 200 ul and store at -70°C until use for transformation.

2. Phenol-Chloroform extraction of DNA and ethanol precipitation

1. DNA was extracted from 50 µl of EDTA whole blood and 50 µl of distilled water that mixed with 500 µl of denature solution, was shaken for 5-10 minutes
2. Add DNA phenol 150 µl and chloroform 150 µl was shaken for 5 minutes.
3. Centrifuge the sample at 13,000 rpm for 5 minutes to separate the phases.
4. Remove about 90% of the upper, aqueous layer to a clean tube, carefully avoiding proteins at the aqueous-phenol interface. At this stage the aqueous phase can be extracted a second time with same procedure.

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5. Repeat 2-4 again.
6. Remove about 90% of the upper, aqueous layer to a clean tube, add isopropanol 550 µl and 0.5 µl of glycogen (20ng/ml), invert gently up side down and keep in – 80°C for 40 minutes
7. Centrifuge at 13,000 rpm for 10 - 15 minutes. Carefully decant the supernatant.
8. To wash the RNA pellet with 75% ethanol. Centrifuge at 13.000 rpm for 5 minutes. Decant the supernatant, and dry the pellet by air.

3. QIA quick gel extraction kit protocol

1. Exise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl)
3. Icubate at 50°C for 10 mins. To help dissolve gel, mix by vortexing the tube every 2-3 minutes during incubation.
4. After the gel slice has dissolve completely, check that color of the mixture is yellow
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. Apply the sample to the QIAquick column, and centrifuge for 1 minute.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. Add 0.75 ml of PE buffer to QIAquick colume and centrifuge for 1 minute.
10. Discard flow-through and place QIAquick column an additional 1 minutes at 13,000 rpm.
11. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
12. To elute DNA, add 50 ul of EB buffer (10 mM Tris-HCl, pH 8.5) to the center of QIAquick column membrane, let the column stand for 1 minute, and centrifuge for 1 minute.

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4. pGEM®-T Easy system protocol

4.1 Ligation protocol

1. Briefly centrifuge the pGEM®-T Easy vector and control insert DNA tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as described below.
3. Mix the reactions by pipetting. Incubate the reactions overnight at 4˚C.

Appendix Table B1 Reaction component of pGEM®-T Easy system.

Reaction component	Standard Reaction	Positive Control	Background Control
2X rapid ligation buffer, T4 DNA ligase	5 µl	5 µl	5 µl
pGEM-T Easy vector (50 ng)	1 µl	1 µl	1 µl
PCR product	X µl	-	-
Control insert DNA	-	2 µl	-
T4 DNA Ligase (3 weiss units/µl)	1 µl	1 µl	1 µl
Nuclease-free water to a final volume of	10 µl	10 µl	10 µl

5. Fast Digest® restriction enzymes protocol

1. Prepare the reaction mixture at room temperature in the order indicated. The reaction is prepared in a total volume of 30 µl; 17 µl distilled water , 10 µl of 0.2 µg PCR product, 2 µl of 10X Fast Digest® buffer and 1 µl Fast Digest® restriction enzyme.
2. Mix gently and spin down
3. Incubate at 37˚C in a heat block or water thermostat for 20 min for PCR product.
4. Inactivate the enzyme by heating for 5 min at 65˚C.

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6. GeneJET™ plasmid miniprep kit protocol

1. Use 1-5 ml of E. coli culture in LB media for purification of high-copy plasmids.
2. Resuspend the pelleted cells in 250 µl of the resuspension solution. Transfer the cell suspension to a microcentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
3. Add 250 µl of the lysis solution and mix thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear.
4. Add 350 µl of the neutralization solution and mix immediately and thoroughly by inverting the tube 4-6 times.
5. Centrifuge for 5 min to pellet cell debris and chromosomal DNA.
6. Transfer the supernatant to the supplied GeneJET™ spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
7. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
8. Add 500 µl of the wash solution to GeneJET™ spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
9. Repeat the wash procedure (step 8) using 500 µl of the wash solution.
10. Discard the flow-through and centrifuge for an additional 1 min to remove residual wash solution. This step is essential to avoid residual ethanol in plasmid preps.
11. Transfer the GeneJET™ spin column into a fresh 1.5 ml microcentrifuge tube. Add 50 µl of the elution buffer to the center of GeneJET™ spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 min at room temperature and centrifuge for 2 min.
12. Discard the column and store the purified plasmid DNA at -20˚C.

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