THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Genetics)

DEGREE

Genetics	Genetics
FIELD	**DEPARTMENT**

TITLE: Molecular Structure of Repetitive Element and Mitochondrial Genome Analysis in the Water Monitor Lizard (*Varanus salvator macromaculatus*, Platynota, Squamata)

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THESIS

MOLECULAR STRUCTURE OF REPETITIVE ELEMENT AND MITOCHONDRIAL GENOME ANALYSIS IN THE WATER MONITOR LIZARD (*Varanus salvator macromaculatus*, Platynota, Squamata)

NAMPECH CHAIPRASERTSRI

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Genetics)
Graduate School, Kasetsart University
2015

Nampech Chaiprasertsri 2015: Molecular Structure of Repetitive Element and Mitochondrial Genome Analysis in the Water Monitor Lizard (*Varanus salvator macromaculatus*, Platynota, Squamata). Master of Science (Genetics), Major Field: Genetics, Department of Genetics. Thesis Advisor: Mister Kornsorn Srikulnath, Ph.D. 113 pages.

The complete mitochondrial genome of *V. salvator macromaculacus* and *V. salvator komaini* contained twenty-two tRNA genes, two rRNA genes, thirteen protein-coding genes, and two control regions. The gene arrangements in which genes from *ND6* gene to $tRNA^{Pro}$ were extensively shuffled with duplicated control regions. These gene organizations belong to type 3 which was a specific feature of *V. salvator macromaculacus*, *V. salvator komaini, V. salvator* and *V. niloticus*. Sequence divergence of partial mitochondrial *ND2* and *COI* genes were conducted to provide DNA barcoding of six varanid lizards (*V. salvator macromaculacus*, *V. salvator komaini*, *V. rudicollis*, *V. dumerilii*, *V. nebolosus* and *V. bengalensis*) in Thailand. The overall mean sequence divergence was 13.2% for *ND2* and 10.5% for *COI*, indicating that the *ND2* barcode could be a better marker to identify varanid lizards. Karyological characterization of *V. salvator macromaculatus* was performed using conventional Giemsa staining. The karyotype was composed of 2 distinct components, 8 pairs of macrochromosome and 12 pairs of microchromosome, and the chromosomal constitution was $2n = 40$. Two novel repetitive DNA sequences, VSAREP1 and VSAREP2, were located at the C-positive heterochromatin in the pericentromeric region. These two sequences only hybridize to genomic DNA of *V. salvator macromaculatus*, but no signal was observed even for other squamate reptiles, including *V. exanthematicus*, which is a closely related species to *V. salvator macromaculatus*. The results suggest that these sequences were differentiated rapidly or were specifically amplified in the *V. salvator macromaculatus* genome.

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Student's signature Thesis Advisor's signature

ACKNOWLEDGEMENTS

I grateful thank and deeply indebted to Dr. Kornsorn Srikulnath, my thesis advisor, for this advice, encouragement and valuable suggestion in writing this thesis. I my gratitude to Associate Professor Dr. Surin Peyachoknagul for being a co-advisor and for her advice and helpful discussion on the research results.

I would like to thank Professor Dr. Akihiko Koga at Primate Research Institute, Kyoto University, Japan for his suggestion, and encouragement throughout my study and research work. I am greatly indebted to Professor Dr. Yoichi Matsuda and Dr.Yoshinobu Uno at Nagoya University, Japan, for their instruction, support and cooperation. I am also in dept to Mr. Kirati Kunya at Nakhon Ratchasima Zoo, Thailand, and Mr. Saranon Charernsuk and Sudarath Baicharoen at Zoological Park Organization of Thailand for their kindness helps on lizard samples collection used in this study.

Financial support of my research was fully granted by the Center for Advanced Studies in Tropical Natural Resources, National Research Kasetsart University, Thailand (CASTNAR, NRU-KU, Thailand), Capability Building Research Fund (CBRF), Research Scholarships and Publication Support Scholarship. I would like to thank the National Research Council of Thailand (NRCT) for funding of my study and research.

My special gratitude is granted to all members in the Laboratory of Animal Cytogenetics & Comparative Genomics, Kasetsart University, for their kind help during the field experiment conduct. Finally, I thank my family for continuous encouragement and support throughout this time.

Nampech Chaiprasertsri

October 2014

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LIST OF ABBREVIATIONS

A, G, C, and T	=	adenine, guanine, cytocine and thymine
ATP	=	adenosine triphosphate
$Ba(OH)_2$	=	barium hydroxide
BLASTn	=	Basic Local Alignment Search Tool-nucleotide
BLASTnx	=	Basic Local Alignment Search Tool-protein
bp	=	base pairs
°C	=	degree Celcius
CO	=	cytochrome c oxidase
CO_2	=	carbon dioxide
CR	=	control region
CSB	=	conserved sequence block
Cyt *b*	=	cytochrome *b*
DAPI	=	4',6-Diamidino-2-Phenylindole
D-loop	=	a displacement loop
DDBJ	=	DNA Data Bank of Japan
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
dUTP	=	deoxyuridine Triphosphate
EDTA	=	ethylenediamine tetraacetic acid
FITC	=	fluorescein isothiocyanate
FISH	=	fluorescence *in situ* hybridization
H-strand	=	heavy strand
HCl	=	hybridization
Kb	=	kilobase pairs
KCl	=	potassium chloride
L-strand	=	light strand
M	=	molar
$MgCl_2$	=	magnesium chloride
min	=	minute
ml	=	milliliter

LIST OF ABBREVIATIONS (Continued)

mM	=	millimolar
mM	=	millimolar
mm	=	millimeter
mRNA	=	messenger ribonucleic acid
mtDNA	=	mitochondrial DNA
NaCl	=	sodium chloride
NaOH	=	sodium hydroxide
NCBI	=	National Center for Biotechnology Information
ND	=	NADH dehydrogenase
ng	=	nanogram
nm	=	nanometer
No.	=	number
NRCT	=	National Research Council Thailand.
Ori H	=	origin of H-strand replication
Ori L	=	origin of L-strand replication
PCR	=	polymerase chain reaction
pH	=	potential of hydrogen ion
pM	=	picomolar
RNA	=	ribonucleic acid
s	=	second
satDNA	=	satellite DNA
sp.	=	species
SDS	=	sodium dodecyl sulfate
SSC	=	standard saline citrate solution
Tm	=	melting temperature
tRNA	=	transfer ribonucleic acid
Tris	=	tris (hydroxyl methyl) aminomethane
U	=	unit
UV	=	ultra violet
VNTRs	=	variable of tandem repeats

LIST OF ABBREVIATIONS (Continued)

w/v	=	weight of a substance of the total volume
µg	=	microgram
µl	=	microliter
µm	=	micrometer

MOLECULAR STRUCTURE OF REPETITIVE ELEMENT AND MITOCHONDRIAL GENOME ANALYSIS IN THE WATER MONITOR LIZARD (*Varanus salvator macromaculatus*, Platynota, Squamata)

INTRODUCTION

Varanid lizards (Varanidae, Platynota) are a group of carnivorous lizards comprising the largest living lizard, such as Komodo dragon and crocodile monitor. In Thailand, there are six varanid lizards in the genus *Varanus* as follows:-*Varanus salvator macromaculatus, V. salvator komaini*, *V. bengalensis*, *V. rudicollis*, *V. nebolosus* and *V. dumerilii*. Notably, morphological characters of varanid lizards are highly similar, thereby making their labeling very difficult through scale and size. Moreover, monitor lizards and their products are important goods in world-spanning trade with live pets and reptile leather. Consequently, all members of the genus *Varanus* are protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). This study is necessary for the serious issues on the context of conservation biology, and has led to an effort to produce a collection of entire diversity of varanid lizards alongside with an accurate taxonomy, and it therefore is important to provide all DNA sequences of these fascinating lizards. Currently, tools or methods to solve this problem are based mainly on molecular technique.

Mitochondrial DNAs (mtDNAs) are a source of molecular genetic data which is conserved in gene content, abundant (multicopies) in a cell, intronless, and free from frequent DNA recombination with gene duplication/deletion. Because of these advantages, molecular evolutionists have frequently chosen orthologous sets of mtDNA sequences to elucidate the evolution and classification of animal species. Additionally, DNA barcoding is a taxonomic approach using a short DNA marker in an organism to identify its species. The mitochondrial gene is a common element that

denominates the barcode initiative, and aims at representing a practical method for species identification.

Karyotype of Varanidae (2*n* = 40) consists of 8 pairs of macrochromosomes and 12 pairs of microchromosomes, and their karyotypic diversification is observed only as a change of macrochromosome morphology in some species. Accordingly, the karyotype of a platynotan lizard showed large C-positive heterochromatin blocks in the centromeric region of both macro- and microchromosomes and the distal region of chromosome 1q. The repetitive elements are considered to be the source of homologous recombination to conceive various categories of chromosomal rearrangements. Physical chromosome mapping of repetitive DNAs can also provide a better landscape of the genome for insufficient detail chromosome map in animal species. Hence, a complete understanding of the relationship between chromosome structure and chromosomal rearrangements is needed to provide the direction of karyotypic evolution in the platynotan lizards.

In Thailand, even though there are six varanid lizards, no genetic analysis has been conducted, and morphological taxonomy of varanid species are not clearly determined, and deep karyotypic information among varanid lizards that might involve the chromosomal rearrangement in this lineage is still unknown. In this study, the genetic differentiation of *Varanus salvator macromaculatus* was investigated, based on complete mitochondrial genome sequence and DNA barcoding. Karyological characterization with repetitive sequences were cloned from a genomic library prepared from its genomic DNA. The nucleotide sequences, chromosomal distributions, genomic organization, and interspecific conservation of these sequences were subsequently analyzed. The results were discussed in relation to the mitochondrial genome structure, genetic isolation with species boundaries, and karyotype evolution in the group of platynotan lizards.

OBJECTIVES

1. To characterize the water monitor (*V. salvator macromaculatus*) karyotype.

2. To investigate site-specific repetitive elements in the water monitor (*V. salvator macromaculatus*) genome.

3. To reveal complete nucleotide sequences of mitochondrial genomes of the water monitor (*V. salvator macromaculatus*) and the black jungle monitor *(V. salvator komaini*).

4. To construct DNA barcodes of six varanid lizards in Thailand.

LITERATURE REVIEW

Monitor lizard

Monitor lizard is systematically classified as follows (Uetz, 2014):-

Kingdom Animalia
Phylum Chordata
Subphylum Vertebrata
Class Reptilia
Order Squamata
Suborder Sauria
Infraorder Platynota
Superfamily Varanoidae
Family Varanidae
Genus *Varanus*

A varanid lizard (*Varanus* sp.) is an ancient group of platynotan lizards that inhabit Afro-Arabia, the west of Southeast Asia, the Indonesian Archipelago, Papua New Guinea, and Australia (Fuller *et al*., 1998; Ast, 2001). Currently, approximately 73 extant species are classified as belonging to Varanidae (Uetz, 2014). In Thailand, there are six varanid lizards: *Varanus salvator macromaculacus* (water monitor), *V. salvator komaini* (black jungle monitor), *V. bengalensis* (bengal monitor), *V. rudicollis* (roughnecked monitor), *V. nebolosus* (clouded monitor), and *V. dumerilii* (red-headed monitor) (Appendix Figure 1–6 and Appendix Table 1), all *Varanus* species in Thailand are protected from hunting, collection, and export without special permits by the Wildlife Preservation and Protection Act, B.E.2503 (A.D.1960) (Komsorn and Thirakhupt, 2001).

Water monitor (*V. salvator macromaculatus*) is found in tropical rain forest, mangrove forest, and freshwater swamp areas (Cota *et al*., 2009). Monitor lizard has certain physical traits in common, such as elongated snout which is one of the most

noticeable traits. It is a carnivorous species using their tongues to pick up scent particles, flicking food in and out, in much the same way like a snake. This tongue action helps them to hunt any organisms, such as fish, frogs, rodents, birds, crabs, and snakes. It is also used in conjunction with the nostrils to detect prey (Smith, 1986). The last major revision of species status in *V. salvator* was that of Mertens (1942). Over the years, the status of certain subspecies of *V. salvator* has been controversial. Some authors have argued against the validity of the elevation of others to species status, but the general consensus is that the taxonomic revision of *V. salvator* group is necessary to revise modern taxonomic (Gaulke and Horn, 2004). The color pattern of various populations of *V. salvator* varies in each region. Juveniles have a distinctive pattern, but in some populations, the pattern becomes obscured with age. Some populations, however, are darker than others, and the dark or melanistic individuals have been reported from there found in Thailand (*komaini*; Nutphand, 1987). Adult black jungle monitor (*V. salvator komaini*) is shorter in length than that of *V. salvator macromaculatus*, having black coloration throughout the body (no spots or bands), and a grayish-purple tongue. It is found along coast and isle in the east side of southern Thailand.

Roughnecked monitor (*V. rudicollis*) is found in the dense evergreen forest at high elevation. In the dry season, it is sometimes found near the watercourses in coffee plantations which are surrounded by tropical rain forest at lower elevations. However, as *V. rudicollis* shares similar life habits as there of *V. dumerilii*, it may be assumed that clearing of primary and secondary forests contribute to local declines of this forest species (Bennett and Lim, 1995; Lauprasert and Thirakhupt, 2001). Red-headed monitor (*V. dumerilii*) is found in the tropical rain forest, rubber plantation within tropical rain forest and peat-swamp forest. Based on locality records (Taylor, 1963; Nabhitabhata *et al*., 2000; Lauprasert and Thirakupt, 2001; Nabhitabhata and Chan-ard, 2005), the geographical distribution of *V. dumerilii* in Thailand extends northward up the length of peninsular Thailand and along the Thai-Burmese border to a point in the northern Kanchanaburi province. The northern known limit of its range is in the Tenasserim region of Myanmar (Mertens, 1942). The range is by no means continuous and is dependent upon suitable habitat (Cota *et al*., 2008).

Bengal monitor (*V. bengalensis*) is found in the tropical rain forest areas, rubber plantation, farmlands near villages, orchard and coconut plantation. Clouded monitor (*V. nebulosus*) was long treated as a subspecies of *V. bengalensis* but is now considered a distinct species. It was found widespread in natural, semi-natural, and agricultural areas of southern Thailand. *V. bengalensis* and *V. nebulosus* often bask on the trees. They are frequently found far away from watercourses. The taxa bengalensis and nebulosus belong to the *V. bengalensis* species group. Böhme and Ziegler (1997) discussed the taxonomic status of nebulosus and bengalensis in view of sympatric overlap of their distribution ranges in northern Thailand and southern Myanmar and suggested (semi-)specific rank for both taxa. Pianka (2004) did not always follow this indication, and listed *V. bengalensis* as a polytypic species with two subspecies based on Auffenberg's report of a continuous clinal variation in scale counts in 1994. Therefore, further investigations are required to solve this taxonomic issue.

Only few large-growing monitor species, particularly *V. salvator*, are commercially harvested for their skins (Appendix Figure 7A) to produce various fashion (Appendix Figure 7B) mainly for demands in the western industrial nations of Europe and North America (Luxmoore and Groombridge, 1990; Shine *et al*., 1996, 1998; Riquier, 1998, Auliya, 2006). The detrimental impact of the skin trade has on local monitor populations, while some populations have even been locally extirpated due to the enormous annual harvest levels for the international reptile leather industry occurring over the last decades (Auffenberg, 1994; Shine *et al*., 1996; Auliya, 2006).

Animal mitochondrial genome

The animal mitochondrial genome is highly conserved in vertebrates. It is commonly composed of 13 protein coding genes: NADH dehydrogenase subunits 1 – 6 (*ND1, ND2, ND3, ND4, ND4L, ND5,* and *ND6*), cytochrome oxidase subunits I–III (*COI, COII,* and *COIII*), ATPase subunits 6 and 8 (*ATPase 6, ATPase 8*), and cytochrome *b* (*cytochrome b*); 2 ribosomal RNA genes (16S rRNA and 12S rRNA); 22 transfer RNA genes ($tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Leu}$, $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$,

$tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$, $tRNA^{Leu}$, $tRNA^{Thr}$, $tRNA^{Glu}$, and $tRNA^{Pro}$), and 1 control region (CR; D-loop) (Boore, 1999). The abundance of small-sized mtDNA molecules compared with genomic DNA together with very low level of recombination, made mtDNA more reliable and easy to use for phylogenetic applications (Elson and Lightowlers, 2006). Moreover, since the high mutation rate of mtDNA of 5–10 times relative to single copy nuclear genes resulted in an accumulation of base substitutions over a long period of time, while the order of mitochondrial genes often remains unchanged, mitochondrial DNA (mtDNA) is therefore a good target for phylogenetic reconstruction at several taxonomic levels (Boore, 1999).

In Varanidae, comparison of complete mitochondrial DNA (mt DNA) structure of *V. komodoensis* and *V. niloticus* revealed different mitochondrial genome (mtgenome) structure with other squamate reptiles (Kumazawa and Endo, 2004; Kumazawa, 2007). Genes between the NADH dehydrogenase subunit 6 (*ND6*) gene and $tRNA^{Pro}$ gene were extensively shuffled, and the control region was duplicated in both two mt genomes. However, in *V. exanthematicus*, *V. rudicollis*, and *V. acanthurus* mt genome was demonstrated the gene arrangement of *Cytb*, $tRNA^{Thr}$, *CR*1, $tRNA^{Glu}$, *ND6*, $tRNA^{Phe}$ and *CR*2. The gene organizations found in these taxa were consistent with those of *V. komodoensis* and *V. niloticus*. Retention of the control region in the canonical position was located between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes (Amer and Kumazawa, 2008) (Appendix Figure 8). Therefore, more mt genome analyses of varanid lizards are required to elucidate the gene organization and phylogenetic distribution of Varanidae.

Squamates reptiles (lizards and snakes) are a group of reptiles that include a variety of species with intriguing evolutionary questions. According to the classtfication of squamate species, they are categorized into three groups, Serpentes (snakes), Amphisbaenia (worm lizards), and Lacertilia (lizards). The mitochondrial genome of lizards and worm lizards are principally conserved to vertebrates except for the control region which has two regions in the genome from the Austalian agamid subfamily Amphibolurinae (Amer and Kumazawa, 2005), and the Komodo dragon (*V. komodoensis*), mtDNA has two control regions separated by three genes (genes for

$tRNA^{Glu}$, ND6, and $tRNA^{Pro}$) (Kumazawa and Endo, 2004). By contrast, the snake mtDNAs have many unusual features, including two duplicated CRs, a compact genome, and an elevated evolutionary rate (Kumazawa *et al*., 1996, 1998). The control region in a typical mitochondrial genome is responsible for initiating replication and transcription. However, there are three major characters which are known to animal mitochondrial genome variation: gene order, position for the origin of light-strand replication (O_L), and secondary structure of tRNA (Macey *et al*., 1997). Moreover, some genes are lost and some genes are rearranged in some taxa (Rest *et al*., 2003). These features provide an opportunity to examine the timing and relative phylogenetics of taxa.

DNA barcoding

DNA barcoding is a taxonomic approach that uses a short genetic marker in an organism's DNA to verify it as belonging to a particular species. It differs from molecular phylogeny in that the main goal is not to determine classification but to identify an unknown sample in terms of a known classification (Kress *et al*., 2005). A desirable locus for DNA barcoding should be standardized, present in most of the interested taxa and sequencable without species-specific PCR primers, short enough to be easily sequenced with current technology (Kress and Erickson, 2008). They also provide a large variation between species yet a relatively small amount of variation within a species. Although several loci have been suggested, a common set of choice is the mitochondrial *COI* gene. Most eukaryotic cells contain mitochondria, and mtDNA has a relatively fast mutation rate, which results in significant variation in mtDNA sequences between species and, in principle, a comparatively small variance within species. Proof of principle for DNA barcoding has been provided by analysis of a 645 base pair fragment of cytochrome c oxidase subunit I (*COI*) sequences among closely related species and across diverse phyla in the animal kingdom (Hebert *et al*., 2003). An important finding is the congruence between morphologic taxonomy and DNA barcode analysis. The results to date provide confidence that mitochondrial sequence divergences are strongly linked to the process of speciation. In Varanoidea, molecular phylogeny using partial mtDNA genome of the 16S rRNA gene, *COI*, *ND1*,

ND2, nine tRNAs ($tRNA^{Leu}$, the "IQM" cluster of $tRNA^{Ile}$, $tRNA^{Gln}$, and $tRNA^{Met}$, and the "WANCY" cluster of $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$) has been reported (Ast, 2001). Three major lineages are recognized within *Varanus*: an African clade basal to the rest of the group, an Indo-Asian clade, and an Indo-Australian clade. Within the last lineage, the endemic Australian dwarf monitors (Odatria) form a clade sister to the large Australian monitors (the *gouldii* group) (Appendix Figure 9). It is likely that *ND2* and *COI* sequence data is a workable solution for understanding the relationship of *Varanus* species.

Karyotype of genus *Varanus*

The extant lizards can be categorized into six infraorders: Iguania, Gekkota, Scincomorpha, Diploglossa, Dibamia, and Platynota (Uetz, 2014) and they also exhibit a large variation in both chromosome number (2n=24 – 46) and chromosome morphology (Olmo and Signorino, 2005). Karyotypes with few or no microchromosomes are found in the Lacertidae family of Scincomorpha and in the Gekkota, whereas karyotypes containing many microchromosomes are found in the Iguania, Platynota, Dibamia, Diploglossa, and Scincomorpha excluding the Lacertidae.

The Varanidae family of Platynota has commonly diploid chromosome number 40 (King and King, 1975). The karyotype is characterized by eight pairs of macrochromosomes and twelve pairs of microchromosomes (FN = 30 for 16 macrochromosomes), and their karyotypic diversification is observed only as a change of macrochromosome morphology in some species. Accordingly, the karyotype of a platynotan lizard (*V. acanthurus*, *V. gouldii*, and *V. rosenbergi*, platynotan lizards of Australia) showed large C-positive heterochromatin blocks in the centromeric region of both macro- and microchromosomes and the distal region of chromosome 1q (King *et al*., 1982; Matsubara *et al*., 2014). This suggests that platynotan lizards might be good models to examine the molecular evolution of heterochromatin and the presence of chromosomal size dependent distribution of repetitive sequences in squamate reptiles.

Repetitive DNA sequences (repetitive element)

Repetitive DNA sequences which are widely distributed in eukaryotic genomes are a large portion of the DNA content in genome, and that the variation in the genome size of different eukaryotes is mostly attributed to difference of these sequences (Brenner *et al*., 1993). They are also important to the structural and functional organization and evolution of the genome (Kidwell and Lisch, 2000; Schueler *et al*., 2001). Physical chromosome mapping of repetitive DNA sequence can also provide a better landscape of the genome in map-poor animal species to investigate the process of karyotype evolution and sex chromosome identification. Repetitive DNA sequences are principally classified into two groups according to genome organization and chromosomal distribution (Singer, 1982). One is the interspersed type of repeated sequences distributed throughout the genome, and the other is the site-specific type of repeated sequences. Site-specific repetitive sequences are mostly satellite DNA (satDNA) comprising highly repetitive sequences and long tandem arrays which evolved rapidly as a consequence of unequal crossing over, gene conversion, transposition, or slippage-replication (Dover, 1982). They are clustered in the heterochromatic regions of chromosomes, such as centromeres, pericentromeric region, telomeres and in the interstitial chromosomal region.

According to the identification of site-specific repetitive DNA sequences in macro- and microchromosomes evidence, this type of repetitive sequences has been reported in various vertebrates (Kalitis and Choo, 1997), but only a few site-specific repetitive sequences have been isolated and characterized in reptiles (Singh *et al*., 1980, Panicker and Singh, 1994, Capriglione *et al*., 1998). The genome of the Australian agamid lizard *Podarcis sicula*, carried out using restriction enzyme analysis followed by identification and cloning of a repetitive DNA fraction (Yamada *et al*., 2005), showed that *P. sicula* generally possesses a quite homogeneous genome composition, with a single tandemly repetitive sequence family. *In situ* hybridization also showed that this satellite is localized in the centromeric region, and that the nucleotide sequences are similar to other species of the same family of lizards (Capriglione *et al*., 1989, 1991, 1994, 1998). Therefore, analyses of such sequences in

additional species of different squamate reptiles should provide more conclusive evidence of the genomic compartmentalization in macro- and microchromosomes.

MATERIALS AND METHODS

1. Sample collections

A male individual of *V. salvator macromaculatus*, which accidentally died, was provided by Nakhon Ratchasima Zoo (Thailand). Its gender was morphologically identified and then confirmed by examining its the internal genital anatomy. Morphological identification of the species was performed as described by Koch *et al*., (2007) and Cota *et al*., (2009). Blood specimens of five *Varanus* spp. (black jungle monitor (*V. salvator komaini*), roughnecked monitor (*V. rudicollis*), red-headed monitor (*V. dumerilii*), bengal monitor (*V. bengalensis*), and clouded monitor (*V. nebulosus*) were collected from Nakhon Ratchasima Zoo, northeast of Thailand and Songkhla Zoo, south of Thailand. These samples were kept in –20°C until DNA extraction.

2. Computer and programs for nucleotide sequence analysis

2.1) Dot matrix analysis; **MAFFT version 6** from http://mafft.cbrc.jp/alignment/server/

2.2) Basic Local Alignment Search Tool - nucleotide; **Blastn** and **Blastx** from http://blast.ncbi.nlm.nih.gov/Blast.cgi

2.3) CAP3 Sequence Assembly Program; **CAP3** from http://pbil.univ-lyon1.fr/cap3.php

2.4) tRNAscan-SE Search Server; **tRNA Scan-SE1.21** from http://lowelab.ucsc.edu/tRNAscan-SE/

2.5) RNA fold web server; **RNA fold** from http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

2.6) Multiple Sequence Alignment; **ClustalW** from http://www.ebi.ac.uk/Tools/msa/clustalw2/

2.7) DNA restriction mapper tool; **NEBcutter V2.0** from http://tools.neb.com/NEBcutter2/

2.8) OligoAnalyzer 3.1; **OligoAnalyzer** from https://sg.idtdna.com/analyzer/applications/oligoanalyzer/

2.9) Reverse Complement; **Reverse Complement** from http://www.bioinformatics.org/sms/rev_comp.html

2.10) ORF Finder (Open Reading Frame Finder); **ORF Finder** from http://www.ncbi.nlm.nih.gov/gorf/

2.11) ExPASy-tranlate tool; **ORF translate** from http://web.expasy.org/translate/

2.12) Codon Usage - Bioinformatics.Org; **Codon Usage** from http://www.bioinformatics.org/sms2/codon_usage.html

3. Genomic DNA extraction

Whole genomic DNA was extracted from all individual of six varanid lizards using a simple salting out method as described by Prakhongcheep *et al*. (2014). The tissues were digested at 55°C for 1 h using 1.6 µg/µl proteinase K in extraction buffer (50 mMTris (pH8.0), 20 mM EDTA, 1% (w/v) SDS). Then, the mixture was extracted with salt sedimentation (0.05 volumes of 5M NaCl) and the DNA was precipitated with 2 volume of isopropanol. After washing in 70% ethanol, the genomic DNAs was air-dried and resuspended in 10 mMTris (pH 8.0), and kept in –80°C. DNA quality and concentration were determined by 1% agarose gel electrophoresis and spectrophotometric analysis.

4. Mitochondrial genome sequencing

PCR primers for mitochondrial genome sequencing were taken from Kumazawa and Endo (2004) and/or designed with conserved sequence of varanid lizard using the alignment of sequence with ClustalW. Nucleotide sequences of the primers used for mt DNA are shown in Appendix Table 2. Fifty nanogram of genomic DNA was taken in to 20 µl of 10× reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 pM specific primers and 0.5 U of *Taq* DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and PCR was performed in the following condition: an initial

denaturation at 94°C for 3 min, following with 35 cycles of 94°C for 30s, 40–60°C for 40s and 72°C for 1 min 30s, and final extension at 72°C for 10 min. The PCR products were examined by electrophoresis on 1% agarose gel. The DNA fragments were subsequently extracted from the ethidium bromide-stained gel and photographed under the UV light using Wizard® Gel/PCR DNA fragments Extraction Kit (Promega, USA). Nucleotide sequences of the DNA fragments were determined by 1stBase DNA sequencing service (Seri Kembangan, Malaysia).

5. Nucleotide sequence annotation and analysis

Sequence assembly was performed to combine all overlapping PCR fragments into one contig strand using Cap 3 sequence assembly program. The nucleotide sequence comparison against the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) database was used to perform using the blastx and the blastn program. For identification of tRNA genes, the nucleotide sequence was searched for regions which can form characteristic secondary structures for mitochondrial tRNA genes using tRNA Scan-SE1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/). All nucleotide sequences were deposited at DNA Data Bank of Japan (DDBJ). The %A+T and %G+C values, and AT-skew and GC-skew (Perna and Kocher, 1995) for the H-strand was calculated for 66 database sequences mtDNA genomes of squamate reptiles and the complete mtDNA genomes of *V. salvator macromaculatus* and *V. salvator komaini* are shown recorded.

6. DNA barcoding

Databases sequence divergences of *ND2* and *COI* sequence are considerred to be the standard barcodes of many squamate reptiles. Here, mitochondrial *ND2* and *COI* genes were selected to perform DNA barcoding. PCR primers of *ND2* and *COI* were designed based on conserved sequence of mtDNA of six *Varanus* spp. using ClustalW (Larkin *et al*., 2007). Nucleotide sequences of the primers used for DNA barcoding are shown in Appendix Table 3. The partial DNA fragments of *ND2* and *COI* genes were amplified using these PCR primers. Fifty nanogram of genomic DNA

was taken in to 20 µl of 10× reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 pM specific primers and 0.5 U of *Taq* DNA polymerase (Vivantis, Selangor Darul Ehsan, Malaysia), and PCR was performed in the following condition: an initial denaturation at 94°C for 3 min, following with 35 cycles of 94°C for 30s, 50–53°C for 40s and 72°C for 1 min, and final extention at 72°C for 10 min. The amplicons were examined by electrophoresis on 1% agarose gel. The DNA fragments were subsequently extracted from the ethidium bromide-stained gel using Gel/PCR DNA fragments Extraction Kit (Geneaid), and directly sequenced by 1stBase DNA sequencing service. The nucleotide sequences were used to search with the National Center for Biotechnology Information database using the blastn and blastx programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Then, *COI* and *ND2* nucleotide sequences of all specimens were aligned using MEGA4 program (Kumar *et al*., 2004). All unalignable sites and gap containing sites were checked carefully before they were removed from these data sets. Sequence divergence among different species was estimated with MEGA4 using the uncorrected pairwise distances (*p*-distance). The phylogenetic trees were reconstructed using maximum likelihood (ML) and Baysian inference methods. The ML trees with Phyml 3.0 (Guindon *et al.*, 2010) by heuristic searches with the tree bisection-reconnection (TBR) branch swapping and 10 random taxon additions. The corresponding best-fit evolutionary model and parameters indicated by Modeltest version 3.7 based on the Akaike Information Criterion (AIC) and PAUP* v. 4.0b10 were used. For Bayesian inference (BI) with MrBayes v3.0b4 (Huelsenbeck and Ronquist 2001) was used with the same model and parameters as mentioned above. The Markov Chain Monte Carlo (MCMC) process was set to run four chains simultaneously for 1 million generations. After the log-likelihood value reached stationarity, sampling procedure was performed at every 100 generations to get 10,000 trees and subsequently provide a majority-rule consensus tree with averaged branch lengths, and Bayesian posterior nodal relationship in the sampled tree population was shown as percentage. The program MEGA4 was subsequently used to construct phylogenetic trees from ML and Bayesian analyses.

7. Cell culture

The heart, lung, and mesentery of *V. salvator macromaculatus* were minced and then cultured in Dulbecco's modified Eagle's medium (Life Technologies-GIBCO, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Life Technologies-GIBCO), 100 μg/ml kanamycin, and 1% antibiotic–antimycotic (Life Technologies-GIBCO). The cultures were incubated at 26 °C in a humidified atmosphere of 5% CO_2 in air. Primary cultured fibroblast cells were harvested using trypsin and then subcultured.

8. Chromosome preparation and C-banding

Fibroblasts cells of heart, lungs, and mesentery at logarithmic phase of the growth cycle were harvested after colcemid treatment (120 ng/ ml) for 45 min, suspended in 0.075 M KCl for 20 min at room temperature, and then fixed with 3:1 methanol/ acetic acid following a standard protocol. After centrifugation, the cell suspension was dropped onto clean glass slides and air-dried. The slides were kept at −80 °C until use. For karyotyping, the chromosome slides were stained with 4% Giemsa solution for 10 min. To examine the chromosomal distribution of constitutive heterochromatin in *V. salvator macromaculatus* chromosomes, C-banding was performed using the standard barium hydroxide/saline/Giemsa (BSG) method (Sumner, 1972) with slight modification: chromosome slides were treated with 0.2 N HCl at room temperature for 5 min and then with 5% $Ba(OH)_2$ at 50 °C for 30 s.

9. Isolation of highly repetitive DNA sequences

A genomic library was constructed using a pCC1FOS Fosmid Library Construction Kit (Epicentre Biotechnologies, Madison, Wisconsin, United States.), following the manufacturer's protocol. Genomic DNA fragments of approximately 35–45 kb, which were mechanically sheared, were inserted into the 8.1 kb fosmid pCC1FOS vector. The 576 colonies from the VSA genomic library were cultured in liquid medium and distributed into 96 well plates. Two microliters of each 100 μl

culture was dotted onto nylon membranes. Genomic DNA of *V. salvator macromaculatus*, mechanically sheared to an approximate size of 20 kb, was directly labeled using the AlkPhos Direct Labelling Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and then hybridized to the membranes at 60 °C. Chemiluminescent signals were detected using the CDP-Star Detection System (GE Healthcare) and exposed to Fuji medical X-ray film (Fujifilm, Tokyo, Japan). The colonies with intense signals were selected, and the nucleotide sequences of inserted DNA fragments were determined with a Big Dye Terminator (version 3.1) Cycle Sequencing Kit (Life Technologies-Applied Biosystems, Carlsbad, California.), using an ABI3130 automated sequencer (Life Technologies-Applied Biosystems). The nucleotide sequences were searched in the National Center for Biotechnology Information (NCBI) database using the Blastx and Blastn programs. Dot matrix analysis of the nucleotide sequences was performed with MAFFT version 6. The clones were also used for fluorescence *in situ* hybridization (FISH) mapping.

10. Fluorescence *in situ* hybridization

Two different repetitive DNA sequences were determined by dual-color FISH as described by Matsuda and Chapman (1995) and Srikulnath *et al*. (2009). Two hundred and fifty nanograms of repetitive DNA fragments were labeled separately with digoxigenin-11-dUTP and biotin-16-dUTP using nick translation (Roche Diagnostics, Basel, Switzerland). After hybridization, the digoxigenin- and biotin-labeled probes were stained with anti-digoxigenin-Rhodamine, Fab fragments (Roche Diagnostics) and avidin labeled with fluorescein isothiocyanate (avidin-FITC; Vector Laboratories), respectively, and then the slides were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The fluorescence hybridization signals were captured using a cooled CCD camera mounted on a Leica DMRA microscope and processed using the 550CW-QFISH application program of Leica Microsystems Imaging Solutions Ltd (Cambridge, UK).

11. Slot-blot hybridization

Slot-blot hybridization was performed to examine the conservation of repetitive sequences among different species. Genomic DNA were collected from 8 species of Squamata: water monitor lizard (*V. salvator macromaculatus*, Varanidae, Sauria), Savannah monitor lizard (*V. exanthematicus*, Varanidae, Sauria), butterfly lizard (*Leiolepis reevesii rubritaeniata*, Agamidae, Sauria), sand lizard (*Lacerta agilis*, Lacertidae, Sauria), Hokou gecko (*Gekko hokouensis*, Gekkonidae, Sauria), Burmese python (*Python molurus bivittatus*, Pythonidae, Serpentes), Japanese four-striped rat snake (*Elaphe quadrivirgata*, Colubridae, Serpentes), and habu (*Protobothrops flavoviridis*, Viperidae, Serpentes). For preparation of slot blots, 200 ng of genomic DNA was denatured with 0.4 N NaOH for 10 min and transferred onto nylon membranes using BIO-DOT SF blotting equipment (Bio-Rad, Hercules, CA). DNA fragments of repetitive sequences were labeled with DIG-11-dUTP using PCR DIG Labeling Mix (Roche Diagnostics) and hybridized to the membranes. Hybridization was carried out at 45 °C overnight in DIG Easy Hyb solution (Roche Diagnostics). After hybridization, the membranes were washed at 45 °C in 0.1% sodium dodecyl sulfate (SDS)/2 × saline-sodium citrate (SSC), 0.1% SDS/1 × SSC, 0.1% SDS/0.5 × SSC, and 0.1% SDS/0.1 × SSC for 15 min each. Chemiluminescent signals were detected using anti-digoxigenin-AP Fab fragments and CDP-Star (Roche Diagnostics) and exposed to BioMax MS autoradiography film (Carestream Health, Rochester, NY).

RESULTS AND DISCUSSION

1. Mitochondrial genome content and organization

The complete mitochondrial genome of *V. salvator macromaculatus* and *V. salvator komaini* were originally characterized, and deposited at DDBJ as AB980995 and AB980996, respectively. The total mitochondrial genome sequences of *V. salvator macromaculatus* and *V. salvator komaini* were 17,536 bp and 17,715 bp in length, respectively, and comprised thirteen protein-coding genes, twenty-two tRNA genes, two rRNA genes, and two non-coding control region (Figures 1–2, Tables 1–2). There were twenty-eight genes encoded in the majority-strand (H-strand) and nine genes in the minority-strand (L- strand). The first and second non-coding control regions were located between $tRNA^{Thr}$ and $tRNA^{Glu}$ genes, and $tRNA^{Pro}$ and $tRNA^{Phe}$ genes, respectively, in the two mitochondrial genomes of moniter lizards. The sequence of the second CR region was similar to the first CR region. The duplicated control regions had a high sequence similarity of 99.21% and 98.90% for *V. salvator macromaculatus* and *V. salvator komaini*, respectively. The relative position and orientation of all the genes, and control region were similar to those of the *V. komodoensis* and *V. niloticus* (Rest *et al*., 2003; Kumazawa, 2004; Kumazawa and Endo 2004; Amer and Kumazawa 2005). However, mechanisms for the maintenance of the apparently redundant control regions, as well as those for the concerted sequence evolution of two control regions within species, remain to be elucidated. Duplicated control regions in the water monitor lizard and the black jungle monitor lizard mtDNA are present in an entirely different arrangement from that in snakes, suggesting that independent duplication events occurred on lineages leading to snakes and both of monitor lizards (Kumazawa *et al*., 1996).

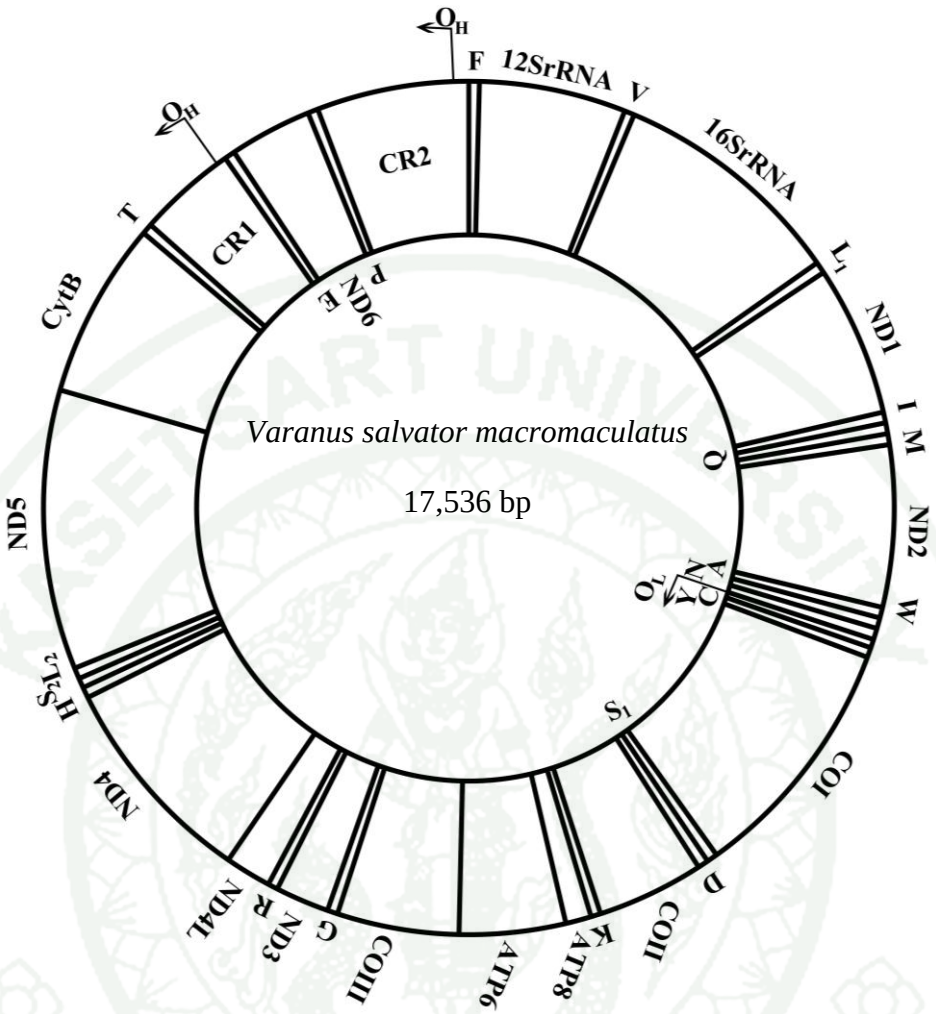


Figure 1 Mitochondrial genome structure of *Varanus salvator macromaculatus*. Genes which are encoded by heavy strand are shown outside the circle, whereas those encoded by light strand are shown inside the circle. CR, O_H, and O_L stand for the control region, the heavy-strand replication origin, and the light-strand replication origin, respectively.

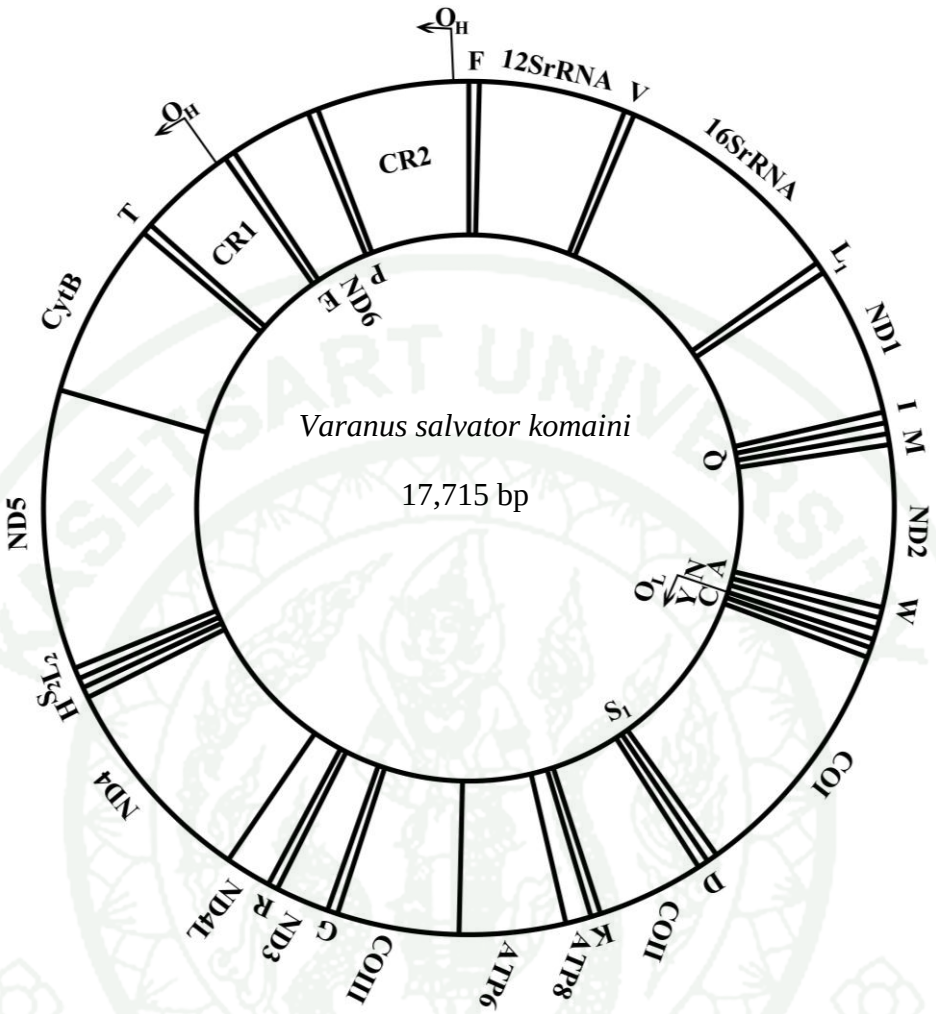


Figure 2 Mitochondrial genome structure of *Varanus salvator komaini*. Genes which are encoded by heavy strand are shown outside the circle, whereas those encoded by light strand are shown inside the circle. CR, O_H, and O_L stand for the control region, the heavy-strand replication origin, and the light-strand replication origin, respectively.

Table 1 Gene organization and features of *Varanus salvator macromaculacus* mitochondrial genome.

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
tRNA-Phe	F	1	67	67				UUC	GAA	H
12SrRNA		68	1012	945						H
tRNA-Val	V	1013	1076	64				GUA	TAC	H
16SrRNA		1077	2589	1513						H
tRNA-Leu1	L	2590	2662	73				UUA	TAA	H
ND1		2664	3629	966	321	ATA	TAA			H
tRNA-Ile	I	3631	3699	69				AUC	GAT	H
tRNA-Gln	Q	3700	3769	70				CAA	TTG	L
tRNA-Met	M	3769	3837	69				AUG	CAT	H
ND2		3838	4874	1037	344	ATT	TAa			H
tRNA-Trp	W	4875	4943	69				UGA	TCA	H
tRNA-Ala	A	4944	5012	69				GCA	TGC	L
tRNA-Asn	N	5014	5086	73				AAC	GTT	L
L-strand rep[1]		5087	5116	30						H
tRNA-Cys	C	5114	5168	55				UGC	GCA	L
tRNA-Tyr	Y	5169	5232	64				UAC	GTA	L

Table 1 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
COI		5243	6829	1596	531	ATG	AGA			H
tRNA-Ser1	S	6823	6893	71				UCA	TGA	L
tRNA-Asp	D	6896	6963	68				GAC	GTC	H
COII		6964	7653	690	229	ATG	TAA			H
tRNA-Lys	K	7657	7719	63				AAA	TTT	H
ATP8		7722	7886	165	54	ATG	TAA			H
ATP6		7877	8560	684	227	ATG	TAA			H
COIII		8560	9344	785	260	ATG	TAa			H
tRNA-Gly	G	9344	9410	67				GGA	TCC	H
ND3		9411	9756	346	114	ATA	Taa			H
tRNA-Arg	R	9758	9821	64				CGA	TCG	H
repeat region		9822	10309							
ND4L		10310	10606	297	98	ATG	TAA			H
ND4		10600	11973	1374	457	ATG	TAA			H
tRNA-His	H	11976	12044	69				CAC	GTG	H
tRNA-Ser2	S	12045	12107	63				AGC	GCT	H

Table 1 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
tRNA-Leu2	L	12107	12177	71				CUA	TAG	H
ND5		12179	13972	1794	597	ATA	TAA			H
Cytb		13981	15113	1133	376	ATG	TAa			H
tRNA-Thr	T	15114	15181	68				ACA	TGT	H
control region1		15182	15816	635						
tRNA-Gln	E	15817	15884	68				GAA	TTC	L
ND6		15890	16420	531	176	ATG	AGG			L
tRNA-Pro	P	16491	16556	66				CCA	TGG	L
control region2		16557	17536	980						

[a] TAA stop codon is completed by the addition of 3'A residues to mRNA.

[1] A putative light-strand replication origin.

Table 2 Gene organization and features of *Varanus salvator komaini* mitochondrial genome.

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
tRNA-Phe	F	1	67	67				UUC	GAA	H
12SrRNA		68	1012	945						H
tRNA-Val	V	1013	1076	64				GUA	TAC	H
16SrRNA		1077	2586	1510						H
tRNA-Leu1	L	2593	2665	73				UUA	TAA	H
ND1		2667	3632	966	321	ATA	TAA			H
tRNA-Ile	I	3634	3702	69				AUC	GAT	H
tRNA-Gln	Q	3703	3772	70				CAA	TTG	L
tRNA-Met	M	3772	3841	70				AUG	CAT	H
ND2		3842	4878	1037	344	ATT	TAa			H
tRNA-Trp	W	4879	4947	69				UGA	TCA	H
tRNA-Ala	A	4948	5016	69				GCA	TGC	L
tRNA-Asn	N	5018	5090	73				AAC	GTT	L
L-strand rep[1]		5091	5120	30						H
tRNA-Cys	C	5118	5172	55				UGC	GCA	L
tRNA-Tyr	Y	5173	5236	64				UAC	GTA	L

Table 2 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
COI		5238	6833	1596	531	ATG	AGA			H
tRNA-Ser1	S	6828	6896	69				UCA	TGA	L
tRNA-Asp	D	6900	6967	68				GAC	GTC	H
COII		6968	7657	690	229	ATG	TAA			H
tRNA-Lys	K	7661	7723	63				AAA	TTT	H
ATP8		7726	7890	165	54	ATG	TAA			H
ATP6		7881	8564	684	227	ATG	TAA			H
COIII		8564	9347	784	260	ATG	Taa			H
tRNA-Gly	G	9348	9414	67				GGA	TCC	H
ND3		9415	9760	346	114	ATA	Taa			H
tRNA-Arg	R	9762	9825	64				CGA	TCG	H
repeat region		9826	10487							
ND4L		10488	10784	297	98	ATG	TAA			H
ND4		10778	12151	1374	457	ATG	TAA			H
tRNA-His	H	12154	12222	69				CAC	GTG	H
tRNA-Ser2	S	12223	12285	63				AGC	GCT	H

Table 2 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Strand
		From	To			start	stop			
tRNA-Leu2	L	12285	12355	71				CUA	TAG	H
ND5		12357	14150	1794	597	ATA	TAA			H
Cytb		14159	15291	1133	376	ATG	TAa			H
tRNA-Thr	T	15292	15359	68				ACA	TGT	H
control region1		15360	15994	635						
tRNA-Gln	E	15995	16062	68				GAA	TTC	L
ND6		16068	16598	531	176	ATG	AGG			L
tRNA-Pro	P	16670	16735	66				CCA	TGG	L
control region2		16736	17715	980						

[a] TAA stop codon is completed by the addition of 3'A residues to mRNA.

[1] A putative light-strand replication origin.

Comparison of genome arrangements in Lacertilia based on mitochondrial genome structure of 68 species including *V. salvator macromaculacus* and *V. salvator komaini* were analyzed. Twenty-two types of gene organization were summarized and shown in Table 3–4. Type 1 represented gene arrangement which was found in the majority of lacertilian species observed. They had similar organization to that of typical vertebrate, and the features of mitochondrial genome represent IQM cluster of $tRNA^{Ile}$, $tRNA^{Gln}$, and $tRNA^{Met}$, WANCY cluster of $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$, and HSL cluster of $tRNA^{His}$, $tRNA^{Ser2}$, and $tRNA^{Leu2}$.

Squamate reptiles were known to contain atypical vertebrate mitochondrial genomes. Three main observations have been made: (1) gene rearrangements, (2) duplicated genes, and (3) duplicated control regions. Gene rearrangements were known in Acrodonta including the lizard families Agamidae and Chamaeleonidae (Macey *et al*., 1997, 2000), and the lizard family Varanidae (Kumazawa and Endo, 2004). Mitochondrial DNA duplications occur in all three types of lizard: diploid nonhybrids, diploid hybrids, and triploid hybrids were known in Cnemidophorus lizards in the family Teiidae (Moritz and Brown, 1986, 1987). The genome included some insertions ranging from 1.2 to 10.4 kb ($tRNA^{Phe}$–12SrRNA–$tRNA^{Val}$–16srRNA–$tRNA^{Leu}$–*ND1*–*ND4*–$tRNA^{His}$–$tRNA^{Ser2}$–$tRNA^{Leu2}$–*ND5*–*ND6*–$tRNA^{Glu}$–*Cytb*–$tRNA^{Thr}$–$tRNA^{Pro}$–*CR* gene) in Heternotia geckoes in the family Gekkonidae (Moritz, 1991), and the duplicated of $tRNA^{thr}$ and $tRNA^{Pro}$ in the lizard family Cordylidae (Kumazawa, 2004). Duplicated control region sequences were independently (Townsend *et al*., 2004) associated with gene rearrangements in the lizard family Varanidae (Kumazawa and Endo, 2004). All of these features are observed as atypical vertebrate state in which *V. salvator macromaculatus* and *V. salvator komaini* were also grouped. This indicates that gene arrangement occurred independently even within the same lacertilian species. The overall nucleotide base composition of mitochondrial genome of H-strand was 30.90% A, 31.20% C, 13.00% G, and 24.90% T for *V. salvator macromaculatus*, and 31.00% A, 31.10% C, 13.00% G, and 24.90% T for *V. salvator komaini* mitochondrial genome. The overall A+T content was 55.80% and 55.90%, respectively. By contrast, the AT-skew was 0.108 and 0.109, respectively. Overall G+C content was 44.20% and 44.10%, respectively, which was

as expected. However, GC-skew was 0.412 and 0.410, respectively, and the correspondence between G+C% value and GC-skew was correlated in Varanidae, which corresponded well with the values found in other vertebrate species (Asakawa *et al*., 1991; Janke and Arnason, 1997; Janke *et al*., 2001) (Table 5). The findings demonstrated that even though the nucleotide frequencies were statistically consequential propotions disparity for individual and all codon position. Consequently, the base composition analysis showed more A-T base pairs than G-C base pairs and more A and C base contents than T and G base contents, which corresponded well with the values found in other lacertilian species and other vertebrate species (Wang *et al*., 2013; Zardoay and Meyer, 1998; Srikulnath *et al*., 2012; Janke *et al*., 2005).

Table 3 Comparison of mitochondrial gene organization in order Squamata based on other lacertilian species.

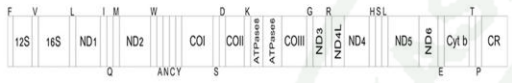
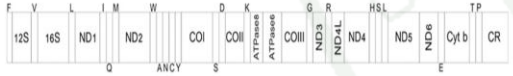
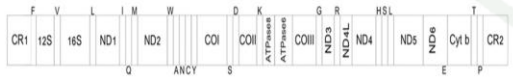
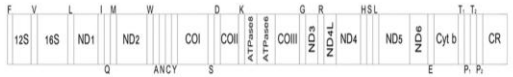
Type	Gene rganization	Gene characteristics	Lacertilian species of each suborder
1		The IQM cluster and WANCY cluster.	**Anguimorpha :** *Heloderma suspectum.* **Scinciformata :** *Lepidophyma flavimaculatum*, *Plestiodon egregius*. **Gekkota :** *Cnemaspis limi*, *Gekko swinhonis*, *Hemidactylus frenatus*, *Gekko gecko*, *Coleonyx variegatus*, *Hemitheconyx caudicinctus*, *Teratoscincus keyserlingii*, *Phyllodactylus unctus*. **Laterata :** *Lacerta agilis*, *Eremias argus*, *Podarcis siculus*, *Podarcis muralis*, *Phoenicolacerta kulzeri*, *Geocalamus acutus*, *Amphisbaena schmidti*, *Blanus cinereus*, *Diplometopon zarudnyi*. **Iguania :** *Sceloporus occidentalis*, *Polychrus marmoratus*, *Chalarodon madagascariensis*, *Iguana iguana*, *Gambelia wislizenii*, *Basiliscus vittatus*, *Oplurus grandidieri*.
2		The $tRNA^{Pro}$ moved from L-strand to H-strand.	**Anguimorpha :** *Anguis fragilis.*
3		The extensive gene shuffling at the *ND6* with *Cyt b* and $tRNA^{Thr}$ genes, and the control region which was duplicated in genomes.	**Anguimorpha :** *Varanus salvator*, *Varanus niloticus, Varanus salvator macromaculatus, Varanus salvator komaini.*
4		The duplication of control region.	**Anguimorpha :** *Shinisaurus crocodilurus*, *Abronia graminea.* **Gekkota :** *Gekko vittatus*. **Laterata :** *Takydromus tachydromoides*.
5		The duplication of $tRNA^{Thr}$ and $tRNA^{Pro}$ gene.	**Scinciformata :** *Smaug warreni.*

Table 3 (Continued)

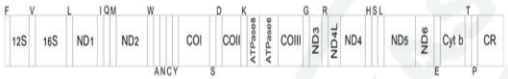
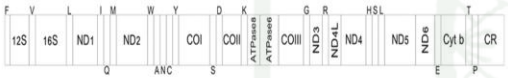
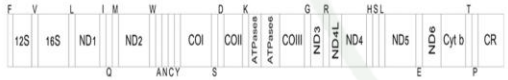
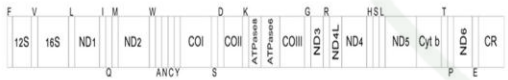
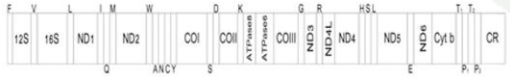
Type	Gene rganization	Gene characteristics	Lacertilian species of each suborder
6		The tRNA Q moved from L-strand to H-strand.	**Gekkota :** *Tarentola mauritanica*.
7		The genome included some the size of insertions ranging from 1.2 to 10.4 kb (F-12S-V-16s-L-*ND1*-*ND4*-H-S-L-*ND5* -*ND6*-E-*Cytb*-T-P-*CR2* gene).	**Gekkota :** *Heteronotia binoei*.
8		The tRNA Y moved from L-strand to H-strand.	**Laterata :** *Takydromus wolteri.*
9		The tRNA M moved from H-strand to L-strand and tRNA C moved from L-strand to H-strand.	**Laterata :** *Eremias brenchleyi.*
10		The $tRNA^{Ser}$ moved from L-strand to H-strand .	**Laterata :** *Lacerta viridis viridis*.
11		The extensive gene shuffling between the *ND6* with $tRNA^{Gln}$ genes.	**Laterata :** *Bipes canaliculatus*, *Bipes tridactylus.*
12		The cluster gene shuffling between the *Cyt b*, $tRNA^{Thr}$ and $tRNA^{Pro}$ genes with the *ND6* and $tRNA^{Gln}$ genes.	**Laterata :** *Rhineura floridana.*
13		The gene shuffling at the ND6 with $tRNA^{Gln}$ genes, and the duplication of $tRNA^{Thr}$ and $tRNA^{Pro}$ gene.	**Laterata :** *Bipes biporus.*

Table 3 (Continued)

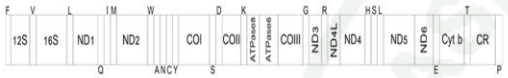
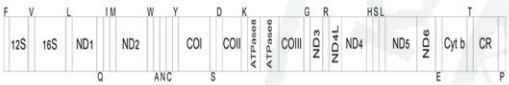
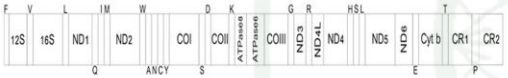
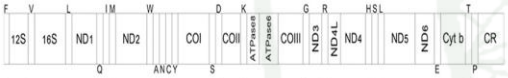
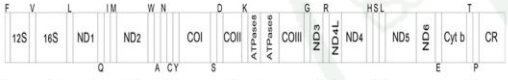
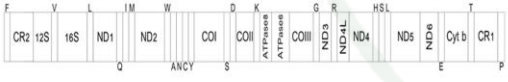
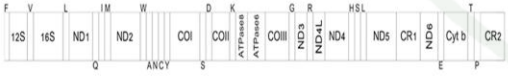
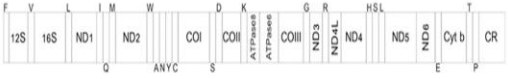
Type	Gene rganization	Gene characteristics	Lacertilian species of each suborder
14		The QIM cluster, and the position of control region located between $tRNA^{Thr}$ and $tRNA^{Pro}$.	**Iguania :** *Xenagama taylori*, *Pseudotrapelus sinaitus*, *Furcifer oustaleti*.
15		The tRNA Y moved from L-strand to H-strand.	**Iguania :** *Chamaeleo calcaricarens*, *Chamaeleo arabicus* *Chamaeleo zeylanicus*, *Chamaeleo monachus*, *Chamaeleo dilepis*, *Chamaeleo africanus*, *Chamaeleo calyptratus*, *Chamaeleo chamaeleon*, *Kinyongia fischeri*.
16		The duplication of control region located between $tRNA^{Pro}$ and $tRNA^{Phe}$.	**Iguania :** *Trioceros melleri*, *Brookesia decaryi.*
17		The QIM cluster and the WANCY cluster.	**Iguania :** *Leiolepis guttata*, *Hydrosaurus amboinensis.*
18		The $tRNA^{Pro}$ moved from L-strand to H-strand.	**Iguania :** *Acanthosaura armata*, *Calotes versicolor*.
19		The tRNA N moved from L-strand to H-strand.	**Iguania :** *Uromastyx benti.*
20		The duplication of control region located between $tRNA^{Thr}$ with $tRNA^{Pro}$ gene and located between $tRNA^{Phe}$ with 12SrRNA.	**Iguania :** *Phrynocephalus mystaceus*, *Phrynocephalus axillaris.*
21		The control region duplication located between *ND5* with *ND6* gene and located between $tRNA^{Pro}$ and $tRNA^{Phe}$.	**Iguania :** *Chlamydosaurus kingie*, *Pogona vitticeps.*
22		The IQM cluster, and the new order is WANYC cluster.	**Iguania :** *Leiocephalus personatus*.

Table 4 Classification, accession numbers and comparison of mitochondrial gene organizations of order squamata. Gene arrangements are presented for the following twenty-two types of order squamata.

Order	Suborder	Family	Species	Accession numbers	Gene organizations
Squamata	Anguimorpha	Varanidae	*Varanus salvator*	EU747731	Type (3)
Squamata	Anguimorpha	Varanidae	*Varanus niloticus*	AB185327	Type (3)
Squamata	Anguimorpha	Helodermatidae	*Heloderma suspectum*	AB167711	Type (1)
Squamata	Anguimorpha	Anguioidea	*Anguis fragilis*	EU443256	Type (2)
Squamata	Anguimorpha	Shinisauridae	*Shinisaurus crocodilurus*	AB080274	Type (4)
Squamata	Anguimorpha	Anguidae	*Abronia graminea*	AB080273	Type (4)
Squamata	Scinciformata	Xantusiidae	*Lepidophyma flavimaculatum*	AB162908	Type (1)
Squamata	Scinciformata	Scincidae	*Plestiodon egregius*	AB016606	Type (1)
Squamata	Scinciformata	Cordylidae	*Smaug warreni*	AB079613	Type (5)
Squamata	Gekkota	Gekkonidae	*Gekko vittatus*	AB178897	Type (4)
Squamata	Gekkota	Gekkonidae	*Heteronotia binoei*	EF626816	Type (7)
Squamata	Gekkota	Gekkonidae	*Cnemaspis limi*	HQ896026	Type (1)
Squamata	Gekkota	Gekkonidae	*Gekko swinhonis*	JQ906550	Type (1)
Squamata	Gekkota	Gekkonidae	*Hemidactylus frenatus*	GQ245970	Type (1)

Table 4 (Continued)

Order	Suborder	Family	Species	Accession numbers	Gene organizations
Squamata	Gekkota	Gekkonidae	*Gekko gecko*	AY282753	Type (1)
Squamata	Gekkota	Eublepharidae	*Coleonyx variegatus*	AB114446	Type (1)
Squamata	Gekkota	Eublepharidae	*Hemitheconyx caudicinctus*	AB610502	Type (1)
Squamata	Gekkota	Sphaerodactylidae	*Teratoscincus keyserlingii*	AY753545	Type (1)
Squamata	Gekkota	Phyllodactylidae	*Tarentola mauritanica*	EU443255	Type (6)
Squamata	Gekkota	Phyllodactylidae	*Phyllodactylus unctus*	HQ896027	Type (1)
Squamata	Laterata	Lacertidae	*Lacerta agilis*	KC990830	Type (1)
Squamata	Laterata	Lacertidae	*Eremias argus*	JQ086345	Type (1)
Squamata	Laterata	Lacertidae	*Podarcis siculus*	FJ460598	Type (1)
Squamata	Laterata	Lacertidae	*Podarcis muralis*	FJ460597	Type (1)
Squamata	Laterata	Lacertidae	*Phoenicolacerta kulzeri*	FJ460596	Type (1)
Squamata	Laterata	Lacertidae	*Takydromus wolteri*	JX181764	Type (8)
Squamata	Laterata	Lacertidae	*Eremias brenchleyi*	EF490071	Type (9)
Squamata	Laterata	Lacertidae	*Lacerta viridis viridis*	AM176577	Type (10)
Squamata	Laterata	Lacertidae	*Takydromus tachydromoides*	AB080237	Type (4)

Table 4 (Continued)

Order	Suborder	Family	Species	Accession numbers	Gene organizations
Squamata	Laterata	Amphisbaenidae	*Geocalamus acutus*	AY605476	Type (1)
Squamata	Laterata	Amphisbaenidae	*Amphisbaena schmidti*	AY605475	Type (1)
Squamata	Laterata	Bipedidae	*Bipes canaliculatus*	AY605482	Type (11)
Squamata	Laterata	Bipedidae	*Bipes tridactylus*	AY605477	Type (11)
Squamata	Laterata	Bipedidae	*Bipes biporus*	AY605480	Type (13)
Squamata	Laterata	Blanidae	*Blanus cinereus*	EU443257	Type (1)
Squamata	Laterata	Rhineuridae	*Rhineura floridana*	AY605473	Type (12)
Squamata	Laterata	Trogonophidae	*Diplometopon zarudnyi*	AY605474	Type (1)
Squamata	Iguania	Agamidae	*Uromastyx benti*	AB114447	Type (19)
Squamata	Iguania	Agamidae	*Leiolepis guttata*	AB476400	Type (17)
Squamata	Iguania	Agamidae	*Hydrosaurus amboinensis*	AB475096	Type (17)
Squamata	Iguania	Agamidae	*Xenagama taylori*	DQ008215	Type (14)
Squamata	Iguania	Agamidae	*Pseudotrapelus sinaitus*	AB262447	Type (14)
Squamata	Iguania	Agamidae	*Acanthosaura armata*	AB266452	Type (18)
Squamata	Iguania	Agamidae	*Calotes versicolor*	AB183287	Type (18)

Table 4 (Continued)

Order	Suborder	Family	Species	Accession numbers	Gene organizations
Squamata	Iguania	Agamidae	*Chlamydosaurus kingie*	EF090421	Type (21)
Squamata	Iguania	Agamidae	*Chlamydosaurus kingie*	AB166795	Type (21)
Squamata	Iguania	Agamidae	*Chlamydosaurus kingie*	KC578685	Type (20)
Squamata	Iguania	Agamidae	*Phrynocephalus axillaris*	KC119493	Type (20)
Squamata	Iguania	Chamaeleonidae	*Furcifer oustaleti*	AB185326	Type (14)
Squamata	Iguania	Chamaeleonidae	*Kinyongia fischeri*	EF222188	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo calcaricarens*	EF222195	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo arabicus*	EF222193	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo zeylanicus*	EF222191	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo monachus*	EF222190	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo dilepis*	EF222189	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo africanus*	EF222196	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo calyptratus*	EF222192	Type (15)
Squamata	Iguania	Chamaeleonidae	*Chamaeleo chamaeleon*	EF222198	Type (15)
Squamata	Iguania	Chamaeleonidae	*Trioceros melleri*	AB474916	Type (16)

Table 4 (Continued)

Order	Suborder	Family	Species	Accession numbers	Gene organizations
Squamata	Iguania	Chamaeleonidae	*Brookesia decaryi*	AB474914	Type (16)
Squamata	Iguania	Iguanidae	*Sceloporus occidentalis*	AB079242	Type (1)
Squamata	Iguania	Iguanidae	*Polychrus marmoratus*	AB266749	Type (1)
Squamata	Iguania	Iguanidae	*Chalarodon madagascariensis*	AB266748	Type (1)
Squamata	Iguania	Iguanidae	*Iguana iguana*	AJ278511	Type (1)
Squamata	Iguania	Iguanidae	*Gambelia wislizenii*	AB218884	Type (1)
Squamata	Iguania	Iguanidae	*Basiliscus vittatus*	AB218883	Type (1)
Squamata	Iguania	Iguanidae	*Oplurus grandidieri*	AB218720	Type (1)
Squamata	Iguania	Iguanidae	*Leiocephalus personatus*	AB266739	Type (22)

Table 5 Comparison of nucleotide contents within the whole mitochondrial genome of *Varanus salvator macromaculacus* and *Varanus salvator komaini* to other vertebrate species.

Order	Species	Percentage of nucleotide within mtDNA (%)						GC	AT
		A	G	C	T	A+T	G+C	skew	skew
Squamata	*Varanus salvator macromaculacus*	30.90	13.00	31.20	24.90	55.80	44.20	0.412	0.108
Squamata	*Varanus salvator komaini*	31.00	13.00	31.10	24.90	55.90	44.10	0.410	0.109
Squamata	*Varanus salvator*	30.93	13.03	31.07	24.91	55.84	44.10	0.409	0.108
Squamata	*Varanus niloticus*	31.77	12.65	28.49	27.09	58.86	41.14	0.385	0.080
Squamata	*Heloderma suspectum*	33.14	12.80	24.20	29.87	63.01	36.99	0.308	0.052
Squamata	*Anguis fragilis*	29.19	16.61	28.25	25.96	55.15	44.85	0.260	0.059
Squamata	*Shinisaurus crocodilurus*	32.01	13.56	27.91	26.53	58.54	41.46	0.346	0.094
Squamata	*Abronia graminea*	33.77	13.14	26.55	26.54	60.30	39.70	0.338	0.120
Squamata	*Lepidophyma flavimaculatum*	30.22	14.87	30.11	24.80	55.02	44.98	0.339	0.099
Squamata	*Plestiodon egregius*	30.48	15.41	28.78	25.33	55.81	44.19	0.303	0.092
Squamata	*Smaug warreni*	31.34	14.23	29.99	24.43	55.77	44.23	0.356	0.124
Squamata	*Gekko vittatus*	32.79	13.54	24.05	29.62	62.41	37.59	0.280	0.051
Squamata	*Heteronotia binoei*	33.28	12.93	30.84	22.95	56.23	43.77	0.410	0.184

Table 5 (Continued)

Order	Species	Percentage of nucleotide within mtDNA (%)						GC skew	AT skew
		A	G	C	T	A+T	G+C		
Squamata	*Cnemaspis limi*	29.89	14.44	32.48	23.20	53.09	46.91	0.385	0.126
Squamata	*Gekko swinhonis*	31.35	14.67	27.71	26.28	57.62	42.38	0.307	0.088
Squamata	*Hemidactylus frenatus*	30.83	15.32	30.53	23.32	54.15	45.85	0.332	0.139
Squamata	*Gekko gecko*	32.32	13.63	27.64	26.41	58.73	41.27	0.340	0.101
Squamata	*Coleonyx variegatus*	32.19	13.87	29.90	24.04	56.23	43.77	0.366	0.145
Squamata	*Hemitheconyx caudicinctus*	29.94	14.90	30.90	24.26	54.20	45.80	0.350	0.105
Squamata	*Teratoscincus keyserlingii*	30.57	14.10	30.04	25.30	55.86	44.14	0.361	0.094
Squamata	*Tarentola mauritanica*	31.64	15.05	29.56	23.74	55.38	44.62	0.325	0.143
Squamata	*Phyllodactylus unctus*	27.85	15.22	33.80	23.13	50.98	49.02	0.379	0.093
Squamata	*Lacerta agilis*	31.29	13.29	26.40	29.02	60.31	39.69	0.330	0.038
Squamata	*Eremias argus*	29.92	13.98	27.63	28.46	58.38	41.62	0.328	0.025
Squamata	*Podarcis siculus*	31.61	12.78	26.42	29.20	60.80	39.20	0.348	0.040
Squamata	*Podarcis muralis*	31.74	12.86	25.69	29.71	61.45	38.55	0.333	0.033
Squamata	*Phoenicolacerta kulzeri*	31.30	13.34	26.19	29.17	60.47	39.53	0.325	0.035

Table 5 (Continued)

Order	Species	Percentage of nucleotide within mtDNA (%)						GC	AT
		A	G	C	T	A+T	G+C	skew	skew
Squamata	*Takydromus wolteri*	31.31	13.50	24.51	30.69	61.99	38.01	0.290	0.010
Squamata	*Eremias brenchleyi*	30.12	14.01	27.57	28.30	58.43	41.57	0.326	0.031
Squamata	*Lacerta viridis viridis*	31.32	13.26	26.96	28.45	59.78	40.22	0.341	0.048
Squamata	*Takydromus tachydromoides*	30.85	13.95	25.69	29.47	60.36	39.64	0.296	0.023
Squamata	*Geocalamus acutus*	31.11	14.71	27.37	26.81	57.92	42.08	0.301	0.074
Squamata	*Amphisbaena Schmidti*	31.26	13.98	30.19	24.57	55.83	44.17	0.367	0.120
Squamata	*Bipes canaliculatus*	30.33	15.96	32.50	21.22	51.54	48.46	0.341	0.177
Squamata	*Bipes tridactylus*	31.91	14.87	29.44	23.78	55.69	44.31	0.329	0.146
Squamata	*Bipes biporus*	30.77	15.28	31.60	22.35	53.12	46.88	0.348	0.159
Squamata	*Blanus cinereus*	32.25	14.13	30.19	23.43	55.68	44.32	0.362	0.159
Squamata	*Rhineura floridana*	33.34	13.70	28.60	24.36	57.70	42.30	0.352	0.156
Squamata	*Diplometopon zarudnyi*	32.40	13.60	27.20	26.80	59.20	40.80	0.333	0.095
Squamata	*Uromastyx benti*	35.78	13.61	30.20	20.41	56.19	43.81	0.379	0.274
Squamata	*Leiolepis guttata*	35.52	13.04	27.88	23.56	59.08	40.92	0.363	0.202

Table 5 (Continued)

Order	Species	Percentage of nucleotide within mtDNA (%)						GC	AT
		A	G	C	T	A+T	G+C	skew	skew
Squamata	*Hydrosaurus amboinensis*	34.96	12.82	29.00	23.22	58.18	41.82	0.387	0.202
Squamata	*Xenagama taylori*	35.60	12.64	26.87	24.88	60.49	39.51	0.360	0.177
Squamata	*Pseudotrapelus sinaitus*	33.12	14.09	29.91	22.88	56.00	44.00	0.360	0.183
Squamata	*Acanthosaura armata*	33.12	13.33	30.75	22.79	55.91	44.09	0.395	0.185
Squamata	*Calotes versicolor*	33.76	13.28	26.98	25.98	59.74	40.26	0.340	0.130
Squamata	*Chlamydosaurus kingii*	34.42	12.43	29.01	24.14	58.56	41.44	0.400	0.176
Squamata	*Pogona vitticeps*	32.97	13.17	29.87	23.99	56.96	43.04	0.388	0.158
Squamata	*Phrynocephalus mystaceus*	35.99	12.50	26.21	25.30	61.29	38.71	0.354	0.175
Squamata	*Phrynocephalus axillaris*	35.98	11.65	24.10	26.46	64.26	35.74	0.348	0.148
Squamata	*Furcifer oustaleti*	35.57	12.00	26.11	26.32	61.89	38.11	0.370	0.150
Squamata	*Kinyongia fischeri*	32.83	14.03	29.76	23.38	56.21	43.79	0.359	0.168
Squamata	*Chamaeleo calcaricarens*	35.36	12.78	26.30	25.56	60.92	39.08	0.346	0.161
Squamata	*Chamaeleo arabicus*	35.59	12.14	26.58	25.69	61.28	38.72	0.372	0.162
Squamata	*Chamaeleo zeylanicus*	36.04	12.08	27.16	24.69	60.77	39.23	0.384	0.187

Table 5 (Continued)

Order	Species	Percentage of nucleotide within mtDNA (%)						GC	AT
		A	G	C	T	A+T	G+C	skew	skew
Squamata	*Chamaeleo monachus*	36.37	11.76	25.92	25.93	62.33	37.67	0.376	0.167
Squamata	*Chamaeleo dilepis*	36.43	12.09	25.05	26.42	62.86	37.14	0.349	0.160
Squamata	*Chamaeleo africanus*	35.72	12.45	26.01	25.83	61.54	38.46	0.353	0.161
Squamata	*Chamaeleo calyptratus*	35.72	12.10	26.58	25.61	61.33	38.67	0.374	0.165
Squamata	*Chamaeleo chamaeleon*	35.95	12.23	26.16	25.66	61.61	38.39	0.363	0.167
Squamata	*Trioceros melleri*	35.31	13.36	25.08	26.25	61.56	38.44	0.305	0.147
Squamata	*Brookesia decaryi*	36.41	12.43	22.94	28.22	64.63	35.37	0.297	0.127
Squamata	*Sceloporus occidentalis*	33.04	14.26	27.95	24.75	57.80	42.20	0.324	0.143
Squamata	*Polychrus marmoratus*	31.59	12.40	29.08	26.93	58.52	41.48	0.402	0.080
Squamata	*Chalarodon madagascariensis*	32.35	12.61	27.88	27.17	59.52	40.48	0.377	0.087
Squamata	*Iguana iguana*	32.33	13.46	31.71	22.49	54.82	45.18	0.404	0.179
Squamata	*Gambelia wislizenii*	33.18	13.74	27.47	25.60	58.79	41.21	0.333	0.129
Squamata	*Basiliscus vittatus*	33.41	13.45	27.28	25.86	59.27	40.73	0.340	0.127
Squamata	*Oplurus grandidieri*	35.43	12.71	25.06	26.79	62.22	37.78	0.323	0.139

Table 5 (Continued)

Order	Species	Percentage of nucleotide within mtDNA (%)						GC skew	AT skew
		A	G	C	T	A+T	G+C		
Squamata	*Leiocephalus personatus*	33.82	13.69	25.15	27.34	61.17	38.83	0.295	0.106
Squamata	*Python molurus molurus*[1]	33.56	12.81	29.38	24.25	57.81	42.19	0.393	0.161
Squamata	*Naja atra*[2]	33.03	13.23	28.39	25.35	58.38	41.62	0.364	0.132
Testudines	*Pelodiscus sinensis*[3]	35.23	11.78	25.73	27.26	62.49	37.51	0.372	0.128
Testudines	*Pelomedusa subrufa*[4]	33.91	12.10	26.60	27.38	61.30	38.70	0.375	0.107
Crocodylia	*Crocodylus siamensis*[5]	32.01	14.77	28.47	24.76	56.77	43.23	0.317	0.128
Crocodylia	*Tomistoma schlegelii*[6]	31.29	14.05	27.65	27.01	58.31	41.69	0.326	0.073
Aves	*Gallus gallus*[7]	30.24	13.52	32.54	23.79	54.03	45.97	0.414	0.119
Aves	*Struthio camelus*[8]	30.47	14.21	30.40	24.92	55.39	44.61	0.363	0.100

[1] HM581978, [2] EU913475, [3] Wang *et al*., (2013), [4] Zardoay and Meyer (1998), [5] Srikulnath *et al*., (2012), [6] Janke *et al*., (2005), [7] KJ778617, [8] AF338715

2. Protein coding genes

The thirteen protein-coding genes in two varanid lizards were 11,397 bp and 11,396 bp, respectively. *ND6* gene encoded in the L-strand, and the other 12 genes encoded in H-strand. The longest one was *ND5* gene (1,794 bp in two varanid lizards), and the shortest one was *ATPase8* (165 bp in the two varanid lizards). The length of protein-coding sequences *COIII* gene was different between two varanid lizards, but the rest of protein-coding sequences were the same in length (Tables 6–7). Several spacers between tRNA genes were identified as indicated by the occurrence of non-coding sequences between corresponding positions of nucleotides in the sense sequences of open reading frames (ORFs).

The most common start codon for two varanid lizards was ATG (nine of thirteen protein-coding genes (Tables 1–2), whereas *ND1*, *ND3* and *ND5* were initiated by ATA codon, and *ND2* was started by ATT codon. Interestingly, all thirteen protein-coding genes (*ND1, ND2, ND3, ND4, ND4L, ND5, ND6, COI, COII, COIII, ATPase 6, ATPase 8,* and *Cytochrome b genes*) had the same initiation codon in the two varanid lizards. Surprisingly, most protein-coding genes in mt DNA of *V. salvator* and *V.* niloticus used ATG as start codons. Proteins in only a few species start with CTG, or ATA. This suggests that start codon of all thirteen genes was conserved among family Varanidae. Regarding to stop codons, most protein-coding genes in two varanid lizards mitochondrial genome end with complete stop codons, TAA (*ND1, COII, ATPase 6, ATPase 8, ND4, ND4,* and *ND5*), AGA (*COI*), AGG (*ND6*), and the other four protein-coding genes end with incomplete stop codons, T (*COIII* and *ND3*) and TA (*ND2* and *Cytb*), which appeared to be created by post transcriptional polyadenylation (Ojaia *et al*., 1981). The nucleotide usage of two varanid lizards protein-coding genes are shown in Tables 6–7. The nucleotide content was generally similar among all genes except for that of *ND6* (Figures 3–4). The average relative order of nucleotide composition was A>C>T>G with the protein-coding genes on H-strand, whereas that of *ND6* gene encoded on L-strand was T>G>A>C. These findings were also consistent with A-T rich of the mitochondrial genome.

Table 6 Nucleotide composition (% of total number) for all protein-coding genes found in mitochondrial genome of *Varanus salvator macromaculacus.*

Gene	Nucleotide, all Positions						Total Number
	A	G	T	C	AT	GC	
ND1	29.19	11.28	24.74	34.78	53.93	46.07	966
ND2	31.28	9.16	22.85	36.16	54.68	45.32	1037
COI	27.63	16.17	26.75	29.45	54.39	45.61	1596
COII	32.32	11.88	24.35	31.45	56.67	43.33	690
ATP8	29.70	6.67	23.03	40.61	52.73	47.27	165
ATP6	28.65	10.53	23.98	36.84	52.63	47.37	684
COIII	28.41	14.39	25.61	31.59	54.01	45.99	785
ND3	28.90	11.85	27.17	32.08	56.07	43.93	346
ND4L	30.30	8.75	24.58	36.36	54.88	45.12	297
ND4	29.69	9.39	25.62	35.30	55.31	44.69	1374
ND5	32.05	10.42	24.30	33.22	56.35	43.65	1794
Cytb	28.60	11.92	26.13	33.36	54.72	45.28	1133
ND6	12.05	33.90	48.33	10.17	55.93	44.07	531
Average[1]	29.73	11.03	24.93	34.27	54.70	45.30	905.58

[1]Average are given for 12 genes except for *ND6*

Table 7 Nucleotide composition (% of total number) for all protein-coding genes found in mitochondrial genome of *Varanus salvator komaini.*

Gene	Nucleotide, all Positions						Total Number
	A	G	T	C	AT	GC	
ND1	29.50	10.77	24.84	34.89	54.35	45.65	966
ND2	31.92	9.16	22.85	36.07	54.77	45.23	1037
COI	27.57	16.23	26.88	29.32	54.45	45.55	1596
COII	32.17	11.88	24.35	31.59	56.52	43.48	690
ATP8	29.70	6.67	23.03	40.61	52.73	47.27	165
ATP6	28.80	10.53	23.54	37.13	52.34	47.66	684
COIII	28.44	14.41	25.64	31.51	54.08	45.92	784
ND3	30.06	11.56	27.17	31.21	57.23	42.77	346
ND4L	30.30	9.43	23.57	36.70	53.87	46.13	297
ND4	29.55	9.32	25.84	35.30	55.39	44.61	1374
ND5	31.94	10.54	24.19	33.33	56.13	43.87	1794
Cytb	28.42	12.09	26.30	33.19	54.72	45.28	1133
ND6	12.05	33.15	44.36	10.55	56.31	43.69	531
Average[1]	29.86	11.05	24.85	34.24	54.72	45.29	905.50

[1]Average are given for 12 genes except for *ND6*

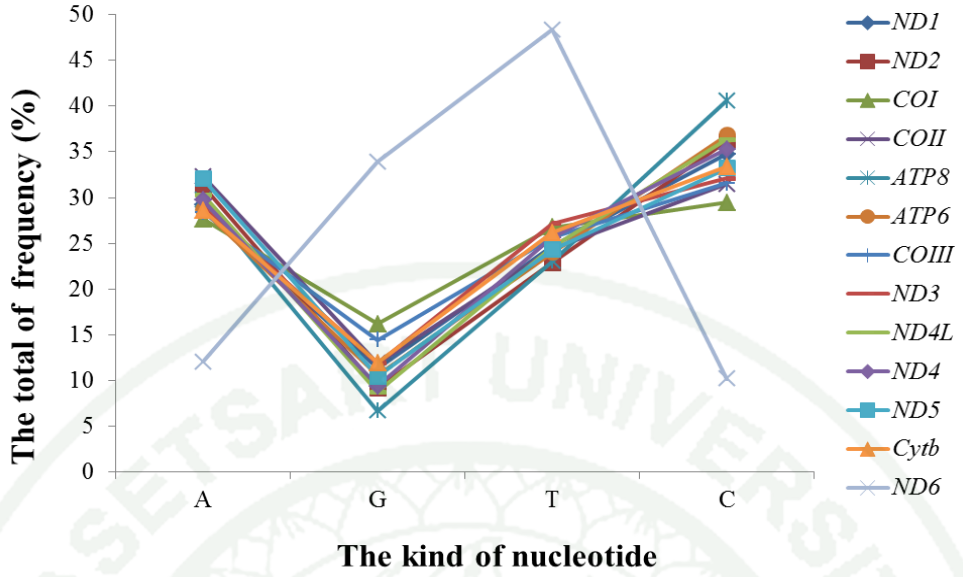


Figure 3 Relationship between total frequency (%) and the kind of nucleotide used in *Varanus salvator macromaculacus.*

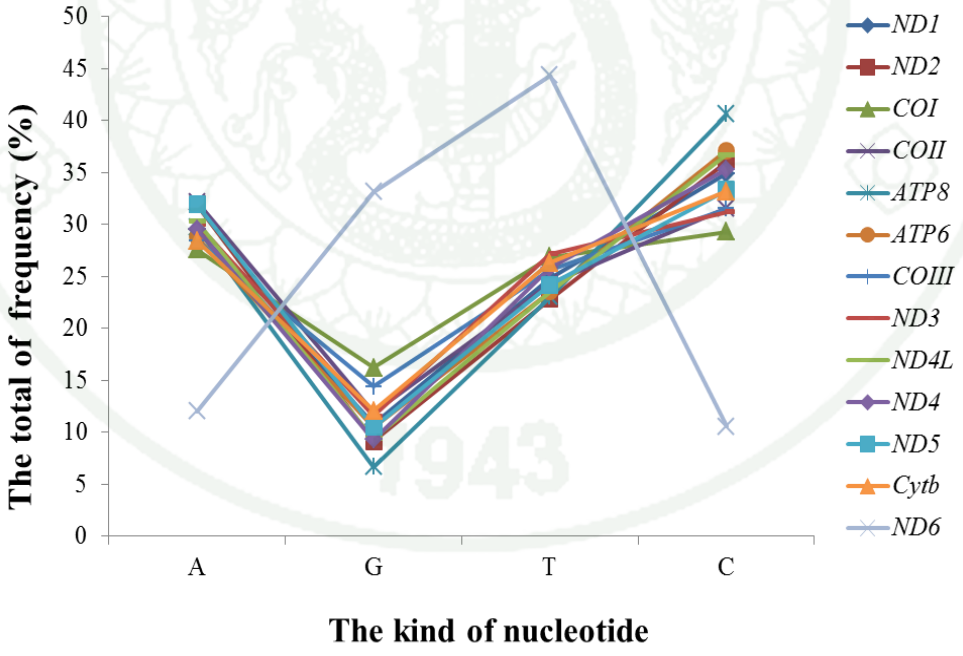


Figure 4 Relationship between total frequency (%) and the kind of nucleotide used in *Varanus salvator komaini*.

Nucleotides of mitochondrial genome were not randomly distributed, and such nucleotides bias was often related to the unequal of synonymous codons as usual. This affirmative action in nucleotide composition toward A-T affected both the codon usage pattern and amino acid composition of proteins. In these two *Varanus* species mitochondrial genome, the most ferquently used codon for translation process was ATC (Amino acid Ile). On the other hand, the least utilized was GTG (Val) for *V. salvator macromaculacus* and CCG (Ala) and GTG (Val) for *V. salvator komaini*, which were selected with low frequently (Table 8 and Appendix Tables 4-5). The base usage of two varanid lizards protein-coding genes was shown in Tables 6–7. Nucleotide C was the most frequent nucleotide at the fourfold degenerate site, followed by T and A. But at the twofold degenerate site, base usage frequency of C was higher than that of T. Similar to other vertebrates, the base composition of *V. salvator macromaculacus* and *V. salvator komaini* for 12 protein-coding genes was bias against G (average content is 11.03–11.05%), respectively, where C was the most frequent nucleotide (average content is 34.27–34.24%), respectively. The observed codon usage showed that the most used codon contained nucleotide AT, whereas the less used codon contained nucleotide CG. These results were agreed with the analysis of nucleotide composition of protein-coding genes, which showed 55.00% AT (Tables 6–7).

The proportion of codon uasge was always decreased if the third position was substituted by G such as Met (4.04% ATA and 0.90% ATG). These results lead us to predict that third codon position mostly reflect mutation bias against G. In squamates reptiles, base usage of *Achalinus meiguensis* mt genome were clarified that the mutational influence was commonly opposed by G at the third codon position (Wang *et al*., 2009). The greater translational efficiency has also been considered to be a potential cause underlying observed codon uasge bias (Ikemura, 1982).

Table 8 Codon pattern composition (% of total number) for all protein-coding genes found in mitochondrial genome of *Varanus salvator macromaculatus* (VSA) and *Varanus salvator komaini* (VSAK).

Amino acid	Codon	Codon composition in total VSA	Codon composition in total VSAK
Ala	GCG	0.14	0.14
Ala	GCA	2.49	2.46
Ala	GCT	0.64	0.66
Ala	GCC	3.41	3.49
Cys	TGT	0.11	0.11
Cys	TGC	0.53	0.55
Asp	GAT	0.33	0.33
Asp	GAC	1.22	1.22
Glu	GAG	0.25	0.28
Glu	GAA	1.99	1.88
Phe	TTT	1.91	1.88
Phe	TTC	3.52	3.54
Gly	GGG	0.36	0.33
Gly	GGA	3.05	3.07
Gly	GGT	0.50	0.50
Gly	GGC	1.50	1.52
His	CAT	0.61	0.64
His	CAC	2.52	2.46
Ile	ATT	2.99	2.93
Ile	ATC	6.12	6.17
Lys	AAG	0.22	0.22
Lys	AAA	2.41	2.49
Leu	TTG	0.11	0.11
Leu	TTA	2.10	2.10
Leu	CTG	0.89	0.86
Leu	CTA	5.79	5.70
Leu	CTT	2.93	3.02
Leu	CTC	5.43	5.40
Met	ATG	0.89	0.91
Met	ATA	4.04	4.04
Asn	AAT	0.83	0.80
Asn	AAC	3.13	3.24
Pro	CCG	0.08	0.08

Table 8 (Continued)

Amino acid	Codon	Codon composition in total VSA	Codon composition in total VSAK
Pro	CCA	2.60	2.60
Pro	CCT	0.80	0.86
Pro	CCC	2.55	2.46
Gln	CAG	0.30	0.30
Gln	CAA	2.35	2.41
Arg	CGG	0.14	0.11
Arg	CGA	0.97	0.97
Arg	CGT	0.11	0.11
Arg	CGC	0.58	0.58
Ser	AGT	0.30	0.28
Ser	AGC	0.91	0.89
Ser	TCG	0.22	0.19
Ser	TCA	2.77	2.74
Ser	TCT	0.72	0.72
Ser	TCC	2.60	2.63
Thr	ACG	0.11	0.14
Thr	ACA	3.68	3.68
Thr	ACT	1.58	1.58
Thr	ACC	5.45	5.37
Val	GTG	0.06	0.08
Val	GTA	1.25	1.22
Val	GTT	0.55	0.58
Val	GTC	1.16	1.14
Trp	TGG	0.33	0.33
Trp	TGA	2.44	2.44
Tyr	TAT	0.70	0.66
Tyr	TAC	1.74	1.74

3. Control region

The first non-coding region and the second non-coding region of *V. salvator macromaculatus* and *V. salvator komaini* were located between $tRNA^{Thr}$ and $tRNA^{Glu}$ gene, and $tRNA^{Pro}$ and $tRNA^{Phe}$ gene, respectively. Their sizes were 635 bp and 980 bp in length, respectively. Another tandem repeat was found at the 3´ end of the second control region. This tandem repeat consists of arrays of an AT-rich repeat unit. The nucleotide composition of the first non-coding region was 25.67% A, 14.49% G, 34.49% T, and 25.35% C for *V. salvator macromaculatus* and 25.67% A, 14.49% G, 34.33% T and 25.51% C for *B. V. salvator komaini*, and the nucleotide composition of the second non-coding region was 29.29% A, 11.84% G, 37.76% T, and 21.12% C for *V. salvator macromaculatus* and 29.39% A, 11.84% G, 37.65% T and 21.12% C for *B. V. salvator komaini*. The sequence of the second non-coding region was similar to the first CR. The duplicated control regions had a high sequence similarity of 99.21% and 98.90%, containing 64.33% and 64.27% A-T content for *V. salvator macromaculatus* and *V. salvator komaini*, respectively. These results agreed with AT content of protein coding genes. However, the relative order of nucleotide content was T>A>C>G in both varanid lizards which showed AT-rich in the sequences. In mammals, the control region was shown to include information necessary for replication and transcription of mtDNAs, such as the origin of heavy-strand replication and initiation of both heavy- and light-strand transcriptions) while conserved sequence blocks (CSBs) I−III were identified as conserved sequence elements in the control region of mitochondrial genome (Clayton, 1992). These two major non-coding regions were judged to be the control region because they contained conserved sequence blocks (CSBs) I and II, as well as extended termination associated sequence 1 (ETAS 1) that have been identified as conserved sequence elements for the control region of mammals (Clayton, 1992 and Sbisa` *et al*., 1997) (Figure 5). CSB II was suggested to be associated with the initiation of heavy-strand replication (Clayton, 1992), whereas ETAS 1 was shown to be a pausing site of nascent heavy-strand synthesis to make a displacement loop (Doda *et al*., 1981). Accordingly, conserved sequence blocks (CSBs) I and II have been found in two varanid lizards and some other lacertilian species for instance *Varanus komodoensis*, *Pogona vitticepes* and

Lacerta viridis viridis mitochondrial genome (Kumazawa and Endo, 2004; Amer and Kumazawa, 2005 and Böhme *et al*., 2007).

Figure 5 Nucleotide sequences of duplicated control regions of water monitor (*Varanus salvator macromaculacus*, VSA) and black jungle monitor (*Varanus salvator komaini*, VSAK) mtDNA. All nucleotide sequences shown correspond to the heavy strand. The sequences are presented with the alignment between comparable regions of two control regions of the Komodo dragon mtDNA (*Varanus komodoensis*) (AB080276). The control region consists of conserved sections, including the extended

Figure 5 (Continued)

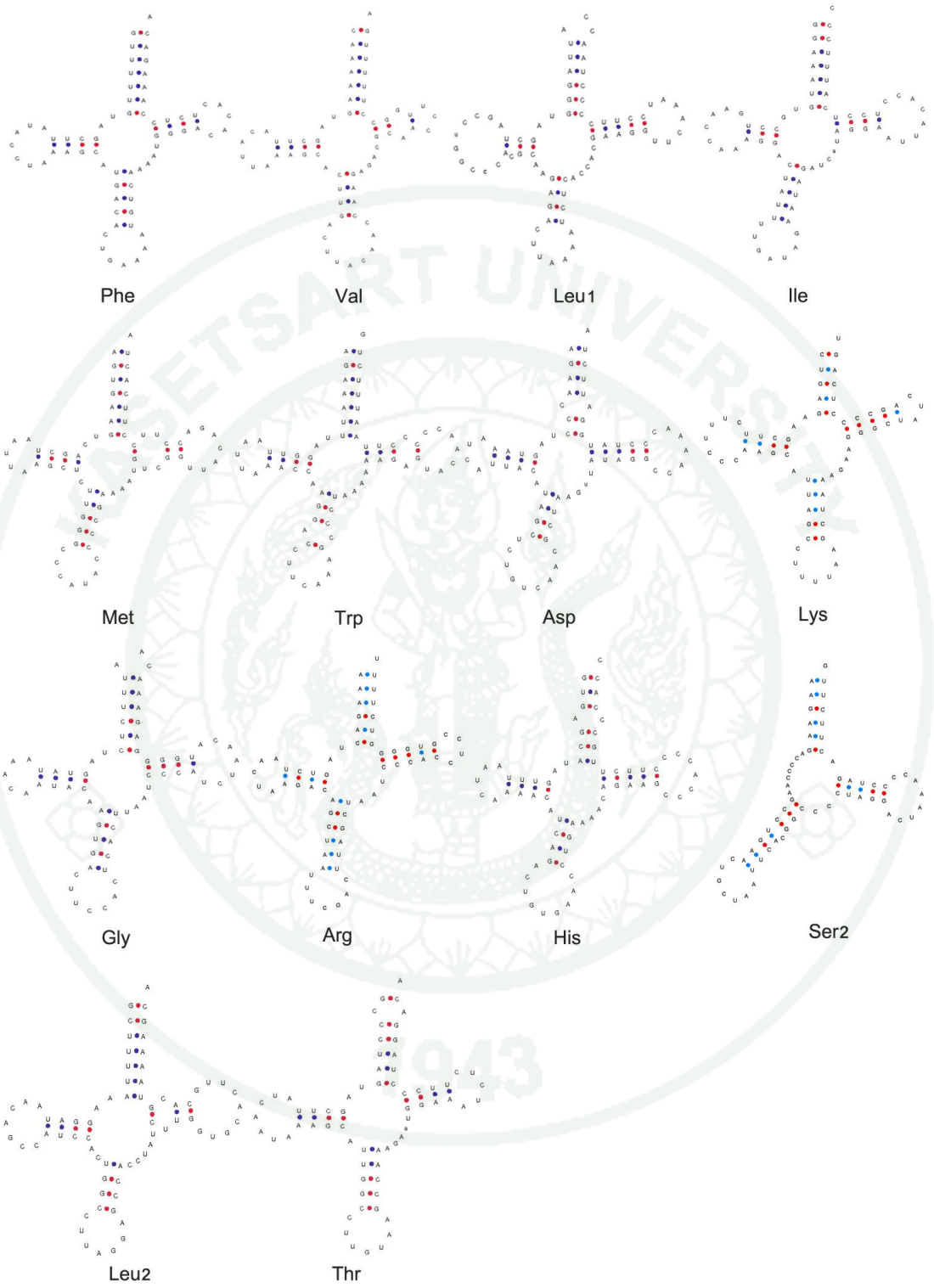
termination associated sequence (ETAS) as shown by red box and conserved sequence blocks 1 and 2 (CSB I-II) shown by yellow and green boxes, respectively.

4. Ribosomal and transfer RNA genes

Two ribosomal RNA (12S rRNA and 16S rRNA) genes were encoded in the two varanid lizards. 12S rRNA and 16S rRNA genes were located between $tRNA^{Phe}$ and $tRNA^{Leu}$. In addition, they were separated by $tRNA^{Val}$. Their length were 945 and 1,510 bp for *V. salvator macromaculacus* and 945 and 1,513 bp for *V. salvator komaini*. Moreover, the nucleotide contents of the two varanid lizard ribosomal RNA genes, where A>C>T>G were similar with the protein-coding gene on H-strand (Tables 6–7).

Twenty-two tRNA sequences ranging from 63–73 bp were determined in the mitochondrial genome of two varanid lizards (Figures 6–7). They interspersed between rRNA and protein-coding genes. The anticodon triplet sequences of these tRNA genes were exactly identical to their counterparts for other vertebrates (Amer and Kumazawa, 2005, Kumazawa and Nishida, 1993 and Kumazawa and Endo, 2004, Srikulnath *et al*., 2011). They all assumed the putative clover-leaf secondary structures in which G–U wobble pairings and occasional mismatches are allowed comparable to other squamata tRNAs. Generally, the secondary structure of tRNA constituted highest variability in its DHU and TΨC loops, and being more conservative in the anticodon and acceptor stems. Most tRNAs were formed into a typical secondary clover leaf structure. The $tRNA^{Cys}$ gene of two varanid lizards appears to lack the DHU stem. This also found in many other acrodont lizards (Macey *et al*., 2000). Likewise, the $tRNA^{Ser1}$ (AGY) of two varanid lizards showed to be lost the DHU stem. This feature has been found in *Crocodylus siamensis* and three butterfly lizards (*Leiolepis reevesii rubritaeniata*, *Leiolepis belliana belliana* and *Leiolepis boehmei*, Agamidae, Squamata) as well (Ji *et al*., 2008; Srikulnath *et al*., 2011).

(A)



(B)

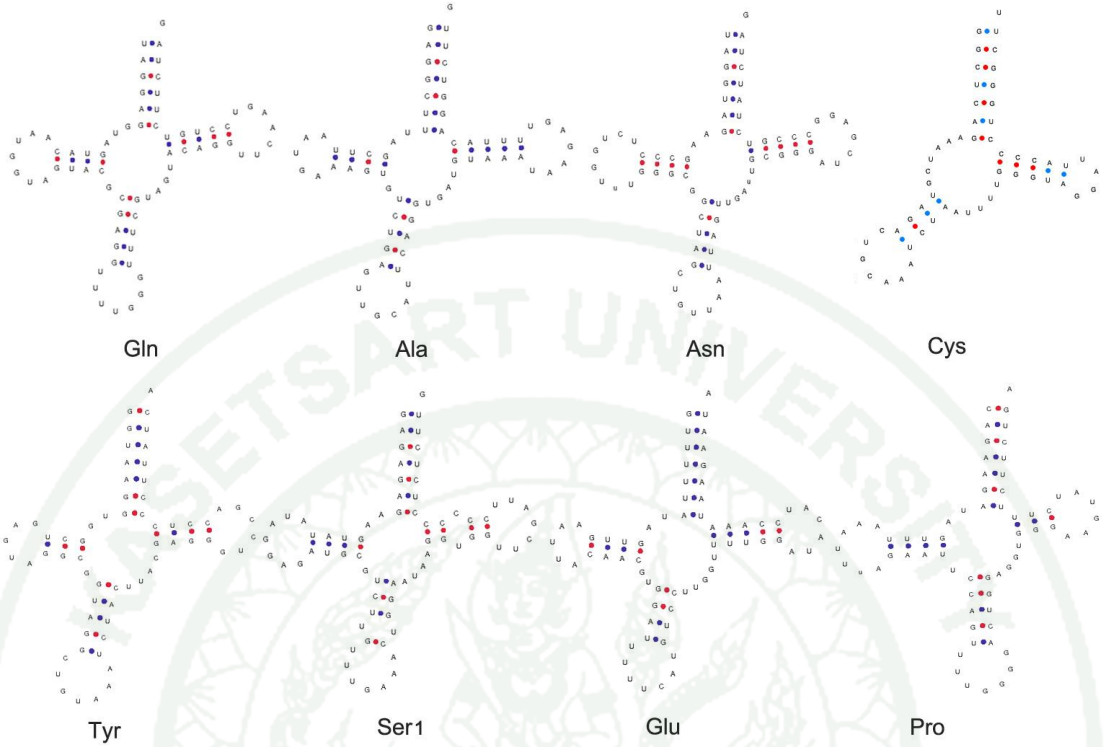
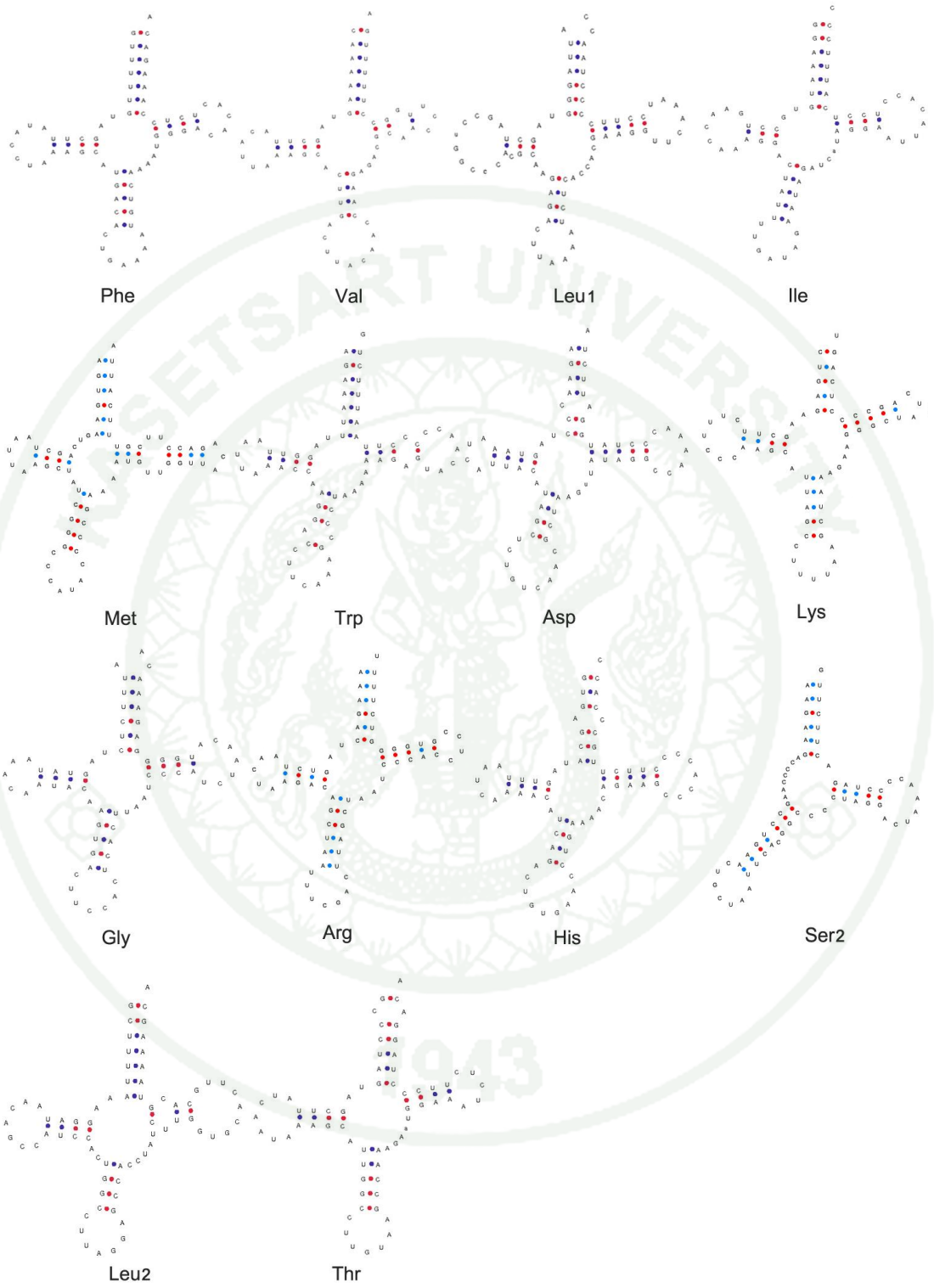


Figure 6 Putative secondary structure folds for the tRNA genes of *Varanus salvator macromaculacus* mt genome**.** Watson-Crick is designated double hydrogen bond by "•", and triple hydrogen bond, "•". (A) Heavy coding strand tRNAs. (B) Light coding strand tRNAs.

(A)



(B)

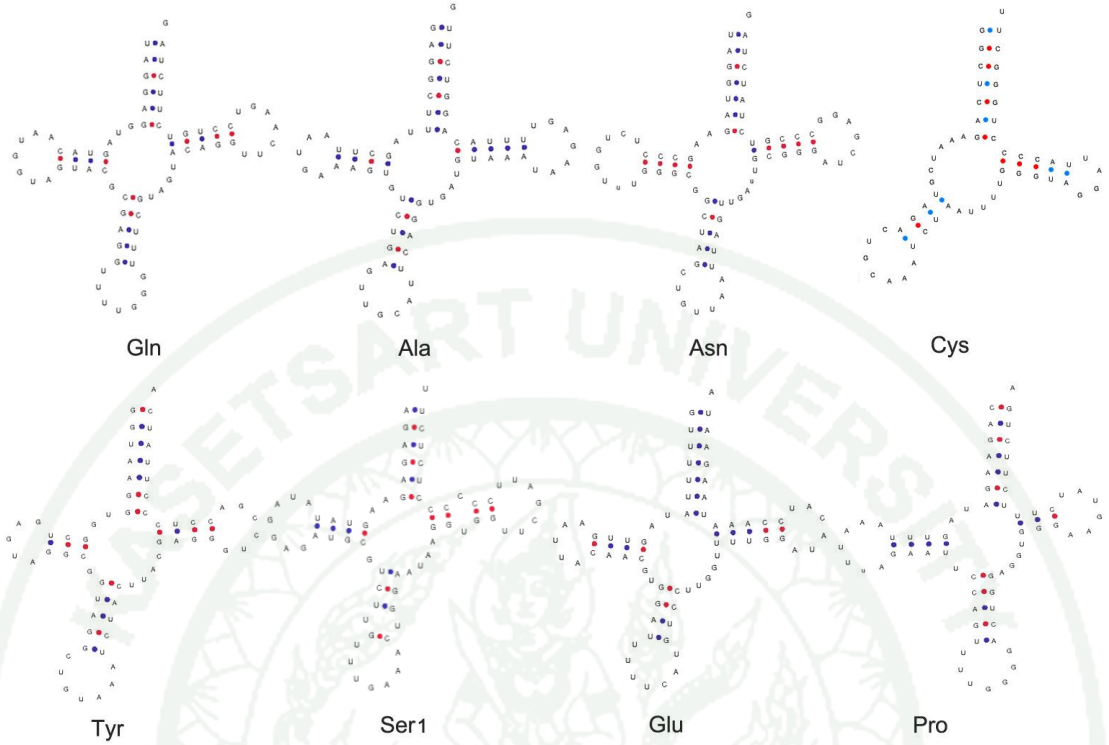


Figure 7 Putative secondary structure folds for the tRNA genes of *Varanus salvator komaini* mt genome**.** Watson-Crick is designated double hydrogen bond by "●", and triple hydrogen bond, "●". (A) Heavy coding strand tRNAs. (B) Light coding strand tRNAs.

5. tRNA^{Cys} and the origin of light-strand replication

A characteristic stem-and-loop feature for the putative origin of the light-strand replication which was found in a typical vertebrate located between tRNA^{Asn} and tRNA^{Cys} genes was also observed in the mitochondrial genome of two varanid lizards (Figure 8). The size of OriL was 24 bp in two varanid lizards. They had the same 9 bp stem and variable 6 nucleotide loop. The African and Asian species of *Varanus* also have the 6-bp stem and 2-bp loop of the T-arm in tRNA^{Cys} (Macey *et al.*, 1999). However, the Australian species generally have shorter stems, varying from 3 to 5 bases, and larger loops, varying from 1 to 7 bases (Ast, 2001). In Acrodonta, the origin of the light-strand replication structure was absent in the mitochondrial genome of *Physignathus vitticepis*, *P. cocincinus*, while most Australasian agamids appear to have lost the light-strand replication origin which may have occurred in multiple lineages of acrodont lizards (Macey *et al*., 2000; Amer and Kumazawa, 2005). In all *Varanus* sequenced, the last three bases of the origin of light-strand replication overlap with the first three bases of the aminoacyl (AA) stem in tRNA^{Cys}, as has been observed throughout squamate reptiles (Macey *et al.*, 1997, 1999). The 3´-GCC-5´ region that initiates light-strand elongation in mouse (Brennicke and Clayton, 1981) was present, and the 3´-GBCCB-5´ consensus sequence suggested by Macey *et al*., (1997) to be related to the 3´- GGCCG-5´ necessary for genome replication in humans (Hixson *et al*., 1986) was different among most *Varanus* sequenced. Surprisingly, the OriL stem-and-loop structure observed in the two varanid lizards were presumably functional since they had the critical features comprising 3´-GCC-5´ heavy-stranded sequence identified as the point of light-stranded elongation in mouse (Brennicke and Clayton, 1981) and the heavy-stranded sequence 3´- GGCCT-5´ or 3´- GGCCC-5´ related to the 3´- GGCCG-5´ sequenced required for *in vitro* replication in humans (Hixson *et al*., 1986) (Table 9).

(a) (b)

Figure 8 Characteristic stem-and-loop feature. The putative origin of replication for light strand (O_L) in (a) *Varanus salvator macromaculacus* and (b) *Varanus salvator komaini*.

Table 9 Aligned consensus structures of stem and loop region of O_L within the putative origin of replication for light strand of *Varanus salvator macromaculacus* and *Varanus salvator komaini* to *Varanus* species. Blue box indicates the 5´-CGG-3´ region demonstrated by Brennicke and Clayton (1981) to initiate light-strand replication in Mouse; yellow box underline indicates the 5-base region reported by Macey *et al*., (1997) to complement the heavy-strand sequence 3´-GGCCG-5´ in $tRNA^{Cys}$ necessary for light-strand replication (Hixson *et al*., 1986). Red word indicates the overlap between the 3´-most OriL stem and $tRNA^{Cys}$.

Species of Varanus	Sequence (5´–3´)	Accession numbers
V. salvator macromaculatus	CTTCTCCCGTTT--------GAGGAAAAACGGGAGAAGCCCAGGG	AB980995
V. salvator komaini	CTTCTCCCGTTT--------GAGGAAAAACGGGAGAAGCCCAGGG	AB980996
V. salvator salvator	CTTCTCCCGTTT--------GAGAAAAAACGGGAGAAGCCCAGGG	AF407526
V. rudicollis	CTTCTCCCGTTT--------GAGGAAAAACGGGAGAAGCCCAGGG	AF407521
V. exanthematicus	CTTCTCCCGTTT--------GGAGAAAAACGGGAGAAGCCCAGGG	AF407496
V. niloticus	CTTCTCCCGTTTG--------GGGAAAAACGGGAGAAGCCCAGGG	AF407514
V. bengalensis nebulosis	CTTTTCCCGTTTGGA----AGGGGGAAAACGGGAGAAGCCCAGGG	AF407492
V. dumerilii	CTTCTCCCGTTTTAAG---AAAAG--AAACGGGAGAAGCCCAGGG	AF407494
V. komodoensis	CTTCTCCCGTTTTTAA--AAGGGAGAAAACGGGAGAAGTCCAGGG	AB080276
V. acanthurus	CTTCTCCCGTTTTCAGGAAAAAAGGAAAACGGGAGAAGCCCAGGG	AF407488

6. Molecular barcoding of varanid lizards in Thailand

The most common gene used to perform DNA barcoding in squamate reptiles is mitochondrial *COI* and *ND2* genes. The number of aligned *ND2* and *COI* data set was 763 and 521 characters, respectively (Figures 9–10). There were two haplotypes in *V. nebolosus*, *V. dumerilii*, *V. rudicollis*, three haplotypes in *V. salvator macromaculacus*, *V. salvator komaini*, and one haplotype in *V. bengalensis* for *ND2* gene with the mean intraspecific uncorrected pairwise distance (*p*-distance) of 1%, 0.6%, 0.4%, 0.8%, 0.2% and 0.1%, respectively. The overall mean sequence divergence was 13.2% (Table 10 and Figure 11). For the result of *COI* data set, both *V. nebolosus*, but *V. dumerilii*, *V. rudicollis*, *V. salvator macromaculacus*, *V. salvator komaini*, *V. bengalensis* had only one haplotype with *p*-distance of 0.6%, 0.8%, 0%, 0.1%, 0% and 0%, respectively. The overall mean sequence divergence was 10.5% (Table11 and Figure 12).

The minimum nucleotide divergences of *ND2* gene was from 0.4% between *V. salvator komaini* and *V. bengalensis* to the maximum value of 22.0% between *V. rudicollis* and *V. dumerilii* in this study. The sequenece divergence in *Anolis* lizards was about 10% for *ND2* gene (Glor and Laport, 2012), and Malagasy ground gecko lizard ranged from 18-31% among *Paroedura* spp. (Main *et al*., 2012). This suggested that the sequence divergence of interspecific variation might be more than 10% for *ND2* gene. The minimum genetic distance of *COI* gene was 0% between *V. salvator komaini* and *V. bengalensis*, and the maximum value was 17.8% between *V. dumerilii* and *V. nebolosus*. The sequence divergence of interspecific variation in vertebrates exceeded 2% for *COI* gene (Avise, 2000; Hebert *et al*., 2003). The sequence divergences of venomous snakes ranged from 3.7% to 21.8% between *Naja siamensis* and *Naja sumatrana* and between *Naja kaouthia* and *Daboia russelii siamensis*, respectively, for *COI* gene (Supikamolseni *et al*., 2011). This suggests that the sequence divergence of *COI* gene might not be suitable to determine the closely related species in varanid lizards. However, genetic distances of *V. salvator komaini* and *V. bengalensis* were not substantially different under speific level of both two genes. It is not yet clear whether *V. salvator komaini* and *V. bengalensis* might be the

intraspecific variation or might be the species that are closely related. Other mitochondrial and nuclear genes for DNA barcoding and more varanid species are required to identify and confirm varanid lizard identification.

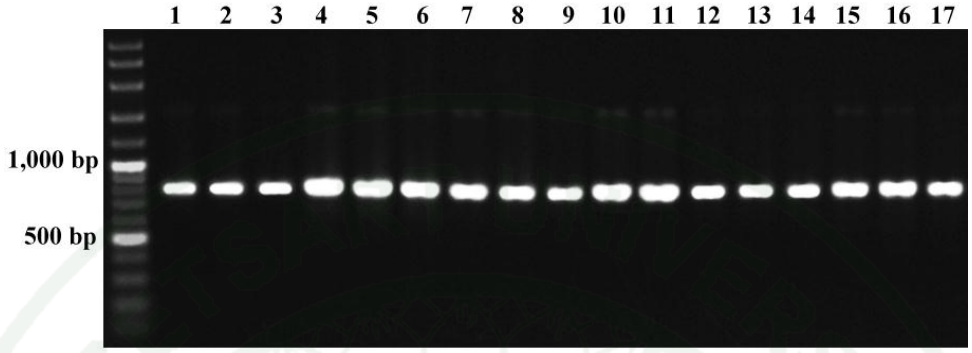


Figure 9 Electrophoretic analysis on PCR products of the short DNA barcode region by B.ND2F and B.ND2R primers from genomic DNA of *Varanus salvator macromaculacus* (lanes 1–3), *Varanus salvator komaini* (lanes 4-6), *Varanus rudicollis* (lanes 7–9), *Varanus dumerilii* (lanes 10–12), *Varanus nebolosus* (lanes 13–15) and *Varanus bengalensis* (lanes 16–17).

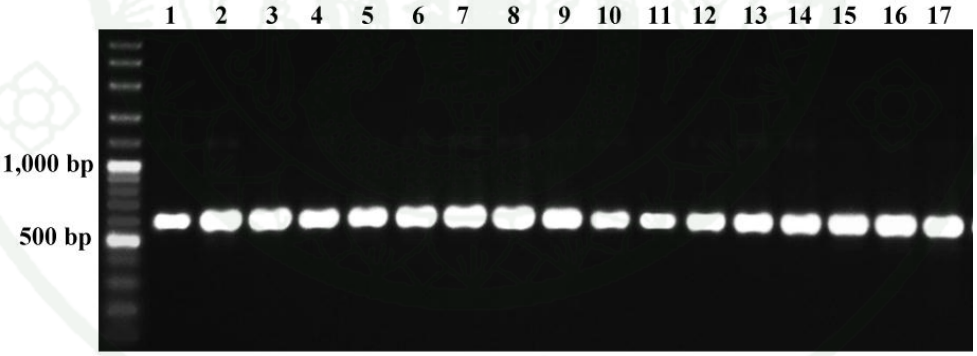


Figure 10 Electrophoretic analysis on PCR products of the short DNA barcode region by B.COIF and B.COIR primers from genomic DNA of *Varanus salvator macromaculacus* (lanes 1–3), *Varanus salvator komaini* (lanes 4–6), *Varanus rudicollis* (lanes 7–9), *Varanus dumerilii* (lanes 10–12), *Varanus nebolosus* (lanes 13–15) and *Varanus bengalensis* (lanes 16–17).

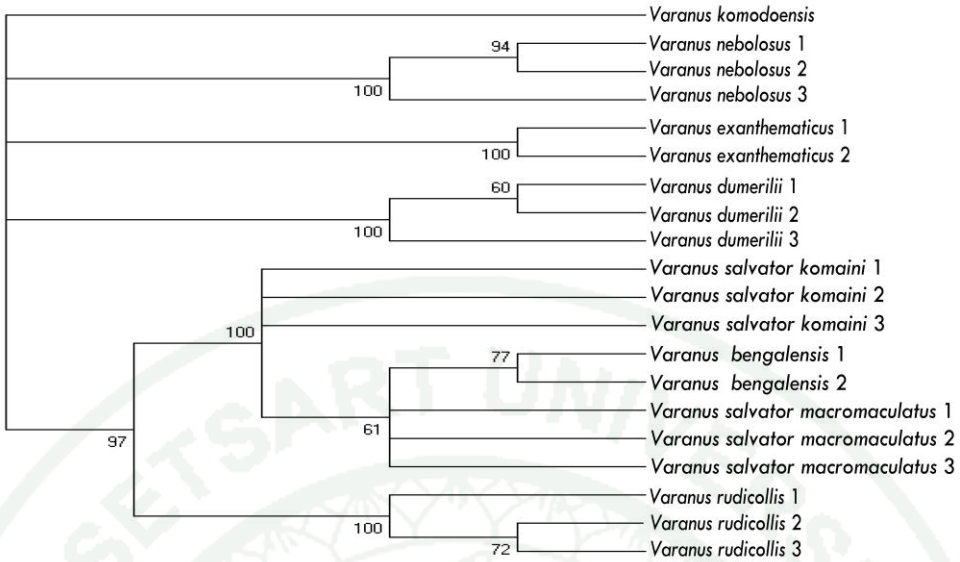


Figure 11 Phylogenetic tree shows the relationship derived from *ND2* gene among varanid lizards (*V. salvator macromaculatus, V. salvator komaini, V. rudicolli*s, *V. dumerilii, V. nebolosus, V. bengalensis, Varanus exanthematicus* and *V. komodoensis*).

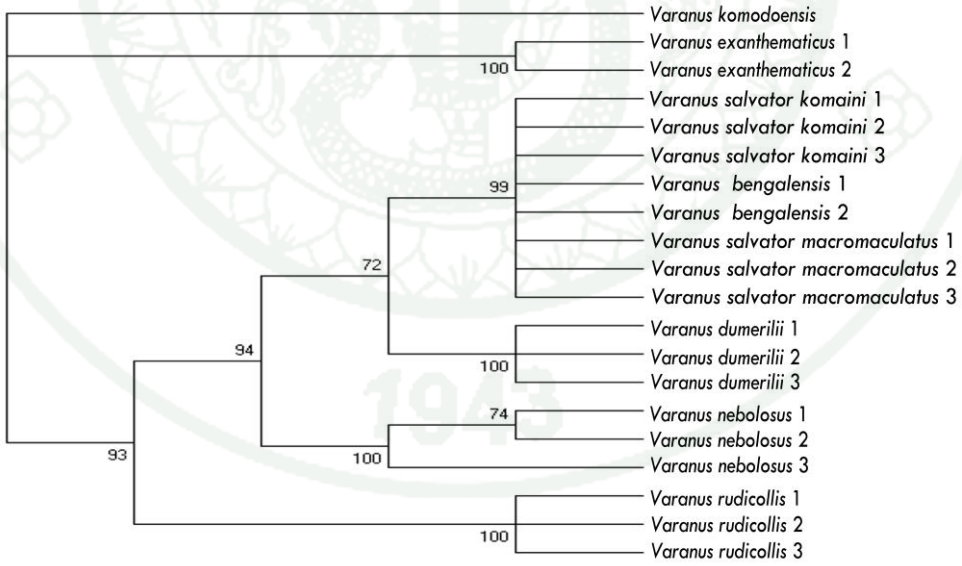


Figure 12 Phylogenetic tree shows the relationship derived from *COI* gene among varanid lizards (*V. salvator macromaculatus, V. salvator komaini, V. rudicolli*s, *V. dumerilii, V. nebolosus, V. bengalensis, Varanus exanthematicus* and *V. komodoensis*).

Table 10 Average sequence divergence (*p*-distance) of mitochondrial *ND2* gene within species and between pairs of varanid lizard species. Diagonal values (in bold) were *p* distance for intra-specific comparisons. The number in parenthesis indicates the number of individual varanid lizard per species.

	1	2	3	4	5	6
1. *V. salvator komaini* (3)	**0.002**	0.141	0.197	0.004	0.171	0.005
2. *V. rudicollis* (3)		**0.004**	0.220	0.143	0.185	0.145
3. *V. dumerilii* (3)			**0.006**	0.197	0.203	0.199
4. *V. bengalensis* (2)				**0.001**	0.173	0.005
5. *V. nebolosus* (3)					**0.010**	0.173
6.*V. salvator macromaculacus* (3)						**0.008**

Table 11 Average sequence divergence (*p*-distance) of mitochondrial *COI* gene within species and between pairs of varanid lizard species. Diagonal values (in bold) are *p* distance for intra-specific comparisons. The number in parenthesis indicates the number of individual varanid lizard per species.

	1	2	3	4	5	6
1. *V. salvator komaini* (3)	**0.000**	0.117	0.152	0.000	0.139	0.001
2. *V. rudicollis* (3)		**0.000**	0.166	0.117	0.152	0.117
3. *V. dumerilii* (3)			**0.008**	0.152	0.178	0.152
4. *V. bengalensis* (2)				**0.000**	0.139	0.001
5. *V. nebolosus* (3)					**0.006**	0.139
6.*V. salvator macromaculacus* (3)						**0.001**

7. Karyotypes of the water monitor lizard (*V. salvator macromaculatus*)

Giemsa-stained karyotype of *V. salvator macromaculatus* showed a diploid chromosome number to be 40 (FN = 30 for 16 macrochromosomes). The macrochromosomes comprises two pairs of large metacentrics (1st and 2nd), two pairs of medium-sized metacentrics (3rd and 4th), one pair of small acrocentrics (5th), three pairs of small submetacentrics (6th–8th), and 12 pairs of indistinguishable microchromosomes (Figure 13a). Chromosome number of *V. salvator macromaculatus* was shown to be 2*n* = 40 and comprises 8 pairs of macrochromosomes and 12 pairs of microchromosomes. This karyotypic feature is conserved throughout the genus *Varanus* (King and King, 1975). A large secondary constriction was located in the proximal region of chromosome 1p. Large C-positive bands were found at the distal region of chromosome 1q, pericentromeric region of chromosome 2q, centromeric region of chromosome 5, and 6 microchromosomes (Figure 13b). These results were different from those of *V. acanthurus*, in which prominent C-positive bands were found at the distal region of chromosome 1q, the pericentromeric regions of chromosomes 1q and 2q, and faint C bands in the centromeric region of chromosome 5 and 2 microchromosomes (King *et al*., 1982). These results suggest that heterochromatin distribution varies among varanid lizard species.

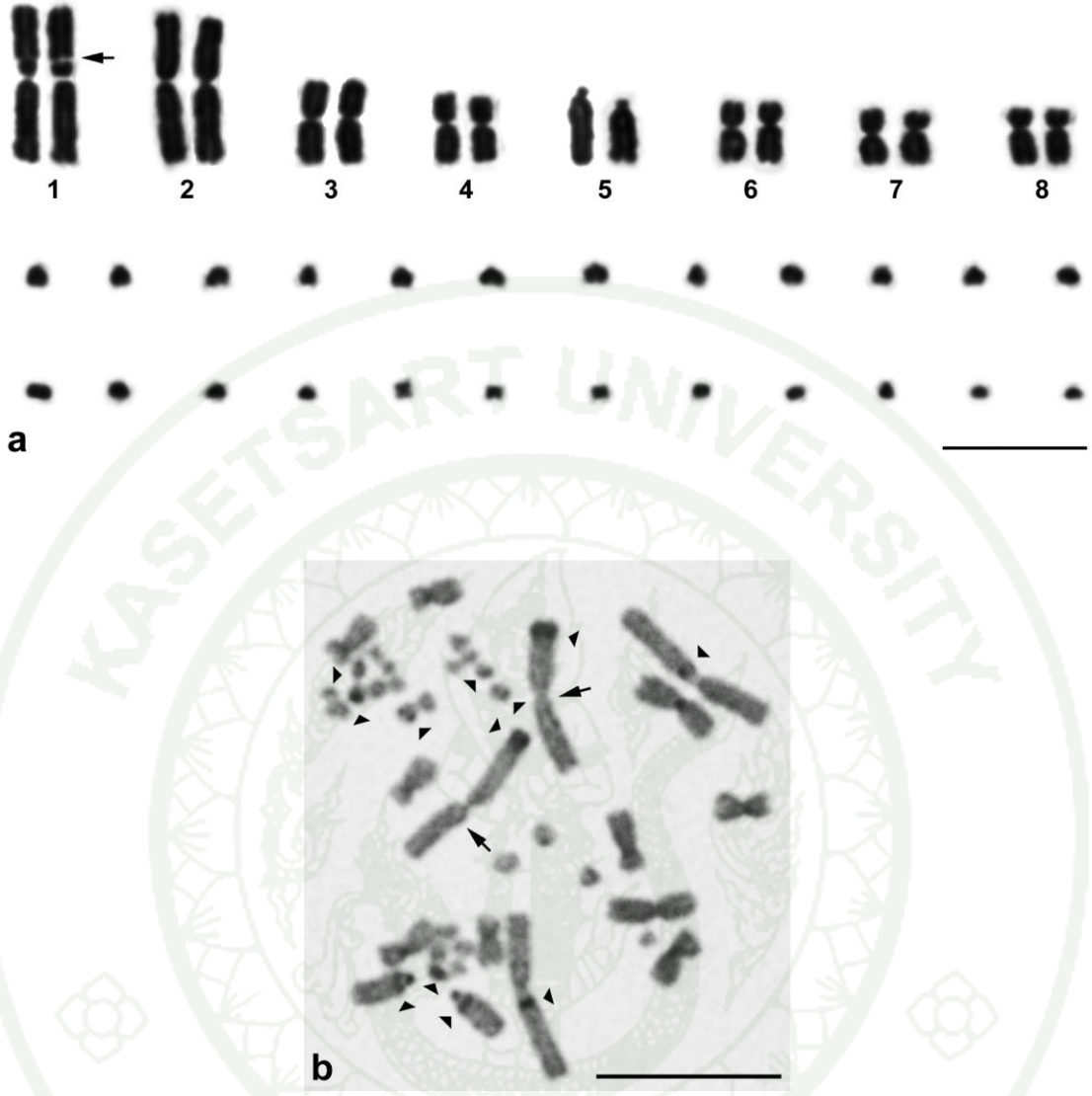


Figure 13 Giemsa-stained karyotype (**a**) and C-banded metaphase spread (**b**) of a male monitor lizard (*Varanus salvator macromaculatus*). Arrows indicate the secondary constriction. Arrowheads indicate C-positive heterochromatin blocks. Scale bars represent 10 μm.

8. Isolation of highly repetitive DNA sequences and their nucleotide sequences

Genomic hybridization with 576 fosmid clones identified 2 prominent clones, pFOSVSA1 and pFOSVSA2. We characterized 500 to 800 bp nucleotide sequences from the terminal ends of the 2 clones. Dot matrix analysis showed that pFOSVSA1 and pFOSVSA2 sequences contained tandem-arrayed repetitive sequences (Figure 14). Five units within pFOSVSA1 insert were each 190 bp, and their GC contents ranged from 56.3% to 57.9%, with an average of 57.5% (Figure 15a–c). The nucleotide sequence homologies among 5 fragments ranged from 88.9% to 96.3%. Five units within pFOSVSA2 insert were each 185 bp, and their average GC content was 59.7% (58.4–60.5%) (Figure 16d–f). Their nucleotide sequence identities ranged from 88.5% to 97.8%. The identity between consensus pFOSVSA1 and pFOSVSA2 sequences was 92.4%, indicating that these sequences were grouped in the same family of repetitive sequences (Figure 17). Interrogation of NCBI database for sequences homologous to any of the families of repetitive sequences failed to reveal any sequences with substantial homology. The identity of nucleotide sequences between VSAREP1 and VSAREP2 was as high as 92%, indicating that these repetitive sequences are grouped into the same family of repetitive sequences but different subfamilies. The centromeric heterochromatin of human chromosomes comprises several α-satellite subfamilies, which are located on different chromosomes (Willard and Waye, 1987; Plohl *et al*., 2008). These results lead us to predict that VSAREP1 and VSAREP2 originated from the same stDNA sequence and that their nucleotide sequences were diversified independently by base substitutions and/or deletions followed by tandem duplication in the same chromosomal regions.

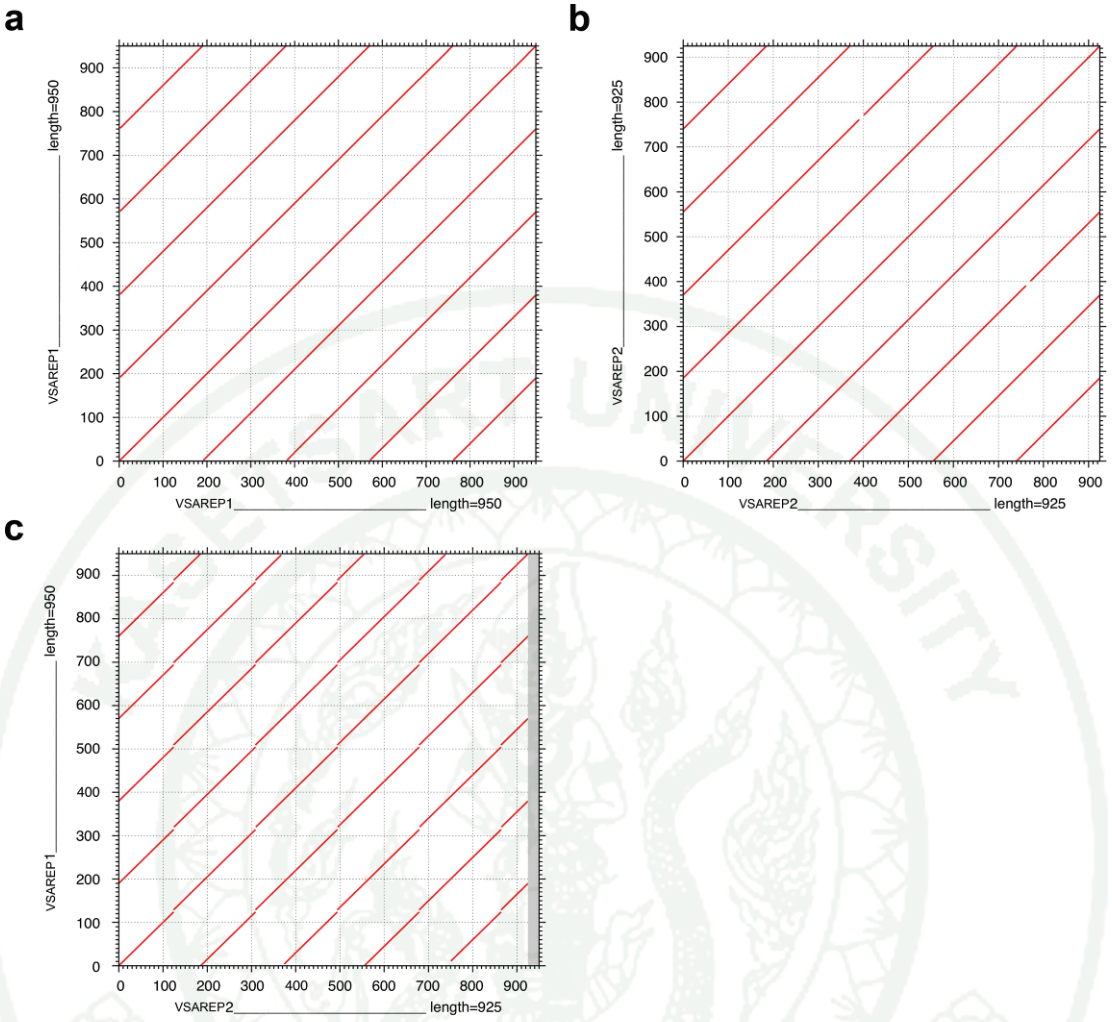


Figure 14 Dot matrix analysis of pFOSVSA1 sequence (**a**), pFOSVSA2 sequence (**b**), and comparison of pFOSVSA1 and pFOSVSA2 sequences (**c**). Sequences were compared with scoring matrix for nucleotide sequences: 200PAM/ K=2, and threshold: score = 39 (*E* = 8.4e-11).

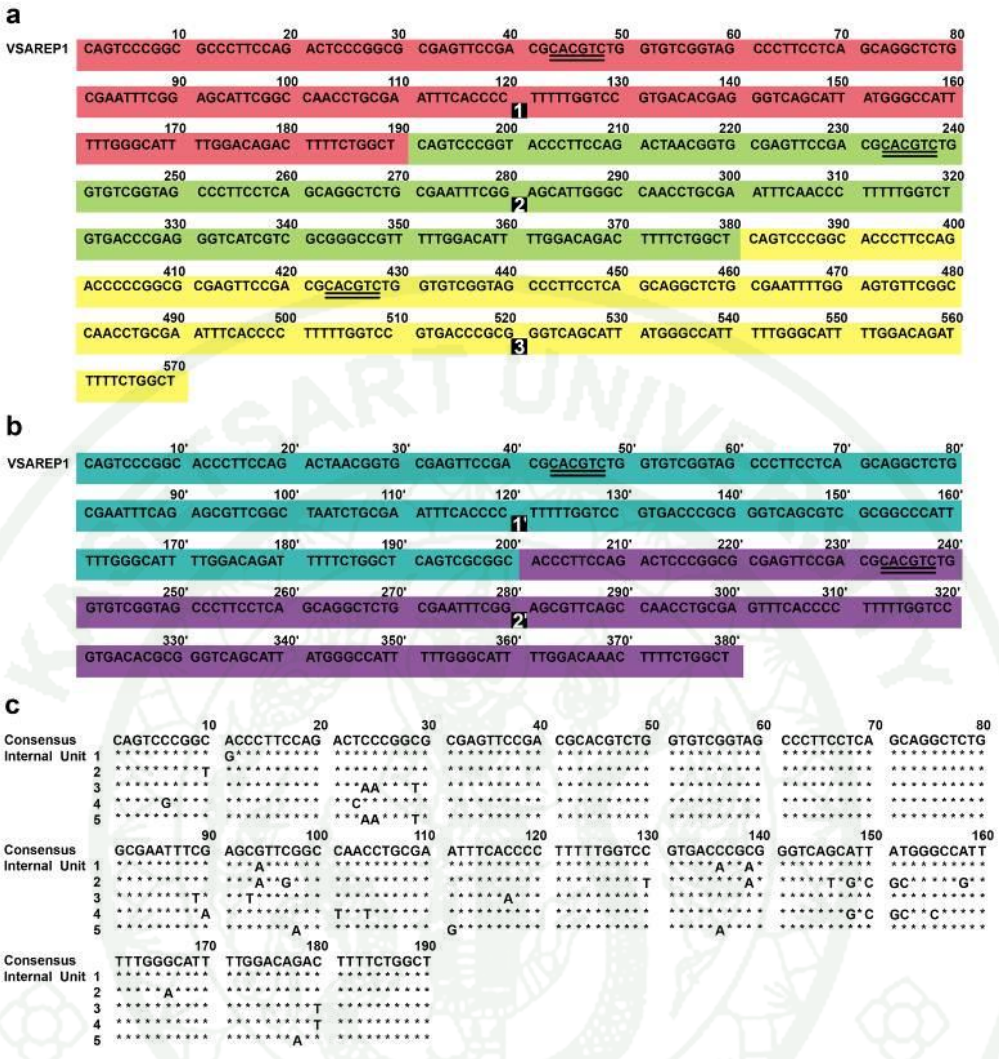


Figure 15 Nucleotide sequences of pFOSVSA1 (**a**–**c**). Nucleotide sequences of 3 VSAREP1 units (**a**) and 2 VSAREP1 units (**b**), which were determined using M13 forward primer and M13 reverse primer, respectively. Internal restriction sites of *Btr*I are represented by double straight lines. (**c**) Comparison of the nucleotide sequences of 5 VSAREP1 units. Asterisks indicate the same nucleotides as those of the consensus sequence of VSAREP1 units at the top.

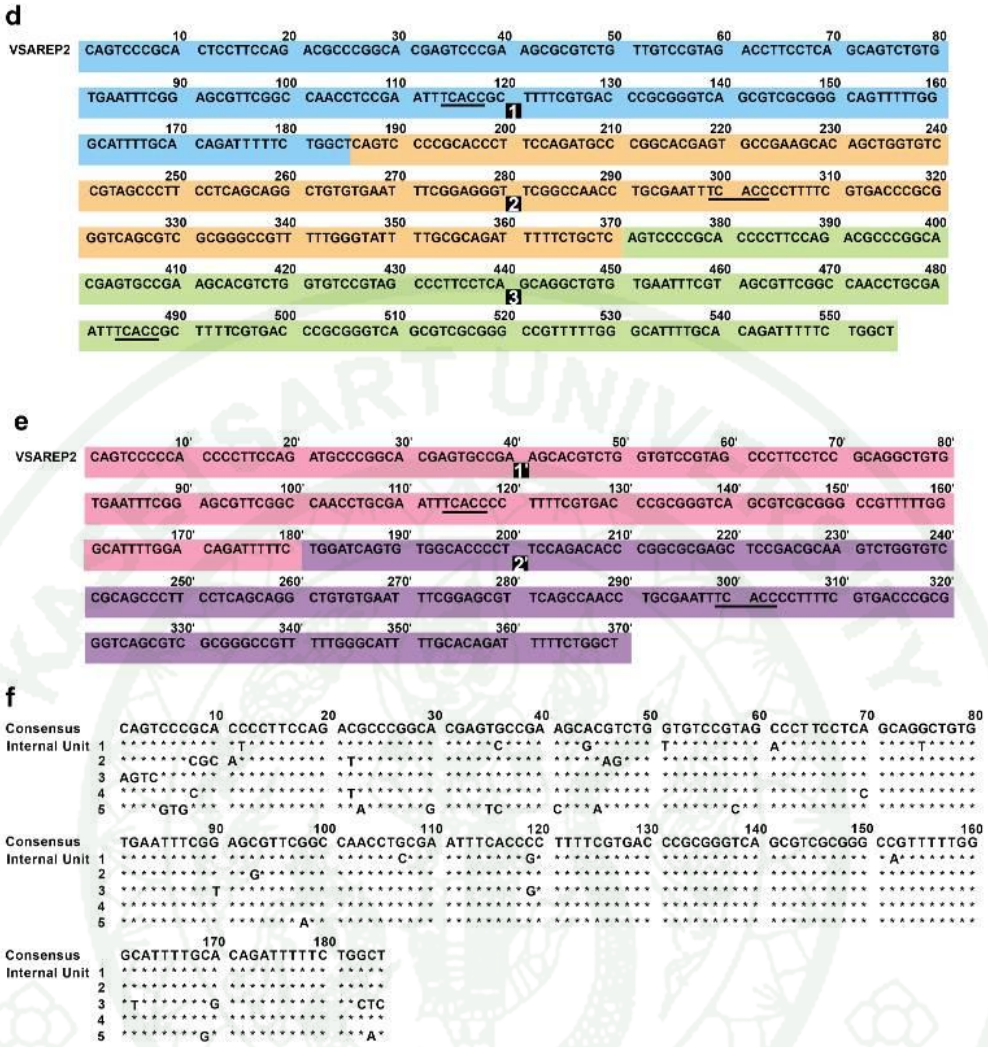


Figure 16 Nucleotide sequences of pFOSVSA2 (**d**–**f**). Nucleotide sequences of 3 VSAREP2 units (**d**) and 2 VSAREP2 units (**e**), which were determined using M13 forward primer and M13 reverse primer, respectively. Internal restriction sites of *Asu*HPI are represented by straight lines. (**f**) Comparison of the nucleotide sequences of 5 VSAREP2 units. Asterisks indicate the same nucleotides as those of the consensus sequence of VSAREP2 units at the top.

```
VSAREP1   CAGTCCCGGCACCCTTCCAGACTCCCGGCGCGAGTTCCGACGCACGTCTGGTGTCGGTAG  60
VSAREP2   ••••••••CAC••••••••••G••••••A•••••G••••A•••••••••••••C••••  60

VSAREP1   CCCTTCCTCAGCAGGCTCTGCGAATTTCGGAGCGTTCGGCCAACCTGCGAATTTCACCCC  120
VSAREP2   ••••••••••••••••G••T•••••••••••••••••••••••••••••••••••••••  120

VSAREP1   TTTTTGGTCCGTGACCCGCGGGTCAGCATTATGGGCCATTTTTGGGCATTTTGGACAGAC  180
VSAREP2   ••••--------••••••••••••••••G•CGC•••••G••••••••••••••C•••••T  175

VSAREP1   TTTTCTGGCT  190
VSAREP2   ••••••••••  185
```

Figure 17 Comparison between the nucleotide sequences of VSAREP1 and VSAREP2 units (accession numbers: AB773867 and AB773868). The same nucleotides as those of VSAREP1 are shown by dots in VSAREP2.

9. Genomic organization of repetitive sequences

Internal restriction sites of *Btr*I were found in pFOSVSA1 sequence and *Asu*HPI sites for pFOSVSA2 sequence (Figure 24a,b and Figure 25d,e). The fosmid vector (pCC1FOS) contains 2 *Bam*HI sites that are located at the boundaries of the cloning sites. Hence, digestion of the fosmid clone with only *Bam*HI produced 2 fragments: an 8.1-kb vector and a larger fragment that did not contain any *Bam*HI recognition sites (Figure 26). By contrast, the dominant product of complete digestion with *Bam*HI and *Btr*I for pFOSVSA1 or with *Bam*HI and *Asu*HPI for pFOSVSA2 was a fragment of about 200 bp, whereas partial digestion with *Btr*I for pFOSVSA1 or *Asu*HPI for pFOSVSA2 produced additional fragments with the sizes of about 400, 600, and 800 bp (Figure 18). These results suggest that the 190-bp sequence with a *Btr*I site and the 185-bp sequence with an *Asu*HPI site are basic repeat units of pFOSVSA1 and pFOSVSA2, respectively (Figure 15a,b and Figure 16d,e). These repeated sequence units comprising pFOSVSA1 and pFOSVSA2 were designated as VSAREP1 and VSAREP2 (accession numbers: AB773867 and AB773868), respectively.

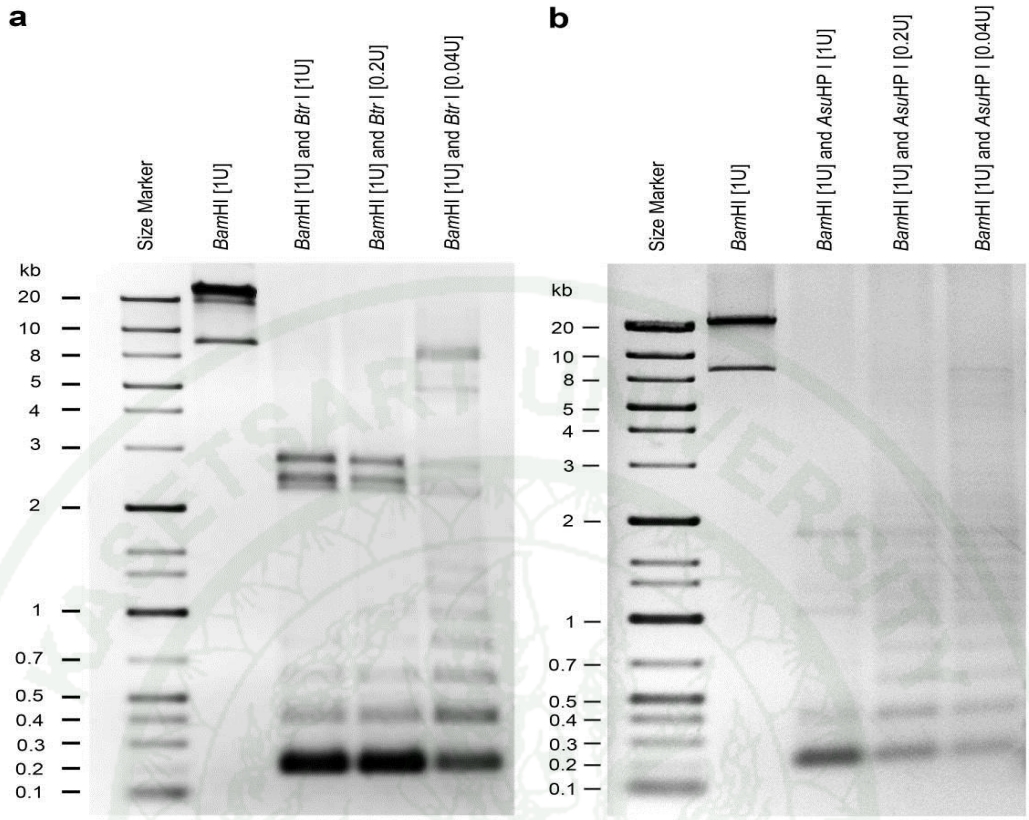


Figure 18 Genomic organization of pFOSVSA1 (VSAREP1) (**a**) and pFOSVSA2 (VSAREP2) (**b**) sequences shown by digestion with restriction endonucleases. The second lanes from left exhibit 2 major DNA bands produced by complete digestion with *Bam*HI; the 8.1- and 35- to 45-kb bands correspond to the vector (pCC1FOS) and the inserted genomic DNA fragments of *Varanus salvator macromaculatus*, respectively. The third lanes show completely digested patterns of pFOSVSA1 with *Bam*HI and *Btr*I (**a**) and pFOSVSA2 with *Bam*HI and *Asu*HPI (**b**), which exhibit ladder bands of the basal VSAREP1 and VSAREP2 units, respectively. The remaining 2 lanes show DNA bands of pFOSVSA1 produced by complete digestion with *Bam*HI and partial digestion with *Btr*I (**a**) and DNA bands of pFOSVSA2 produced by complete digestion with *Bam*HI and partial digestion with *Asu*HPI (**b**), which exhibit the same bands found in the third lanes and several additional larger ladder bands.

10. Chromosomal distribution of repetitive sequences

VSAREP1 and VSAREP2 sequences were arranged in tandem arrays and localized to the C-positive heterochromatin blocks in the pericentromeric region of chromosome 2q, the centromeric region of chromosome 5, and 3 pairs of microchromosomes (Figure 19). However, they did not hybridize to the C-positive heterochromatin in the distal region of chromosome 1q. Faint signals of the VSAREP1 sequence were also found in the pericentromeric region of chromosome 1q. This suggests that the two sequences should be categorized as stDNA sequences (Singer, 1982). Centromeric repetitive sequences are good cytogenetic markers to investigate chromosomal size-dependent genomic compartmentalization in birds and reptiles. Microchromosome-specific repetitive sequence families have been isolated from several species of Galliformes and Struthioniformes (Matzke *et al*., 1990, 1992; Tanaka *et al*., 2000; Yamada, Nishida-Umehara *et al*., 2002; Yamada, Shibusawa *et al*., 2002). These species are located at the basal position of avian phylogenetic tree and exhibit a typical avian karyotype with a large number of microchromosomes (Takagi and Sasaki, 1974; Belterman and De Boer, 1984, 1990; Nishida-Umehara *et al*., 2007). Three different types of microchromosome-specific repetitive sequence have also been found in Chinese soft-shelled turtle (*P. sinensis*) (2*n* = 66) (Yamada *et al*., 2005). These findings suggest that the repetitive sequences are separately homogenized between macrochromosomes and between microchromosomes but not between macro- and microchromosomes in these species. By contrast, the two novel stDNA sequences of *V. salvator macromaculatus*, VSAREP1 and VSAREP2, were located in the pericentromeric and/or centromeric regions of 2 macrochromosome pairs and 3 microchromosome pairs. This suggests that these centromeric repetitive sequences have evolved in a concerted manner across macro- and microchromosomes without chromosome size-dependent genomic compartmentalization. However, it is still unknown if this type of repetitive sequence is common in squamate reptiles. Molecular cloning of heterochromatin related sequences and their characterization are required for Serpentes (snake) and Iguania in order to obtain a better understanding of molecular evolution of heterochromatin associated with karyotype evolution in amniotes.

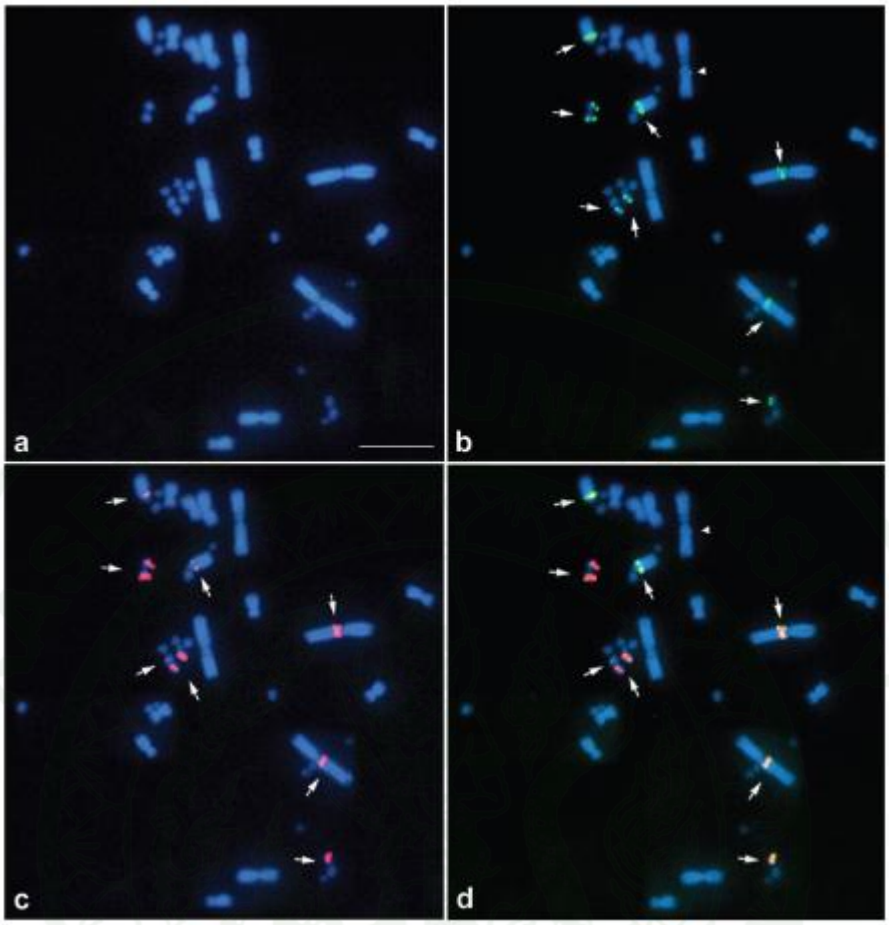


Figure 19 Chromosomal distribution of VSAREP1 and VSAREP2 sequences on a DAPI-stained metaphase spread prepared from a male *Varanus salvator macromaculatus*. (**a**) Fluorescent DAPI-stained pattern of chromosomes. (**b**–**d**) Chromosomal distribution of FITC-labeled VSAREP1 (**b**) and rhodamine-labeled VSAREP2 (**c**), and their cohybridization pattern (**d**). Arrows indicate strong hybridization signals. The arrowheads indicate a weak hybridization signal on chromosome 1q. Scale bar represents 10 μm.

11. Nucleotide sequence conservation of repetitive sequences

Nucleotide sequence conservation of VSAREP1 and VSAREP2 sequences was examined by slot-blot hybridization of 8 species representing 7 families of squamate reptiles (Figure 20). Intense hybridization signals were observed in *V. salvator macromaculatus*, whereas no signals were found in the other squamate reptilian species analyzed: *L. reevesii rubritaeniata*, *L. agilis*, *G. hokouensis*, *P. molurus bivittatus*, *E. quadrivirgata*, and *P. flavoviridis*. Centromeric repetitive sequences have been reported in two squamate reptiles, *Podarcis sicula* and *Lacerta graeca* (Lacertidae, Scincomorpha), which has only 1 pair of microchromosomes (Capriglione *et al*., 1989, 1991, 1994, 1998). The repetitive sequences of both squamate species were AT rich, located in the centromeric regions of all chromosomes, and highly conserved throughout the lacertid lizards. However, stDNA sequences isolated from *V. salvator macromaculatus* in the present study were not found in any of the other squamate reptiles tested. These included *V. exanthematicus*, a close relative of *V. salvator macromaculatus*. This result suggests that the copy number of the sequences in other squamate species may be too low to be detected by slot-blot hybridization because the sequences rapidly diversified or were specifically amplified in the lineage of *V. salvator macromaculatus*.

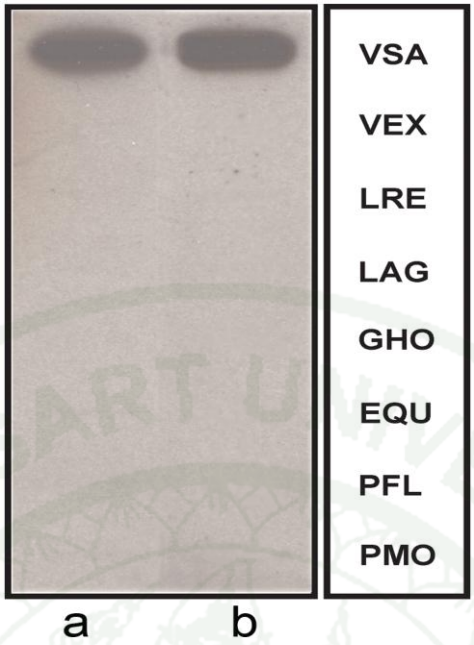


Figure 20 Slot-blot hybridization probed with VSAREP1 (**a**) and VSAREP2 (**b**) sequences. Genomic DNAs used in this experiment were as follows: *Varanus salvator macromaculatus* (VSA), *Varanus exanthematicus* (VEX), *Leiolepis reevesii rubritaeniata* (LRE), *Lacerta agilis* (LAG), *Gekko hokouensis* (GHO), *Elaphe quadrivirgata* (EQU), *Protobothrops flavoviridis* (PFL), and *Python molurus bivittatus* (PMO) of Leipidosauromorpha.

CONCLUSION

1. Complete mitochondrial genome of *V.salvator macromaculacus* and *V.salvator komaini* was 17,536 bp and 17,715 bp in length. Genome structures contained thirteen protein-coding genes, twenty-two tRNA genes, two rRNA genes, and two control region. The relative position and orientation of all composition were similar to those of most vertebrates.

2. Complete mitochondrial genome from 68 species in other lacertilian species from NCBI database comprising 5 groups (Anguimorpha, Scincoidea, Gekkota, Lacertoidea and Iguania) was analyzed and compared the genome organization. The type of gene organization was categorized into twenty-two types with the translocation of tRNA. However, there are three major characters which are known to animal mitochondrial genome variation: gene duplication, control region duplication and gene rearrangements. Type 3 represented the gene arrangement in two varanid lizards.

3. Two major non-coding regions were judged to be the control region because they contained conserved sequence blocks (CSBs) I and II, as well as extended termination associated sequence 1 (ETAS 1) that have been identified as conserved sequence elements for the control region of mammals. CSB II was suggested to be associated with the initiation of heavy-strand replication, whereas ETAS 1 was shown to be a pausing site of nascent heavy-strand synthesis to make a displacement loop.

4. A characteristic stem-and-loop structure for the putative origin of the light-strand replication found in mammals between asparagine and cysteine tRNA genes was obseaved in mitochondrial genome of two varanid lizards.

5. Sequence divergence of partial mitochondrial *ND2* and *COI* genes were conducted to provide DNA barcoding of six varanid lizards in Thailand. The overall mean sequence divergence was 13.2% for *ND2* and 10.5% for *COI*, indicating that *ND2* barcode could be the better marker to identify varanid lizards.

6. Chromosome number of *V. salvator macromaculatus* was composed of two distinct chromosomal components, macrochromosomes and microchromosomes: two pairs of large metacentrics (1st and 2nd), two pairs of medium-sized metacentrics (3rd and 4th), one pair of small acrocentrics (5th), three pairs of small submetacentrics (6th–8th), and 12 pairs of indistinguishable microchromosomes described as 2n = 40 (FN = 30 for 16 macrochromosomes). Moreover, a large secondary constriction was located in the proximal region of chromosome 1p.

7. Fluoresence signal of two novel heterochromatin-related repetitive sequences, VSAREP1 and VSAREP2, from the genomic library of *V. salvator macromaculatus* was observed. Both sequences were arranged in tandem arrays and localized to the C-positive heterochromatin blocks in the pericentromeric region of chromosome 2q, the centromeric region of chromosome 5, and 3 pairs of microchromosomes. This suggests that the two sequences should be categorized as stDNA sequences.

8. Homology of nucleotide sequences between VSAREP1 and VSAREP2 was as high as 92%, indicating that these repetitive sequences are grouped into the same family of repetitive sequences but different subfamilies.

9. Slot-blot hybridization to identify VSAREP1 and VSAREP2 sequences isolated from *V. salvator macromaculatus* in the present study were not found in any of the other squamate reptiles tested. These included *V. exanthematicus*, a close relative of *V. salvator macromaculatus*.

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APPENDIX

Appendix Table 1 Varanid lizards found in Thailand.

Species	Local names	Location
Varanus salvator macromaculatus	Hiea (เหี้ย), Laan Dok-mai (แลนดอกไม้), Tua Nguen Tua Tong (ตัวเงินตัวทอง)	throughout Thailand.
Varanus salvator komaini	Hiea Dam (เหี้ยดำ), Laan Kiam (แลนเคี้ยม), Mongkorn Dam (มังกรดำ)	Isle in the east side of southern part of Thailand.
Varanus rudicollis	Ngu-hao Chang (งูเห่าช้าง), Kor Lung (คอลั้ง), Hao Dong (เห่าดง), Hao Chang (เห่าช้าง)	Phuket mountain ranges, Nakhon Si Thammarat and San Kara Kiri mountain ranges
Varanus dumerilii	Tut-too (ตุ๊ดตู่), Hao Chang Kao (เห่าช้างขาว)	Nakhon Si Thammarat and San Kara Kiri mountain ranges from Surat Thani to Narathiwat provinces.
Varanus bengalensis	Ta-kuat-Nuer (ตะกวดใต้), Laan (แลน), Kuat Prow (กวดพร้าว)	Phu Khiew Wildlife Sanctuary, Chaiyaphum province, northeastern Thailand.
Varanus nebulosus	Ta-kuat-Tai (ตะกวดเหนือ)	throughout all mountain ranges of southern Thailand.

Appendix Table 2 Primers used for the amplification of mitochondrial genes in this study.

No	Primer name	Direction	Sequence (5´–3´)	Tm	Size (bp)	Source
1.	rPhe-1L	F	AAAGCACGGCACTGAARATGC	57°C	700	Kumazawa and Endo (2004)
2.	H1858	R	TCGATTATAGRACAGGCTCCTCTAG			Kumazawa and Endo (2004)
3.	rl2S-1L	F	AGGATTAGATACCCTACTA	49°C	650	Kumazawa and Endo (2004)
4.	r16S-3H	R	CAKKTTTCCCTTGCGGTACT			Kumazawa and Endo (2004)
5.	ul2S-1L	F	GCGYACAYAYCGCCCGTC	45°C	600	Kumazawa and Endo (2004)
6.	rl6S-5H	R	TTTATYRRGYAACCAGCTATC			Kumazawa and Endo (2004)
7.	rl6S-3L	F	AACCCYYGTACCTYTTGCATCATG	52°C	900	Kumazawa and Endo (2004)
8.	rl6S-lH	R	TYCACAGGGTCTTYTCGTC			Kumazawa and Endo (2004)
9.	r16S-2L	F	CRACTGTTTACCAAAAACAT	42°C	550	Kumazawa and Endo (2004)
10.	l6sbr-H	R	CCGGTCTGAACTCAGATCACGT			Kumazawa and Endo (2004)
11.	rl6S-4L	F	TACTCCAGGGATAACAGCGC	45°C	750	Kumazawa and Endo (2004)
12.	rND1-lH	R	GCRTATTTTGAGTTKGAKGCTCA			Kumazawa and Endo (2004)
13.	rND1-1L	F	TACATRCAACTWCGAAAAGG	49°C	550	Kumazawa and Endo (2004)
14.	rND1-2H	R	TCAAATGGKGCTCGRTTDGTTTC			Kumazawa and Endo (2004)
15.	rND1-2L	F	CAAACMATCTCMTAYGAAGT	49°C	700	Kumazawa and Endo (2004)
16.	rMet-2H	R	GGTATGGGCCCRAWAGCTT			Kumazawa and Endo (2004)

Appendix Table 2 (Continued)

No	Primer name	Direction	Sequence (5´–3´)	Tm	Size (bp)	Source
17.	rND1-3L	F	CGATTCCGATAYGACCAACT	49°C	900	Kumazawa and Endo (2004)
18.	rND2-2H	R	ATTGATGAGWAKGCTATRATTTTTCG			Kumazawa and Endo (2004)
19.	rND2-5L	F	TTACCCWCGAGCAACWGAAGC	52°C	1100	Kumazawa and Endo (2004)
20.	rAsn-lH	R	TGGGYGKTTAGCTGTTAAYTA			Kumazawa and Endo (2004)
21.	rND2-1L	F	GCCCCMYTMCACTTCTGA	46°C	1100	Kumazawa and Endo (2004)
22.	rCOl-3H	R	GTAYAGGGTGCCRATRTCTTT			Kumazawa and Endo (2004)
23.	rTrp-lL	F	TAAACCARGRGCCTTCAAAG	46°C	1050	Kumazawa and Endo (2004)
24.	rCOl-2H	R	GGGTGKCCAAARAATCAGAA			Kumazawa and Endo (2004)
25.	rCOl-lL	F	ATCGGCGGRTTYGGAAACTG	52°C	900	Kumazawa and Endo (2004)
26.	rCOl-1H	R	TAGTGGAARTGKGCTACTAC			Kumazawa and Endo (2004)
27.	rCOl-2L	F	TCWGCCACAATAATYATYGC	46°C	900	Kumazawa and Endo (2004)
28.	rCO2-1H	R	TGGAAGTGWARTAGYTCTTCTAT			Kumazawa and Endo (2004)
29.	rCOl-4L	F	TACTCAGACTACCCAGAYGC	57°C	900	Kumazawa and Endo (2004)
30.	uCO2-1H	R	CCGCAGATTTCTGAGCATTG			Kumazawa and Endo (2004)
31.	uCO2-lL	F	GGMCAYCAATGATACTGA	43°C	600	Kumazawa and Endo (2004)
32.	rAT6-3H	R	AAGYTTAKGGTCATGGTCA			Kumazawa and Endo (2004)

Appendix Table 2 (Continued)

No	Primer name	Direction	Sequence (5´–3´)	Tm	Size (bp)	Source
33.	Varanus CO2F	F	TACATAGCTCTGTCAACGCT	53°C	2250	This study
34.	Varanus CO2R	R	AAGAATGTTGATCCAAATACGCT			This study
35.	uCO3-1L	F	ATAGTWGACCCMAGCCCATGACC	58°C	900	Kumazawa and Endo (2004)
36.	uND3-2H	R	GGGTCRAAKCCRCATTCRTA			Kumazawa and Endo (2004)
37.	Varanus CO3F	F	AGCGTATTTGGATCAACATTCTT	53°C	1500	This study
38.	Varanus CO3R	R	CGGGCTGTTGCTACTAG			This study
39.	rND4L-1L	F	TGCATTGAARGYATAATACT	40°C	800	Kumazawa and Endo (2004)
40.	uND4-2H	R	CTACRTGKGCTTTTGGKARTCA			Kumazawa and Endo (2004)
41.	rND4L-2L	F	TAACCTTCTCMGCMTGYGAAGC	53°C	900	Kumazawa and Endo (2004)
42.	rND4-2H	R	GATGTTAAKCCGTGGGCRATTAT			Kumazawa and Endo (2004)
43.	rND4-3L	F	CCAAAAGCCCAYGTAGARGC	53°C	850	Kumazawa and Endo (2004)
44.	rCUN-1H	R	CTTTTACTTGGADTTGCACC			Kumazawa and Endo (2004)
45.	rHis-2L	F	AACAAAAACAYTAGRCTGTG	43°C	850	Kumazawa and Endo (2004)
46.	rND5-1H	R	ACWACTATTGTGCTKGAGTG			Kumazawa and Endo (2004)
47.	rND5-1L	F	TCCAAGCMATYATCTAYAACCG	45°C	650	Kumazawa and Endo (2004)
48.	rND5-2H	R	ATWGYGTCTTTTGAGTARAAKCC			Kumazawa and Endo (2004)

Appendix Table 2 (Continued)

No	Primer name	Direction	Sequence (5´–3´)	Tm	Size (bp)	Source
49.	V.ND5_F	F	GCTTTTAAAGGATAAYAGCCATC	55°C	6000	This study
50.	V.12S_R	R	CACCGTCAAGTCTTTTGAG			This study
51.	Vnil-ND5-2L	F	GAACAAGACCTGCGTAACATAGG	48°C	3000	Amer and Kumazawa (2008)
52.	V-ND6-1H	R	GGGATGGTYGTWGTWTTTGC			Amer and Kumazawa (2008)
53.	V.ND6_F	F	GCACAATTTGGTCTCCAC	56°C	3000	This study
54.	V.Phe_R	R	GTCATGCTTTAGGTATAAGCTAC			This study
55.	rThr-2L	F	YAAAGCMTTGRTCTTGTAA	42°C	1200	Kumazawa and Endo (2004)

Appendix Table 3 Primers used for the amplification of mitochondrial *ND2* and *COI* genes of six *Varanus* spp.

No	Primer name	Direction	Sequence (5´–3´)	Tm	Size (bp)	Source
1.	B.ND2F	F	AACYGAAGCYWCAACAAAATA	45°C	800	This study
2.	B.ND2R	R	GTTTRWADCGTCATGTGTTTTT			This study
3.	B.COIF	F	GCCGGAATAATTGGAACCGCCATAA	61°C	600	This study
4.	B.COIR	R	TAAAGGATTGGGTCYCCTCCACC			This study

Some variable sites in the mitochondrial genome are labeled with standard one-letter code: R = A, G; Y = C, T; W = A, T; D = A, G, T.

Appendix Table 4 Codon pattern composition (% of total number) for each protein-coding genes found in the mitochondrial genome of *V. salvator macromaculatus*.

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Ala	GCG	5	0.14	2
Ala	GCA	90	2.49	37
Ala	GCT	23	0.64	10
Ala	GCC	123	3.41	51
Cys	TGT	4	0.11	17
Cys	TGC	19	0.53	83
Asp	GAT	12	0.33	21
Asp	GAC	44	1.22	79
Glu	GAG	9	0.25	11
Glu	GAA	72	1.99	89
Phe	TTT	69	1.91	35
Phe	TTC	127	3.52	65
Gly	GGG	13	0.36	7
Gly	GGA	110	3.05	56
Gly	GGT	18	0.50	9
Gly	GGC	54	1.50	28
His	CAT	22	0.61	19
His	CAC	91	2.52	81
Ile	ATT	108	2.99	33
Ile	ATC	221	6.12	67
Lys	AAG	8	0.22	8
Lys	AAA	87	2.41	92
Leu	TTG	4	0.11	1
Leu	TTA	76	2.10	12

Appendix Table 4 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Leu	CTG	32	0.89	5
Leu	CTA	209	5.79	34
Leu	CTT	106	2.93	17
Leu	CTC	196	5.43	31
Met	ATG	32	0.89	18
Met	ATA	146	4.04	82
Asn	AAT	30	0.83	21
Asn	AAC	113	3.13	79
Pro	CCG	3	0.08	1
Pro	CCA	94	2.60	43
Pro	CCT	29	0.80	13
Pro	CCC	92	2.55	42
Gln	CAG	11	0.30	11
Gln	CAA	85	2.35	89
Arg	CGG	5	0.14	8
Arg	CGA	35	0.97	54
Arg	CGT	4	0.11	6
Arg	CGC	21	0.58	32
Ser	AGT	11	0.30	4
Ser	AGC	33	0.91	12
Ser	TCG	8	0.22	3
Ser	TCA	100	2.77	37
Ser	TCT	26	0.72	10
Ser	TCC	94	2.60	35
Thr	ACG	4	0.11	1

Appendix Table 4 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Thr	ACA	133	3.68	34
Thr	ACT	57	1.58	15
Thr	ACC	197	5.45	50
Val	GTG	2	0.06	2
Val	GTA	45	1.25	41
Val	GTT	20	0.55	18
Val	GTC	42	1.16	39
Trp	TGG	12	0.33	12
Trp	TGA	88	2.44	88
Tyr	TAT	25	0.70	28
Tyr	TAC	63	1.74	72

Appendix Table 5 Codon pattern composition (% of total number) for each protein-coding genes found in the mitochondrial genome of *V. salvator komaini*.

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Ala	GCG	5	0.14	2
Ala	GCA	89	2.46	36
Ala	GCT	24	0.66	10
Ala	GCC	126	3.49	52
Cys	TGT	4	0.11	17
Cys	TGC	20	0.55	83
Asp	GAT	12	0.33	21
Asp	GAC	44	1.22	79
Glu	GAG	10	0.28	13
Glu	GAA	68	1.88	87
Phe	TTT	68	1.88	35
Phe	TTC	128	3.54	65
Gly	GGG	12	0.33	6
Gly	GGA	111	3.07	57
Gly	GGT	18	0.50	9
Gly	GGC	55	1.52	28
His	CAT	23	0.64	21
His	CAC	89	2.46	79
Ile	ATT	106	2.93	32
Ile	ATC	223	6.17	68
Lys	AAG	8	0.22	8
Lys	AAA	90	2.49	92
Leu	TTG	4	0.11	1
Leu	TTA	76	2.10	12

Appendix Table 5 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Leu	CTG	31	0.86	5
Leu	CTA	206	5.70	33
Leu	CTT	109	3.02	18
Leu	CTC	195	5.40	31
Met	ATG	33	0.91	18
Met	ATA	146	4.04	82
Asn	AAT	29	0.80	20
Asn	AAC	117	3.24	80
Pro	CCG	3	0.08	1
Pro	CCA	94	2.60	43
Pro	CCT	31	0.86	14
Pro	CCC	89	2.46	41
Gln	CAG	11	0.30	11
Gln	CAA	87	2.41	89
Arg	CGG	4	0.11	6
Arg	CGA	35	0.97	55
Arg	CGT	4	0.11	6
Arg	CGC	21	0.58	33
Ser	AGT	10	0.28	4
Ser	AGC	32	0.89	12
Ser	TCG	7	0.19	3
Ser	TCA	99	2.74	37
Ser	TCT	26	0.72	10
Ser	TCC	95	2.63	35
Thr	ACG	5	0.14	1

Appendix Table 5 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Thr	ACA	133	3.68	34
Thr	ACT	57	1.58	15
Thr	ACC	194	5.37	50
Val	GTG	3	0.08	3
Val	GTA	44	1.22	40
Val	GTT	21	0.58	19
Val	GTC	41	1.14	38
Trp	TGG	12	0.33	12
Trp	TGA	88	2.44	88
Tyr	TAT	24	0.66	28
Tyr	TAC	63	1.74	72

Appendix Figure 1 Juvenile of *Varanus salvator macromaculatus* from the Chao Phraya River flood plain of central Thailand.

Source: Cota *et al*. (2009)

Appendix Figure 2 Juvenile of *Varanus salvator komaini* from small islands and areas near the coastline in southwestern Thailand**.**

Source: Dwyer and Perez (2007)

Appendix Figure 3 A juvenile of *Varanus rudicollis*. Characteristic for this monitor species are the enlarged nuchal scales.

Source: Koch *et al*. (2013)

Appendix Figure 4 Juveniles of *Varanus dumerilii* are very colorful. The attractive coloration of juvenile *Varanus dumerilii* fades in adult specimens.

Source: Koch *et al*. (2013)

Appendix Figure 5 *Varanus bengalensis* basks on tree trunk in direct sunlight to gain heat in the morning.

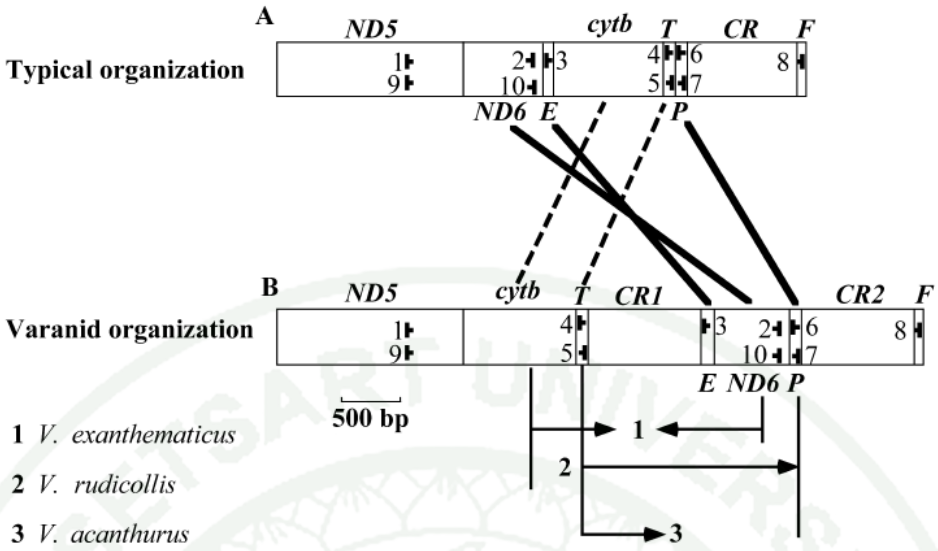
Source: Duengkae and Chuaynkern (2009)

Appendix Figure 6 Juveniles of *Varanus nebulosus* was seen with the front half of its body outside a hole at about 3.5m off the ground on a tree.

Source: Thomas (2013)

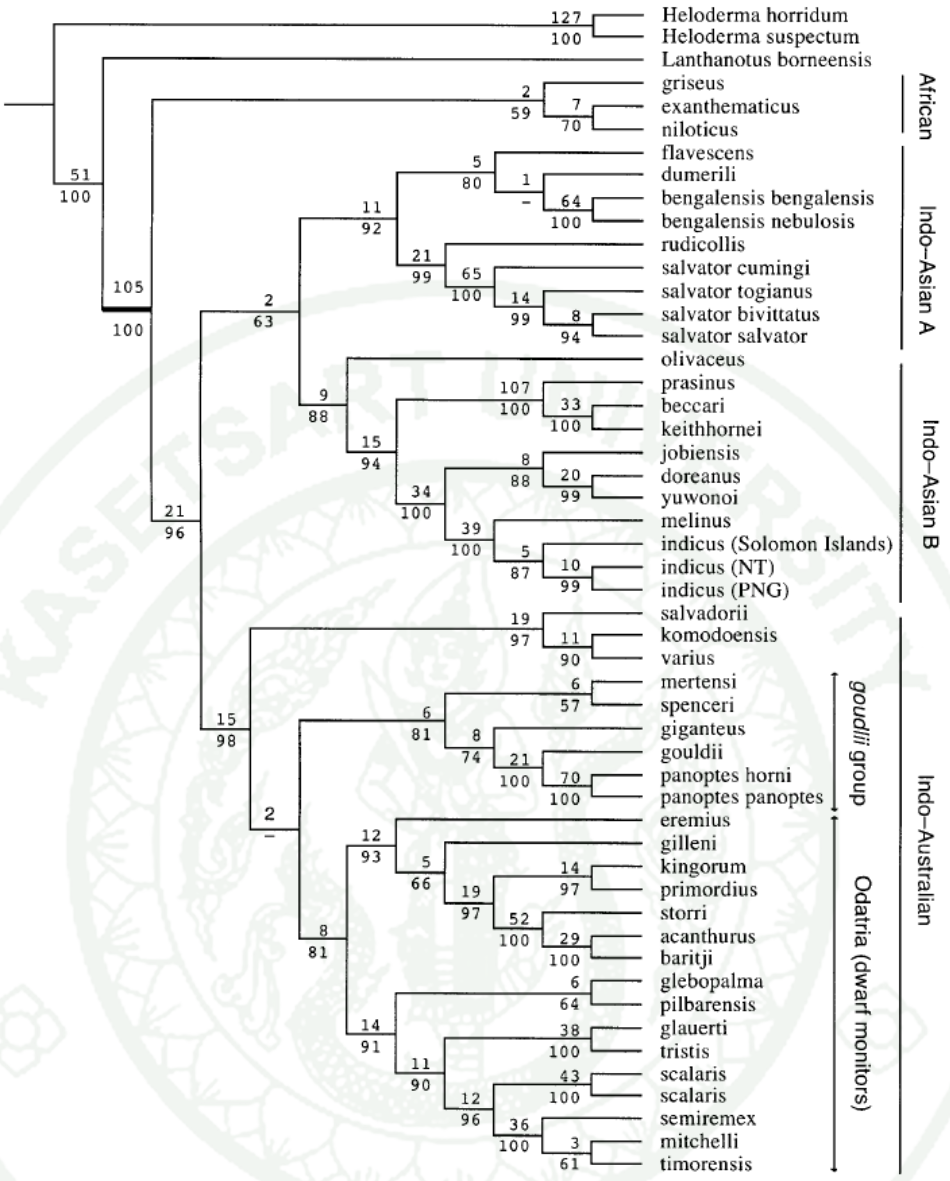
Appendix Figure 7 Thousands of Water Monitor Lizards (*Varanus salvator* ssp.) are cruelly harvested and killed each year for their skins (A), and a seized reptile leather bag made of *Varanus salvator* skin (B).

Source: Koch *et al*. (2013)



Appendix Figure 8 Relative arrangements of several genes and control regions (CRs) in the typical gene organization of vertebrate mtDNAs (A) and in the rearranged organization of the Komodo dragon (*Varanus komodoensis*) and Nile monitors (*Varanus niloticus*) (B). Correspondence of genes between the two organizations is shown with solid or dotted lines. The arrows below refer to the fragments that were sequenced for *Varanus exanthematicus* (1), *Varanus rudicollis* (2) and *Varanus acanthurus* (3).

Source: Amer and Kumazawa (2008)



Appendix Figure 9 Within *Varanus*, three major lineages (African, Indo Asian, and Indo-Australian) are delimited. The African species form a group sister to the rest of *Varanus*, while the Indo-Asian clade is sister to the Indo-Australian clade. The Indo-Asian group, weakly supported as monophyletic, comprises two distinct clades, labeled A and B.

Source: Ast (2001)

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PROCEEDING : Chaiprasertsri, N., A. Supikamolseni, S. Kamolnorranath, K. Kunya, S. Peyachoknagul and K. Srikulnath. 2013. Molecular barcoding of varanid lizards in Thailand. **Proceedings of the 39th Congress on Science and Technology of Thailand (STT39).** Bangkok. Thailand.