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THESIS

CYTOGENETIC CHARACTERIZATION AND MITOCHONDRIAL GENOME ANALYSIS IN THE BUTTERFLY LIZARD (*Leiolepis reevesii rubritaeniata*, Agamidae, Squamata)

KORNSORN SRIKULNATH

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Cytogenetic characterization of the butterfly lizard (*Leiolepis reevesii rubritaeniata*) was determined by conventional Giemsa staining, Ag NOR-banding, FISH with the 18S-28S and 5S rRNA genes and telomeric $(TTAGGG)_{20}$ sequences. The karyotype was composed of two distinct components, macrochromosomes and microchromosomes, and the chromosomal constitution was 2n=2x=36 ($L_4^m + L_2^{sm} + M_2^m + S_4^m$ + 24 microchromosomes). NORs and the 18S-28S rRNA genes were located at the secondary constriction of the long arm of chromosome 1, and the 5S rRNA genes were localized at the pericentromeric region of chromosome 6. Comparison to other two Thai butterfly lizards, *L. belliana belliana* and *L. boehmei*, showed similar major and minor ribosomal gene positions. However, hybridization signals of $(TTAGGG)_{20}$ sequences were observed at the telomeric ends of all chromosomes and interstitially at the same position as the 18S-28S rRNA genes in *L. reevesii rubritaeniata* and *L. boehmei*, suggesting that in the Leiolepidinae tandem fusion probably occurred between chromosome 1 and a microchromosome where the 18S-28S rRNA genes are located. Homologues of six chicken Z-linked genes (*ACO1*/*IREBP*, *ATP5A1*, *CHD1*, *DMRT1*, *GHR*, *RPS6*) were all mapped to *L. reevesii rubritaeniata* chromosome 2p in the same order as that on the snake chromosome 2p. The complete mitochondrial genome of these three butterfly lizards showed twenty-two tRNA genes, two rRNA genes, thirteen protein-coding genes and a control region in their mitochondrial genomes. The deletion of sequences approximately 47 bp in 12S rRNA gene has been revealed in *L. reevesii rubritaeniata* and *L. boehmei*, suggesting that it might occur in the lineage of *Leiolepis* spp. before the divergence of *L. reevesii rubritaeniata* and *L. boehmei*. Molecular phylogenetics comprising nuclear gene (*RAG1*, C-*mos*, *α-Enol* and *GAPD* genes) and concatenate 12 proteins coding mitochondrial gene also suggested that the most primitive among three butterfly lizards might be *L. belliana belliana*, which was more related to *L. reevesii rubritaeniata* while *L. boehmei* was the most recent species.

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Kornsorn Srikulnath

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LIST OF ABBREVIATIONS

Aco1/IREBP	=	soluble aconitase 1/iron-respinsive element binding protein
ATP5A1	=	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle
BrdU	=	5-bromodeoxyuridine
CCD camera	=	charge coupled device camera
cDNA	=	complementary deoxyribonucleic acid
CGH	=	comparative genomic hybridization
CHD1	=	chromodomain helicase DNA binding potein 1
C-mos	=	cellular maloney murine sarcoma
DAPI	=	4', 6-diamidino-2-phenylindole
DMRT1	=	*doublesex* and *mab-3* related transcription factor 1
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
EDTA	=	ethylene diamine tetraacetic acid
FISH	=	fluorescence *in situ* hybridization
GAPD	=	glyceraldehydes-3-phosphate dehydrogenase
GHR	=	growth hormone receptor
GSD	=	genetic sex determination
ITS	=	interstitial telomeric site
MYA	=	million years ago
NF	=	fundamental (arm) number
PCR	=	polymerase chain reaction
PEG	=	polyethylene glycol
PI	=	propidium iodide
RAG1	=	recombination activating gene-1
rDNA	=	ribosomal deoxyribonucleic acid
RNA	=	ribonucleic acid
RPS6	=	ribosomal protein S6
rRNA	=	ribosomal ribonucleic acid

LIST OF ABBREVIATIONS (Continued)

RT-PCR	=	reverse transcription-polymerase chain reaction
SSC	=	standard saline citrate solution
TE	=	Tris EDTA
Tm	=	melting temperature
Tris	=	Tris (hydroxymethyl) aminomethane
TSD	=	temperature sex determination
TSS	=	transformation and storage solution
UV	=	ultraviolet
α-Enolase	=	2-phospho-D-glycerate hydrolase
bp	=	base pair
ºC	=	degree celcius
g	=	gram
kb	=	kilobase pair
µg	=	microgram
µl	=	microlitre
µM	=	micromolar
mg	=	milligram
ml	=	millilitre
mM	=	millimolar
mm	=	millimeter
min	=	minute
×g	=	times gravity

CYTOGENETIC CHARACTERIZATION AND MITOCHONDRIAL GENOME ANALYSIS IN THE BUTTERFLY LIZARD (*Leiolepis reevesii rubritaeniata*, Agamidae, Squamata)

INTRODUCTION

Butterfly lizards (Leiolepidinae, Agamidae) are burrow digger inhabiting in the Southeast Asia, and show a great variety of karyotypes and sexual systems. In Thailand, there are three species (*Leiolepis belliana*, *L. reevesii rubritaeniata* and *L. boehmei*). They are hunted for food which results in population decrease in some regions, especially the northeast. Even though they are native species and commonly found in Thailand, the genetic system and genetic relationship are not comprehensively studied.

Recently, genetic linkage map of chicken revealed the absence of homology between human XY and chicken ZW sex chromosomes, suggesting that mammalian and avian sex chromosomes were derived from different common ancestor autosomes. Completion of chicken whole genome sequencing in 2004 provided new breakthrough and perspective for comparative genomics between Aves and Reptilia, and it was enabled to directly compare chromosome structures between the two taxa by comparative gene mapping. Fossil and molecular evidences demonstrated that the reptiles and birds shared a common ancestor and the phylogenetic tree of birds with reptilian group (bird are a group of reptiles). Thus, sex chromosome between birds and reptiles might have the same origin. However, the comparative mapping of reptilian homologues of chicken Z-linked genes has been revealed in some representative of reptiles except in iguanian lizard. To elucidate the genetic system and genetic relationship of *Leiolepis* spp. in Thailand and mapping of Z-linked homologues genes of chicken in iguanian lizard, this thesis has been conducted using conventional and molecular cytogenetic, and molecular phylogenetic approach. Chromosomal constitution of *Leiolepis* spp. was characterized by meiotic configurations, karyotype analysis, Ag-NOR-banding, fluorescence *in situ*

hybridization (FISH) with probes specific for the 18S and 5S ribosomal DNA and telomeric sequences $(TTAGGG)_{20}$ for *L. reevesii rubritaeniata*. The chromosomal constitution of *L. reevesii rubritaeniata* by molecular cytogenetics was also compared with *L. belliana belliana* and *L. boehmei*. Comparative genomic hybridization (CGH) was performed to find out the cryptic sex chromosomes of *L. reevesii rubritaeniata*. Six Z-linked genes (*ACO1*/*IREBP*, *ATP5A1*, *CHD1*, *DMRT1*, *GHR* and *RPS6*) of chicken were cloned and mapped on *L. reevesii rubritaeniata* chromosomes to reveal the homology of the chicken and other reptilian on chromosomes. The complete mitochondrial genome and nuclear genes (*RAG-1*, C-*mos*, *α-Enolase* and *GAPD*) of three species were cloned, sequences and phylogenetic reconstructd to gain mitochondrial genome organization and phylogenetic relationships among *Leiolepis* spp. in Thailand. DNA markers for discriminating *Leiolepis* spp. were constructed as well. The basic knowledge from this thesis would be useful to the Royal Initiative project of Her Royal Highness Princess Maha Chakri Sirindhorn for the conservation of the butterfly lizard in Thailand.

OBJECTIVES

1. To identify sex chromosomes of *L. reevesii rubritaeniata* through karyotype and CGH analysis of male and female

2. To compare the chromosomal homology of *L. reevesii rubritaeniata* with chicken sex chromosomes and other sex chromosomes of reptilians using Z-linked genes of chicken

3. To characterize chromosomal constitution of the butterfly lizard, *L. reevesii rubritaeniata* through karyotype analysis, and 18S rDNA, 5S rDNA and $(TTAGGG)_{20}$ sequences as the probe for FISH analysis

4. To compare chromosomal constitution of the other *Leiolepis* spp. (*L. belliana belliana* and *L. boehmei*) located in Thailand using 18S rDNA, 5S rDNA and $(TTAGGG)_{20}$ sequences as the probe for FISH analysis

5. To sequence complete mitochondrial genome of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*, and analyze the genetic relationship among butterfly lizards in Thailand using concatenate protein coding sequence mitochondrial DNA sequence and four nuclear gene sequences

LITERATURE REVIEW

1. Squamata and the butterfly lizard

Squamata is the most diverse reptilian order that has been traditionally classified into three suborders: Serpentes (snakes), Amphisbaenia (worm lizards) and Lacertilia (lizards). The extant lizards can be further categorized into five infraorders (Iguania, Gekkota, Scincomorpha, Diploglossa, Dibamia and Platynota). Butterfly lizards classified into infraorder Iguania are burrow digger and have habitat in Southeast Asia. Their systematics are classified based on Uetz (2009) as follows:

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Class	Reptilia
Order	Squamata
Suborder	Lacertilia
Infraorder	Iguania
Family	Agamidae
Subfamily	Leiolepidinae
Genus	*Leiolepis*

There are seven species in the Leiolepidinae (*Leiolepis belliana*, *L. reevesii*, *L. guttata*, *L. peguensis*, *L. triploida*, *L. guentherpetersi* and *L. boehmei*) which are distinctly different from each other by their typical scale and skin color (Peter, 1971). In Thailand, there are three species comprising *L. belliana* (*L. belliana belliana* and *L. belliana ocellata*), *L. reevesii rubritaeniata* and *L. boehmei* (Aranyavalai, 2003). *L. belliana belliana* is widely found in all region of Thailand, whereas *L. belliana ocellata* is located in the upper northern of Thailand. *L. reevesii rubritaeniata* only distribute in the northeast, and *L. boehmei*, of which all individuals are females, are in Songkhla and Nakhon Si Thammarat Provinces, the southern of Thailand.

2. The cytogenetics and sexual systems of the butterfly lizard

Butterfly lizards exhibit a great variety of karyotypes and sexual systems. Bisexual butterfly lizard has been identified in *L. belliana belliana* (2n=2x=36), *L. belliana ocellata* (2n=2x=34), *L. reevesii revesii* (2n=2x=36), *L. reevesii rubritaeniata* (2n=2x=36), *L. guttata* (2n=2x=36) and *L. peguensis* (Satrawaha and Tarpsipare, 1982; Kupriyanova, 1984; Sollender and Schmid, 1988; Aranyavalai, 2003; Srikulnath et al., 2009). Unisexual triploid butterfly lizard has been reported in *L. triploida* (2n=3x=54) and *L. guentherpetersi* (2n=3x=54), and putatively unisexual diploid butterfly lizard has been reported in *L. boehmei* (2n=2x=34) (Hall, 1970; Darevsky and Kupriyanova, 1993; Aranyavalai, 2004). Basically, the karyotype of diploid butterfly lizards was composed of ten metacentric macrochromosomes, two submetacentric macrochromosomes and 24 microchromosomes. This karyotype feature was also conserved in Iguania species (Gorman, 1973; Paull *et al.,* 1976; Olmo, 1986; Olmo and Signorino, 2005).

As for the sex determination, iguanian lizards have been revealed to be temperature sex determination (TSD), genetic sex determination (GSD) type comprising XY and ZW system, as well as homomorphic sex chromosome (Olmo and Signorino, 2005). By contrast, no heteromorphic sex chromosomes were morphologically identified in the butterfly lizards and the sex determination system has not been investigated in *Leiolepis* species yet.

3. Comparative genomic hybridization (CGH)

Comparative genomic hybridization (CGH) method effectively reveals changes of any DNA sequence (gains, amplification and losses) by a single hybridization of whole genomic DNA and makes mapping of these changes to normal chromosomes. CGH was originally developed to detect molecular differences between genome of normal and cancer cells at the cytogenetic level (Kallioniemi *et al*. 1992). Even though CGH could not detect the balanced chromosomal rearrangement, inversions or reciprocal translocations, this technique has been

successfully adapted to identify molecularly differentiated sex-specific regions (Traut *et al*., 2001). Especially in reptile species, heterogametic micro-sex chromosomes have been reported in a ZZ/ZW lizard, *Pogona vitticeps* (Ezaz *et al*., 2005), and a XX/XY turtle, *Chelodina longicollis* (Ezaz *et al*., 2006) and a ZZ/ZW turtle, *Pelodiscus sinensis* (Kawai *et al*., 2007) by CGH analysis.

4. 18S-28S rRNA and 5S rRNA genes

In higher eukaryotes, ribosomal RNA gene (rRNA) is organized into two distinct gene families. The major family encoding 18S, 5.8S and 28S rRNA are generally located at the nucleolar organizing region (NOR), whereas the other gene family encoding 5S rRNA as minor family. Both gene families are tandemly arrayed repeats and considered to evolve in a concerted manner (Arnheim *et al*., 1980; Liao, 1999). They are essentially species-specific providing a useful karyotypic marker as NOR banding and FISH mapping. In the conserved karyotype of Iguania, the 18S-28S rRNA genes are generally located on a pair of microchromosomes or chromosome 2 (Porter *et al*., 1991). Interestingly, lizards in the genus *Tropidurus* (Tropiduridae) have secondary constrictions and NORs in the long arm of chromosome 6 (Kasahara *et al*., 1987). However, the locations of 5S rRNA gene have not been reported in squamate reptiles.

5. Telomeric (TTAGGG)*n* sequences repeat

Telomeres are responsible for preserving the integrity and stability of the eukaryotic chromosomes and the distribution of telomeric sequences provides information on the process involving in karyotype evolution. The telomeric repeats are widely conserved among vertebrates and comprised the tandemly TTAGGG repeated sequence (Meyne *et al*., 1989, 1990). This repeated sequence has been detected not only in telomeres but also in interstitial and chromosomal centromeric region in a variety of vertebrate species (Nanda and Schmid, 1994; Abuin *et al*., 1996; Ocalewicz *et al*., 2004). Although the origin of non-telomeric sites named interstitial sites (ITSs) has not been investigated in detail, it might be remnants of chromosomal

rearrangement as fusion or inversion occurring in the course of genome evolution (Nanda and Schmid, 1994; Go *et al*., 2000). In Squamata, the ITSs are detected at subtelomeric and pericentromeric region in genus *Gonatodes* (Schmid *et al*., 1994), and pericentromeric region in *Leposoma* (Pellegrino *et al*., 1999).

6. The bird Z chromosome and chicken Z-linked gene homologues in reptile

Birds (avian species) have very high chromosome number and unique karyotype including macrochromosome and microchromosome. Generally, birds have chromosome number of 2n = 76-84 (Rodoinov, 1997). In most species, the largest nine pairs of chromosome are considered to be macrochromosome (Ladjadi *et al*., 1993). By contrast, microchromosomes vary in the number. The ratile contain 62 or 64 microchromosomes whereas chicken has 2n = 78 with 60 microchromosomes.

All species of birds exhibit ZW female heterogamety. The Z chromosome is very uniform in size, either the fourth or fifth largest; however, the W chromosome is strikingly different in the size. Chromosome painting using DNA from flow-sorted chicken Z chromosome demonstrates that the Z chromosome is genetically homologous across birds (Shetty *et al*., 1999; Schmid *et al*., 2000; Shibusawa *et al*., 2004; Itoh *et al*., 2005), and gene mapping showed the remnant of the Z chromosome to W chromosome. Z chromosome are degraded to a certain extent in different bird lineages (Mizuno *et al*., 2002). However, a comparison of 13 different Galliform species, analyzed by comparative chromosome painting and FISH mapping, showed that the arrangement of gene order on the Z chromosome is well conserved among Galliforms with only minor inversions (Suzuki *et al*., 1999) and between Galliform and Struthioniformes (Shibusawa *et al*., 2004; Itoh *et al*., 2006) with multiple inversions. These data collectively suggested that Z chromosome has the same gene composition, but different gene order in the lineage of birds.

According to the fossil and molecular evidence, the reptiles and birds shared a common ancestor and the phylogenetic tree of birds with reptilian groups (birds are a group of reptiles) (Kumazawa and Nishida, 1999; Janke *et al*., 2001; Benton and

Donoghue, 2007). This hypothesis is further supported by chromosome mapping. Six chicken Z-linked gene homologues, *ACO1/IREBP-RPS6-DMRT1-CHD1-GHR-ATP5A1* (soluble aconitase 1/iron-respinsive element binding protein (*ACO1/IREBP*), ribosomal protein S6 (*RPS6*)), *doublesex* and *mab-3* related transcription factor 1 (*DMRT1*), chromodomain helicase DNA binding protein 1 (*CHD1*), growth hormone receptor (*GHR*) and ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle (*ATP5A1*) are highly conserved in the order of genes from centromere to the distal end in the Z and W sex chromosome of the Hokou gecko (*Gekko houkouensis*) (Kawai *et al*., 2009). By contrast, the six chicken Z-linked genes were localized on chromosome 6 of the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and short arm of chromosome 2 of the Japanese four-striped rat snake (*Elaphe quadrivirgata*) (Matsuda *et al*., 2005; Matsubara *et al*., 2006; Kawai *et al*., 2007). However, the six chicken Z-linked genes were found on chromosome 3p of Siamese crocodile (*Crocodylus siamensis*) except the *ACO1/IREBP* and *RPS6* that were mapped on chromosome 3q (Kawai *et al*., 2007). These findings suggested that gene linkage and gene order are highly conserved since the common ancestor of Archosauromorpha (crocodiles, birds and turtle) and Lepidosauria (lizard, snake and tuatara) first appeared around 260–290 MYA (Kumazawa and Nishida, 1999; Janke *et al*., 2001; Benton and Donoghue, 2007; Kumazawa, 2007).

7. Mitochondrial genome and nuclear gene sequencing in Squamata

Animal mitochondrial genome is a haploid genome which is double-stranded circular DNA comprising heavy strand (H-strand) and light strand (L-strand). Generally, it is highly conserved for structural organization and composition due to the compact without introns in mitochondrial genes, and clear orthology of homologous gene sequences in vertebrates. It is commonly composed of 13 protein coding genes (Cytochrome C oxidase subunit I (*COI*), *COII*, *COIII*, Cytochrome b (*Cytb*), ATPase subunit 6 (*ATPase6*), ATPase subunit 8 (*ATPase8*) and NADH dehydrogenase subunit (*NADH*) 1, 2, 3, 4, 4L, 5 and subunit 6), 2 ribosomal RNA genes (*12s rRNA* and *16srRNA*), 22 transfer RNA genes ($tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Leu}$(UUR), $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$,

$tRNA^{Tyr}$, $tRNA^{Ser}$(UCN), $tRNA^{Asp}$, $tRNA^{Lys}$, $tRNA^{Gly}$, $tRNA^{Arg}$, $tRNA^{His}$, $tRNA^{Ser}$(AGY), $tRNA^{Leu}$(CUN), $tRNA^{Glu}$, $tRNA^{Thr}$ and $tRNA^{Pro}$), and one control region (CR) that contains signals for replicating the heavy strand for transcription (Boore, 1999). 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*., 1997a; 1997b). 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 phylogenetic positions of taxa.

According to the classification of squamate species, they are categorized into three groups, Lacertilia (lizards), Serpentes (snakes) and Amphisbaenia (worm 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 (Amer and Kumazawa, 2005). By contrast, the snake mitochondrial genome comprising two control regions, and translocation of the $tRNA^{Leu}$ is notable features (Dong and Kumazawa, 2005; Kumazawa, 2007). These findings implied the significance to construct the Squamata phylogeny for example *Pogona vitticepes* (Amer and Kumazawa, 2005), *Calotes versicolor* (Amer and Kumazawa, 2007), *Lacerta viridis viridis* (Böhme *et al*., 2007) and *Anolis cybotes* (Okajima and Kumazawa, 2009).

The nuclear genes are also used to speculate the squamate phylogeny. They are effectively capable for resolving relationships among intermediately diverged taxa. In Squamata, the most nuclear genes analyzed data are recombination activating gene-1 (*RAG-1*), cellular maloney murine sarcoma (C-*mos*), *α-Enolase*, and glyceraldehydes-3-phosphate dehydrogenase (*GAPD*).

C-*mos*, a candidate nuclear gene, is a proto-oncogene encoding a serine/threonine kinase expressed at high levels in germ cells, which the protein regulates cell maturation and tubulin formation (Yew *et al*., 1993). *RAG1*, recombination activating gene-1, is a nuclear gene encoding components of

recombinase enzyme which involves in V(D)J recombination of T-receptor and immunoglobulin genes (Schatz *et al*., 1989; Oettinger *et al*., 1990). Both C-*mos* and *RAG1* genes are single-copy, without introns. Beside a few insertions and deletions, there are no repetitive sequences that make complication of sequence alignment among species. They have also been found in the genome of vertebrates. These attributes make them particularly useful for reconstructing deep phylogenetic relationships within a number of vertebrate groups, especially in Squamata (Saint *et al*., 1998; Townsend *et al*., 2004; Vidal and Hedges, 2004).

α-Enolase (2-phospho-D-glycerate hydrolase, EC no. 4.2.1.11) catalyses the dehydration of 2-phospho-glycerate to phosphoenolpyruvate during glycolysis (Lebioda and Stec, 1988; Kim *et al*., 1991). It was found that the gene coding for *α-Enolase* comprise 12 exons (1731 bp) and 11 introns of varying lengths in the Peking duck (*Anas platyrhynchos*) (Kim *et al*., 1991). *GAPD* (glyceraldehydes-3-phosphate dehydrogenase, EC No. 1.2.1.12) is a glycolytic enzyme that catalyses the reversible reduction of D-glyceraldehyde-3-phosphate to 1,3-diphospho-glyceric acid (Stone *et al*., 1985). To alternate nuclear portion analysis, selectional constraints on introns are relaxed relative to section of the genome coding for functional proteins resulting in relatively high variabilities (Palumbi and Baker, 1994). Both *α-Enolase* and *GAPD* genes have the blocks of sequence which are conserved across species. The conserved exons flanking the introns were identified (Friesen *et al*., 1997). These characteristics make them very amenable to PCR amplification from genomic DNA and direct sequencing of PCR products, especially in squamate reptiles (Friesen *et al*., 1997; Benavides *et al*., 2009; Crottini *et al*., 2009).

For Leiolepidinae lizards in Thailand, no genetic system and genetic relationship have been conducted. Likewise, it is still unknown whether they exhibit genotypic sex determination (GSD) or environmental sex determination (ESD). In this thesis, to characterize chromosomal constitution of Leiolepidinae lizards, we conducted karyotype analysis, meiotic configurations, Ag-NOR-banding, and fluorescence *in situ* hybridization (FISH) using probes specific for 18S-28S and 5S ribosomal RNA genes and telomeric $(TTAGGG)_{20}$ sequences for identification of *L.*

reevesii rubritaeniata. The chromosomal constitution of *L. reevesii rubritaeniata* from molecular cytogenetics with *L. belliana belliana* and *L. boehmei* was also compared. Comparative genomic hybridization (CGH) was also performed to identify cryptic sex chromosomes. In addition, we molecularly cloned homologues of six chicken Z-linked genes (*ACO1*/*IREBP*, *ATP5A1*, *CHD1*, *DMRT1*, *GHR* and *RPS6*), and subsequently mapped them to *L. reevesii rubritaeniata* chromosomes to identify the conserved linkage homology with the chicken Z sex chromosome and other reptilian chromosomes. Furthermore, the complete mitochondrial genome and nuclear genes (*RAG-1*, C-*mos*, *α-Enolase* and *GAPD*) of three species were cloned, sequenced and analysed for phylogenetic data to gain mitochondrial genome organization and phylogenetic relationships among *Leiolepis* species in Thailand.

MATERIALS AND METHODS

Materials

Specimen

A mature male and female of *Leiolepis reevesii rubritaeniata* were captured in Nakon Ratchasima province, northeast of Thailand. Four adult males of *L. belliana belliana* were captured in Chonburi province, east of Thailand, and four adult females of *L. boehmei* were captured in Songkla province, southern Thailand. Their sex were morphologically determined, and then confirmed by the internal genital anatomy. All experimental procedures with the animals were conformed to the guidelines established by the Animal Care Committee, Hokkaido University, Sapporo, Japan. Although *L. belliana ocellata* used to be found in Thalinad, it was not available in this study.

Methods

1. Cell culture

All butterfly lizard specimens were intraperitoneally injected with pentobarbital. Heart, lung and mesentery were subsequently dissected from each individual and used for cell culture. The tissues of *L. reevesii rubritaeniata* and *L. boehmei* were minced, and cultured in the Dulbecco's modified Eagle's medium (Invitrogen-GIBCO, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Invitrogen-GIBCO), 100 µg/ml kanamycin and 1% antibiotic-antimycotic (penicillin-streptomycin-amphotericin; PSA) (Invitrogen-GIBCO). All cultures were incubated at 30°C in an incubator with 5% CO_2. Primarily cultured fibroblast cells were collected and rinsed with 0.25% trypsin in PBS and then subcultured.

2. Chromosome preparation

Fibroblast cells of heart, lung and mesentery at log phase were harvested after 40 ng/ml colcemid treatment for 2 h. Cells were collected and rinsed with 0.25% trypsin in PBS followed by centrifugation at 447 ×g for 5 min. The supernatant was discarded and the pellets were suspended in 5 ml 0.075 M KCl for 10 min at room temperature and fixed with 5 ml 3:1 methanol/acetic acid followed by centrifugation at 447 ×g for 5 min. The supernatant was discarded and the pellets were suspended in 10 ml 3:1 methanol/acetic acid and subjected to another round of centrifugation. After that, cells were dispersed and resuspended in 3:1 methanol/acetic acid, then they were dropped onto cleaned glass slides and air-dried. For karyotype analysis, the mitotic chromosomes were stained with 5% Giemsa solution in phosphate buffer (0.07 M Na_2HPO_4, 0.07 M NaH_2PO_4, pH 6.8) for 15 min at room temperature. For CGH analysis, the chromosome slides were kept at -80 ◦C until use.

For FISH chromosome mapping of six chicken Z-linked gene, replication R-banding was performed according to Matsuda and Chapman (1995) to identify the position of probes on a specific chromosome. Fibroblast cell cultures were supplemented with 12 µg/ml BrdU (Sigma-Aldrich, St. Louis, MO, USA) at the late replication stage for 8 h, added with 30 ng/ml colcemid for another 2 h, then the cells were collected, and chromosome preparation was performed as mentioned above. Slides were dried for 2–3 days at room temperature. After staining with Hoechst 33258 (1 µg/ml) for 10 min, R-bands were obtained by heating at 65°C for 3 min and exposing to UV light at 65°C for an additional time of 6 min. The slides were kept at –80◦C until use.

Meiotic chromosomes were prepared followed the procedure described by Imai *et al*. (1981) with slight modification. The testes of *L. reevesii rubritaeniata* and *L. belliana belliana* were cut into two or three pieces, then placed in a 4 cm Petri dish, added with 2 ml of 0.075 M KCl hypotonic solution, minced, and left at room temperature for 30 min. Subsequently, they were transferred to 2 ml of fixative solution (3:1 methanol: glacial acetic acid) and left at room temperature for 10 min.

The cell suspension was transferred into a centrifuge tube, added with 8 ml of fixative solution and centrifuged at 447 ×g for 10 min. Supernatant was discarded and cell pellets were resuspended in fixative solution, then they were dropped onto cleaned glass slides, left slide dried and stained with 5% Giemsa solution as mentioned above.

3. Karyotype analysis

All chromosomes were photographed from five metaphase cells per sex and subsequently measured using scale Vernier Calliper. Only macrochromosomes were characterized by total chromosome length (TL), short arm length (p), long arm length (q) and relative length (RL) (Turpin and Lejeune, 1965). The relative length (RL) was the average relative length (five metaphases) determined based on RL = TL/∑TL in each metaphase. The order of macrochromosome was arranged based on the chromosome length. The biggest to the smallest macrochromosomes were categorized into the first macrochromosome and the last macrochromosome, respectively.

The sizes were classified to be large (L), medium (M) and small (S) as followed:

L : chromosome was TL > (the biggest macrochromosome + the smallest macrochromosome)/2

M : chromosome was TL < L

S : chromosome was TL < (TL of the biggest macrochromosome)/2

The morphological classification of chromosomes was calculated from the centromeric index (CI) according to Levan *et al*. (1964). The centromeric index (p/TL) was the ratio between the length of the short arm (p) and the total chromosome length (TL). Accordingly, chromosomes were classified as metacentric (CI) = 0.5-0.38, submetacentric (CI) = 0.37-0.26, subtelocentric (CI) = 0.25-0.13 and telocentric (CI) = 0.12-0.

4. DNA extraction

Whole genomic DNA to be used as a template for PCR and CGH analysis was extracted from 5 mm^3 liver from all individuals following the standard phenol-chloroform-isoamyl alcohol protocol (Sambrook and Russell, 2001) with slight modification. After homogenization, tissues were digested at 37°C overnight using 500 µl of 25 µg/µl proteinase K and 500 µl 0.5% (w/v) SDS in 9 ml STE buffer (0.1 M NaCl, 50 mM Tris and 1 mM EDTA, pH 8.0). Then, the mixture was extracted by adding 10 ml phenol-chloroform-isoamyl alcohol (25:24:1), and mixed slowly by shaker. The sample was centrifuged at 1,006 ×g for 10 min at 4°C, and 9-10 ml of supernatant was transferred to a clean tube. After that, DNA was precipitated with 0.05 volume of 0.2 M NaCl and 2.5 volume of absolute ethanol. The solution was gently mixed and kept at -80 °C for 20 min, and then DNA was precipitated by centrifugation at 112 ×g, 447 ×g and 1,006 ×g each for 10 min, respectively. The supernatant was discarded, and the pellet was washed with 5 ml of 70% ethanol followed by centrifugation at 112 ×g for 10 min. The genomic DNA was air-dried and resuspended in TE buffer (50 mM Tris, pH 8.0, 100 mM EDTA), and kept at -20 °C until use.

5. Comparative genomic hybridization (CGH)

To identify sex specific region, CGH was performed according to the procedure described by Srikulnath *et al*. (2009). The 250 ng of male and female genomic DNA of *L. reevesii rubritaeniata* was labeled with FITC-dUTP (Invitrogen-Molecular Probes, Carlsbad, CA, USA) and Cy3-dUTP (GE Healthcare, Buckinghamshire, UK), using a nick translation kit (Roche Diagnostics, Basel, Switzerland) following the manufacture instruction. Labeled DNAs were precipitated by 2.5 volume of absolute ethanol and 10 mg/ml salmon sperm DNA and *E. coli* tRNA. The solution was gently mixed and kept at -80 °C for 20 min, and then the labeled DNA precipitants were precipitated by centrifugation at 11,337 ×g for 20 min. The labeled DNAs were resuspended in 10 µl 100% formamide. Slides were hardened

at 65 °C for 2 h, denatured at 70 °C for 2 min in 70% formamide/2× SSC, and dehydrated in 70% ethanol for 5 min and absolute ethanol for 5 min.

A 20 µl mixture containing FITC-labeled male genomic DNA and Cy3-labeled female genomic DNA, 50% formamide, 2× SSC, 10% dextran sulfate and 2 µg/µl BSA was hybridized onto male chromosome slides or female chromosome slides at 37 °C for three days. After hybridization, slides were washed in 4× SSC, 1% Nonidet P-40/4× SSC and 2× SSC for 5 min each at room temperature, and then they were counterstained with 1 µg/ml DAPI (4', 6-diamidino-2-phenylindole). The fluorescence hybridization signals were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments, Lurgan, UK) mounted on a Leica DMRA microscope, and analysed using the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd (Cambridge, UK).

6. Comparison of chicken Z-linked gene homologues

The results of homologues *L. reevesii rubritaeniata* chicken Z-linked genes were cloned and used for chromosome mapping: *ACO1/IREBP*, *ATP5A1*, *CHD1*, *GHR*, *DMRT1* and *RPS6*. PCR primers of the six genes used in this study were kindly provided by Kawai *et al*. (2009). Testes of *L. reevesii rubritaeniata* were homogenized and lysed with TRIzol Reagent (Invitorgen, Carlsbad, CA, USA), and total RNA was extracted following the manufacture instruction.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the homologues of the chicken Z-linked genes according to Kawai *et al*. (2009). For the cDNA synthesis, 3 µg of total RNA was mixed with 0.5 µg Oligo (dT)12–18 Primer (Invitrogen) and incubated for 10 min at 70 °C. After cooling on the ice, the solution was mixed with 1× first strand buffer, 0.1 M DTT, 10 mM deoxyribonucleoside triphosphate (dNTP), and 200 U of SuperScript II RNase H− reverse transcriptase (Invitrogen). Reactions were performed for 50 min at 42 °C and terminated by incubating for 15 min at 70 °C. The cDNA amplification was

carried out in 20 µl of 1× ExTaq buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5.0 µM degenerated primers, and 0.25 U of TaKaRa Ex Taq (Takara Bio, Japan). The PCR conditions were performed as follows: an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 35 s; and a final extension at 72 °C for 5 min. Nucleotide sequences of the primers used for cDNA cloning of the genes are shown in Appendix Table 1.

PCR products were examined by electrophoresis on 1% agarose gel. The DNA fragments were extracted using a QIAquick Gel Extraction Kit (Qiagen) from the ethidium bromide-stained gel according to the manufacture instruction and ligated using pGEM-T Easy Vector System I (Promega, USA). The ligated DNAs were transformed into *E.* coli DH5α competent cells following the manufacture instruction. Nucleotide sequences of the DNA fragments were determined using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) on the ABI PRISM3100 DNA Analyzer (Applied Biosystems, U.S.A.). Nucleotide sequences of *L. reevesii rubritaeniata* homologues of the six chicken Z-linked genes in the equivalent regions were compared with four reptilian species, Chinese soft-shelled turtle (*Pelodiscus sinensis*), Japanese four-striped rat snake (*Elaphe quadrivirgata*), Siamese crocodile (*Crocodylus siamensis*) (Matsuda *et al*., 2005; Kawai *et al*., 2007) and Hokou gecko (*Gekko hokouensis*) (Kawai *et al*., 2009), chicken (*Gallus gallus*), and human (*Homo sapiens*) using blastx and blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

7. Competent cell preparation

Competent cells were prepared using the method described by Chung *et al*. (1989). About 500 µl of an overnight culture of *E. coli* was added to 50 ml of LB broth and incubated at 11.25 ×g, 37 °C until *E.coli* reached the early exponential phase (OD_{600} at 0.3-0.4). Then, the culture was transferred to a 50 ml centrifuge tube and incubated on ice for 30 min. The cells were subsequently harvested by centrifugation at 1,073 ×g, 4 °C for 5 min and resuspended in 5 ml ice-cold transformation and storage solution (TSS: LB broth containing 10% PEG (MW 8000), 5% (v/v) DMSO

and 50 mM $MgCl_2$, pH 6.5). Cells were harvested again and resuspended in 2 ml ice-cold TSS. Aliquots in 100 µl cell suspension were made and stored at -80 ◦C until use.

8. FISH mapping

Fluorescence *in situ* hybridization (FISH) was performed for localization of the 18S-28S rDNA, the 5S rDNA, telomeric sequences $(TTAGGG)_{20}$ in three butterfly lizard species and six chicken Z-linked gene homologues in *L. reevesii rubritaeniata* as described previously (Matsuda and Chapman, 1995; Srikulnath *et al*., 2009). The partial 1.8 kb fragment (pCSI1) of the 8.2 kb fragment (EU727190) of the 18S-28S rDNA and the 99 bp pCSI5S fragment (EU723235) of the 5S rDNA which were isolated from *C. siamensis* of Thailand (Srikulnath *et al*., unpublished data), a commercial biotin-labeled 42-bp oligonucleotide probe complementary to $(TTAGGG)_{20}$ sequences (Sigma-Aldrich, USA), and cDNA fragments of *L. reevesii rubritaeniata* homologues of chicken Z linked gene were used as probes.

For chromosome mapping of 18S-28S rRNA genes or 5S rRNA genes, the 250 ng DNA fragments were labeled with biotin-12-dUTP (Invitrogen-Molecular Probes) using a nick translation kit following the manufacture instruction. Labeled DNAs were precipitated by 2.5 volume of absolute ethanol and 10 mg/ml salmon sperm DNA and *E. coli* tRNA. The solution was gently mixed and kept at -80 ◦C for 20 min, and then the labeled DNAs were precipitated by centrifugation at 11,337 ×g for 20 min, and prepicipitant was resuspended in 10 µl formamide. Slides were hardened at 65 ◦C for 2 h, denatured at 70 ◦C for 2 min in 70% formamide/2× SSC, and dehydrated in 70% ethanol for 5 min and absolute ethanol for 5 min. A 20 µl mixture containing biotin-12-dUTP, 50% formamide, 2× SSC, 10% dextran sulfate and 2 µg/µl BSA was hybridized to a male/female chromosome slide at 37 ◦C for 16 h. After hybridization, slides were washed in 50% formamide/2× SSC, 2× SSC, 1× SSC and 4× SSC for 15 min each at room temperature. Then, they were added with FITC-avidin (Vector Laboratories, Burlingame, CA, USA) in 1% BSA/4 × SSC at 37◦ C for 60 min. After that, they were washed in 4× SSC, 1% Nonidet P-40/4× SSC, 4×

SSC and 2× SSC for 5 min each at room temperature, and subsequently stained with 0.75 µg/ml propidium iodide (PI). The fluorescence hybridization signals were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments, Lurgan, UK) mounted on a Leica DMRA microscope, and processed using the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd. (Cambridge, UK).

For chromosome mapping of 18S-28S, 5S rRNA genes and telomeric sequences $(TTAGGG)_{20}$, dual-color FISH was performed to compare the chromosomal locations of telomeric sequences $(TTAGGG)_{20}$ with those of the 18S-28S and 5S rDNA. The 250 ng 18S-28S rDNA and 5S rDNA probes were labeled with digoxigenin-11-dUTP (Roche Diagnostics, Switzerland) using a nick translation kit following the manufacture instruction, and then hybridized with biotin-labeled 42 bp TTAGGG repeats. After hybridization, the excess probes were washed as mentioned above and incubated with anti-digoxigenin-Rhodamine, Fab fragments (Roche Diagnostics, Switzerland) and FITC-avidin (Vector Laboratories, USA), and then chromosomes were counterstained with 1 µg/ml DAPI (4', 6-diamidino-2-phenylindole). The fluorescence signals were captured using a cooled CCD camera (MicroMAX 782Y, Princeton Instruments, Lurgan, UK) mounted on a Leica DMRA microscope, and processed using the 550CW-QFISH application program of Leica Microsystems Imaging Solution Ltd (Cambridge, UK).

For chromosome mapping of chicken Z-linked gene homologues, cDNA fragments isolated from *L. reevesii rubritaeniata* obtained from this study were used. Two concatenated cDNA fragments were used as probes for chromosome mapping of *ACO1/IREBP* and *ATP5A1* to cover wide regions of the genes. The 250 ng DNA fragments were labeled with biotin-12-dUTP (Invitrogen-Molecular Probes) by nick translation following the manufacture instruction. After hybridization, slides were washed as previously done and the probe DNA on chromosome slide was incubated with goat anti-biotin antibody (Vector Laboratories) in 1% BSA/4× SSC at 37 °C for 60 min. After that, the slides were washed in 4× SSC, 1% Nonidet P-40/4× SSC and 4× SSC for 5 min each at room temperature, and subsequently stained with Alexa

Fluor 488 rabbit anti-goat IgG (H+L) conjugate (Invitrogen-Molecular Probes) in 1% BSA/4 × SSC at 37°C for 60 min. Then, they were washed in 4× SSC, 1% Nonidet P-40/4× SSC, 4× SSC and 2× SSC for 5 min each at room temperature, and subsequently stained with 0.75 µg/ml propidium iodide (PI). The hybridization signals were observed under a Nikon fluorescence microscope using Nikon filter sets B-2A, FITC and UV-2A (Nikon, Tokyo, Japan), and the FISH images were microphotographed with DYNA HG ASA100 film (Kodak, Rochester, NY, USA).

9. Ag-NOR banding

For visualization of the nucleolar organizer regions (NORs) after chromosomal localization of the 18S-28 rDNA in FISH mapping, the same chromosome slides were stained with silver nitrate ($AgNO_3$) following the procedure of Howell and Black (1990). The mixture solution of 50% $AgNO_3$ and 2% gelatin solution with 1% formic acid (2:1) was poured onto chromosome and incubated at 65° C for 45s. Then, the samples were rinsed with water and dried.

10. PCR condition of mitochondrial genome and nuclear gene sequencing, and DNA marker for discriminating *Leiolepis* spp.

Twenty-five nanogram of genomic DNA was taken into 20 µl of 1× ThermalPoll reaction buffer (1.5 mM $MgCl_2$), 0.2 mM dNTPs, 5 pM specific primers and 0.25 U of NEB *Taq* polymerase (New England Biolabs, Ipswich, England), 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, 50-60 °C for 30s and 72 °C for 45s, and final extension of 72 °C for 10 min.

11. Mitochondrial genome and nuclear gene sequencing

For mitochondrial genome sequencing, PCR primers was designed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) using the nucleotide sequence data of the following *Leiolepis* spp.: *L. reevesii reevesii* (AF215262),

L. reevesii (EU428188, EU428189), *L. guentherpetersi* (EU428187), *L. guttata* (AF378377) and *L. belliana* (AB031986) for the 16S rRNA gene, and *Leiolepis* sp. (AF215132) and *L. belliana* (AB031969) for the 12S rRNA gene, and *Pogona vitticepes* (AB166795), *Calotes versicolor* (AB183287) and *Chlamydosaurus kingie* (EF090421) for whole mitochondrial genome. All nucleotide sequences of the primers used are shown in Appendix Table 2. PCR was performed in the three butterfly lizards as previously described the PCR condition. Then, the PCR products was subjected to electrophoresis on 1% agarose gel, the DNA fragments were subsequently extracted with a QIAquick Gel Extraction Kit (Qiagen) from the ethidium bromide-stained gel and ligated using pGEM-T Easy Vector System I (Promega, Madison, WI, USA), and was transformed into *Escherichia coli* DH5α competent cells following the manufacture instruction. Nucleotide sequences of the DNA fragments were determined by Tech Dragon (China).

For nuclear gene sequencing, the sequence of PCR primers was taken from San Mauro *et al*. (2004) for *RAG-1* gene, Godinho *et al*. (2006) for C-*mos* gene, Friesen *et al*. (1997) for *α-Enolase* and *GAPD* genes. The PCR products and nucleotide sequencing were performed as mentioned above. All nucleotide sequences of the primers used are shown in Appendix Table 3.

12. DNA marker for discriminating *Leiolepis* spp.

The nucleotide sequences of mitochondrial DNA 16S rRNA, *ND1*, *ND2*, *COI* and *ND5* genes from three *Leiolepis* spp. were obtained and aligned using the default parameters of ClustalX (Thompson *et al*. 1997). All pairs of species-specific primers were designed based on the distinctive sites of the sequence among three butterfly lizard species. PCR products were obtained based on the as previously described PCR condition. All nucleotide sequences of the primers used are shown in Appendix Table 4.

13. Sequence analysis

All nucleotide sequences were checked and edited by Chromas program (V. 1.43). For complete mitochondrial genome sequence, sequence assembly was performed to combine all of the overlapping PCR fragments into one contig strand using Cap 3 sequence assembly program (http://deepc2.psi.iastate.edu/aat/cap/cap.html). All nucleotide sequences comparison against the National Center for Biotechnology Information (NCBI) database were performed using the blastx and the blastn program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The location of protein-coding genes, exon and intron were determined by comparing DNA or amino acid sequences with known sequences from other squamate species. For identification of tRNA genes in complete mitochondrial genome, 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/) and RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). All nucleotide sequences were deposited in DDBJ (DNA data bank of Japan, http://www.ddbj.nig.ac.jp/submission-e.html).

14. Phylogenetic analysis

Complete mitochondrial genome sequence and some nuclear genes of the three butterfly lizard species were aligned using the default parameters of clustalX (Thompson *et al*., 1997) for data set. Concatenate protein coding gene in mitochondrial genome was aligned to 32 other squamate reptiles and 2 other reptilian and avian species as out group taken from the NCBI database (Table 20). *RAG1* and C-*mos* gene nucleotide sequences were aligned to 46 other squamate species, and 6 other reptilian and avian species as outgroup taken from the NCBI database (Table4). *α-Enol* and *GAPD* gene nucleotide sequences were also aligned to 13 acrodont lizards and 1 other lizard as outgroup taken from the NCBI (Table 7).

Phylogenetic analyses were conducted with six data sets comprising concatenate protein coding gene data set, *RAG1* data set, C-*mos* data set, combined

RAG1/C-*mos* data set, *α-Enol* data set, *GADP* data set and combined *α-Enol* /*GAPD* data set. All unalignable sites and gap-containing sites were carefully checked before removing from these data sets. Base composition for individual and all codon positions for each nucleotide data set were measured by PAUP* v. 4.0b10 (Swofford, 2002). A chi-square (χ^2) test of base heterogeneity was calculated for individual and for all codon positions, as implemented in PAUP*. Nucleotide saturation was also analyzed for individual and for all codon positions in each nucleotide protein coding data set by plotting the total number of transitions (Ts) + transversion (Tv) against genetic distance values which were based on alternative models implemented with Modeltest version 3.7 (Posada and Crandall, 1998) using the program MEGA4 (Kumar *et al*., 1993) and PAUP*. The level of incongruence between two genes in combined data set was examined using PAUP*. This approach used the incongruence length difference (ILD) test with parsimony criterion (Farris *et al*., 1995), and one hundred randomizations were performed.

The phylogenetic trees were reconstructed by four different methods: maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ) and Baysian inference (BI). The ML trees were generated with PHYML v.2.4.4 (Guindon and Gascuel, 2003) using non-parametric bootstrapping with 1000 pseudoreplicates. The model and parameters indicated by Modeltest 3.7 based on the Akaike Information Criterion (AIC) (Posada and Crandall, 1998) were used. For BI, 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 done at every 100 generation to get 10,000 trees to provide a majority-rule consensus tree with averaged branch lengths. All sample points prior to reaching convergence were discarded as burn-in, and Bayesian posterior nodal relationship in the sampled tree population was shown as percentage of Bayesian posterior probability (BPP) obtained from a majority-rule consensus tree. MP and NJ were carried out using PAUP* v. 4.0b10 by heuristic searches with the tree bisection-reconnection branch swapping (TBR) and 10 random taxon additions, and the non-parametric bootstrap analyses with 1,000

pseudorepricates were performed to obtain estimates of support for each node of the MP and NJ trees. NJ analysis of nucleotide sequence data sets was used with the corresponding best-fit evolutionary models.

RESULTS AND DISCUSSION

1. Karyotype of *Leiolepis reevesii rubritaeniata*

Karyotypes of both male and female in *L. reevesii rubritaeniata* were composed of two distinct chromosomal components, macrochromosomes and microchromosomes: two pairs of large metacentric macrochromosomes (1st, 3rd), one pair of large submetacentric macrochromosomes (2nd), one pair of medium-sized metacentric macrochromosomes (4th), two pairs of small metacentric macrochromosomes (5th, 6th) and 12 pairs of microchromosomes (7th–18th) described as 2n=2x=36 ($L_4^m + L_2^{sm} + M_2^m + S_4^m$ + 24 microchromosomes; NF=24 for macrochromosomes) (Table 1, Figure 1). There was no difference in chromosomal morphology in these two sexes. This karyotype feature was also identical to that of *L. reevesii rubritaeniata* as reported by Satrawaha and Tarpsipare (1982) and indistinguishable from the other subspecies of *L. reevesii reevesii* (Sollender and Schmid, 1988). The karyotype of *L. reevesii rubritaeniata* comprising 12 biarmed macrochromosomes and 24 microchromosomes is known to be conserved through the suborder Iguania (Gorman, 1973; Paull *et al*., 1976; Olmo, 1986; Olmo and Signorino, 2005). It is also similar to those of most of snake species, which exhibit the karyotypes of 2n=2x=36 (16 macrochromosomes + 20 microchromosomes) (Singh, 1972), suggesting that *L. reevesii rubritaeniata* may retain the ancestral form of karyotypes of Squamata. However, a large secondary constriction was also located in the subtelomeric region of the long arm of chromosome 1 in the karyotype of *L. reevesii rubritaeniata*. This feature was dissimilar to the common karyotype of iguanian lizards in which the secondary constriction was identified in the subtelomeric region of the long arm of chromosome 2 (Olmo and Signorino, 2005).

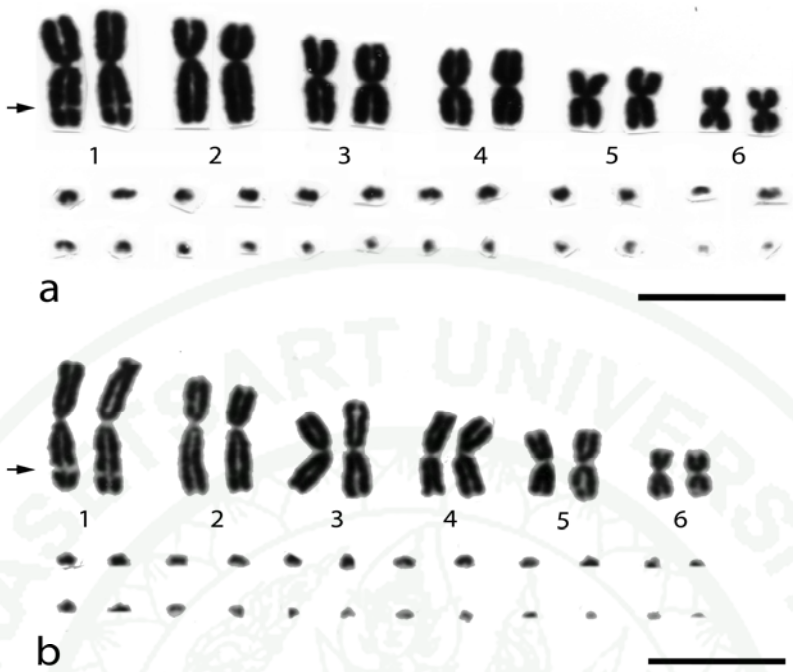

Figure 1 Giemsa-stained karyotypes of *L. reevesii rubritaeniata*. (a) female. (b) male. Arrows indicate the secondary constrictions. Scale bars represent 10 μm.

Table 1 Relative length, centromeric index and size of *L. reevesii rubritaeniata* macrochromosomes.

Chromosome	RL[1]	CI[2]	Size[3]
1	0.26	0.46	L
2	0.21	0.39	L
3	0.17	0.47	L
4	0.16	0.48	M
5	0.12	0.48	S
6	0.09	0.49	S

[1]RL: relative length

[2]CI: centromeric index

[3]L, large; M, medium; S, small

2. Meiotic configuration

Meiotic configuration of spermatocytes of *L. reevesii rubritaeniata* showed 18 bivalents (6 of macrochromosomes and 12 of microchromosomes) at diakinesis-metaphase I (MI), and 18 chromosomes at metaphase II (MII) (Figure 2), confirming that this species has diploid genome. It is different from the triploid species, *L. triploida* (2n=54) and *L. guentherpetersi* (2n=54) (Hall, 1970; Darevsky and Kupriyanova, 1993). There was no detection of diakinesis-MI cells with partially paired bivalents that are speculated to be heteromorphic X and Y chromosomes, and no MII cells with condensed chromosomes that are speculated to be the Y chromosome. These results collectively suggested that *L. reevesii rubritaeniata* might have different sex chromosome other than that of the XY system. However, to determine whether the heteromorphic sex chromosome of *L. reevesii rubritaeniata* is a ZW system or even a cryptic sex chromosome, it has to rely mainly on molecular cytogenetic approach.

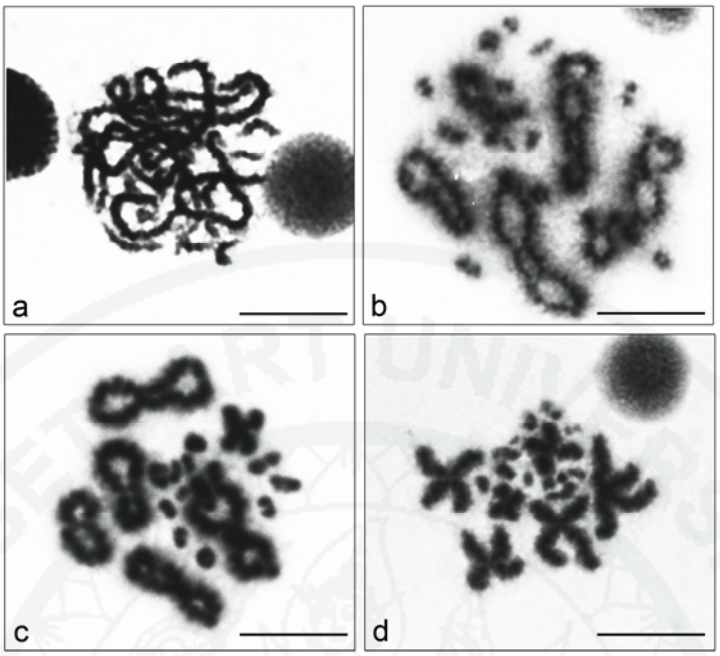

Figure 2 Meiotic configurations of *L. reevesii rubritaeniata*. (a) pachytene. (b) diakinesis. (c) metaphase I (MI). (d) metaphase II (MII). Scale bars represent 10 μm.

3. Comparative genomic hybridization (CGH)

Heterogametic micro-sex chromosomes have been reported in a ZZ/ZW lizard, *Pogona vitticeps* (Ezaz *et al*., 2005), a XX/XY turtle, *Chelodina longicollis* (Ezaz *et al*., 2006) and a ZZ/ZW turtle, *Pelodiscus sinensis* (Kawai *et al*., 2007) by CGH analysis. In *L. reevesii rubritaeniata*, co-hybridization patterns of FITC-labeled male-derived DNA and Cy3-labeled female-derived DNA were compared between female and male metaphase spreads (Figure 3). It is interesting to find that male- and female-derived probes were equally hybridized to whole chromosomal regions in one female and one male, and no sex-specific region was found. Moreover, no heteromorphic sex chromosome was morphologically identified in *L. reevesii rubritaeniata*. No partial synapsis of differentiated chromosomal pair was observed at diakinesis-MI of primary spermatocytes, and no MII cells with condensed chromosomes. Although sex determination systems have not been investigated in *Leiolepis* spp., the absence of

sex-specific chromosomal region in *L. reevesii rubritaeniata* leads us to predict that this species has TSD system or exhibits GSD with morphologically undetectable cryptic sex chromosomes. Further studies with sex determination system of *L. reevesii rubritaeniata* are necessary to indicate this inconclusive result.

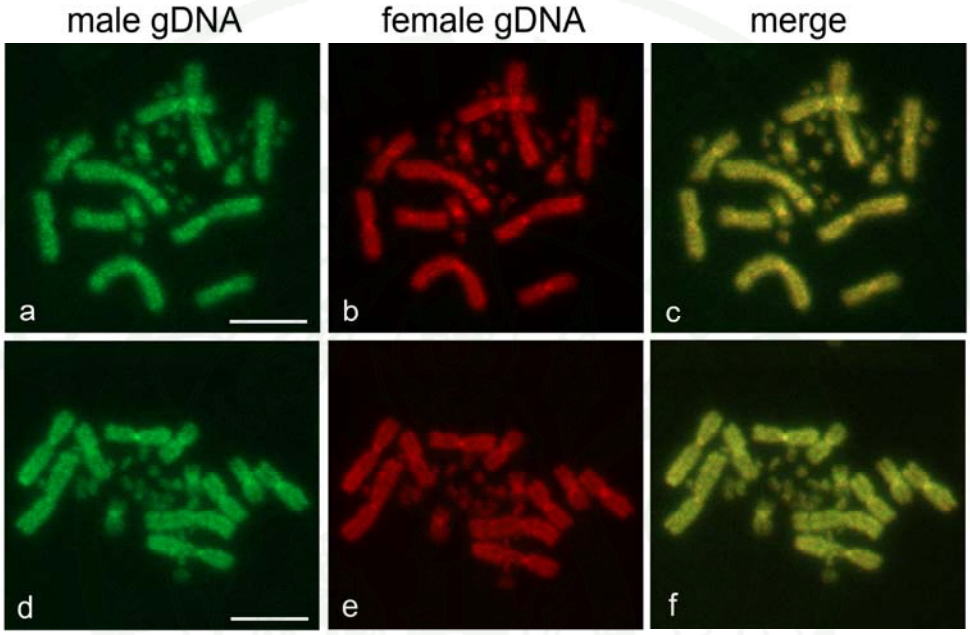

Figure 3 Comparative genomic hybridization patterns of FITC-labeled male genomic DNA (green) and Cy3-labeled female genomic DNA (red) on metaphase chromosome spreads of *L. reevesii rubritaeniata*. (a–c) female. (d–f) male. Scale bars represent 10 μm.

4. Molecular cloning and nucleotide sequences of *L. reevesii rubritaeniata* homologues of chicken Z-linked genes, and their chromosomal locations

L. reevesii rubritaeniata homologues of six chicken Z-linked genes were molecularly cloned (Table 2), and their nucleotide sequences in the equivalent regions were compared with those of human, chicken and five reptilian species (Table 3). The highest sequence identities were observed for five genes, *RPS6* (90.8%), *CHD1* (88.3%), *ACO1*/*IREBP* (85.0%), *DMRT1* (81.0%), and *GHR* (77.3%) between *L. reevesii rubritaeniata* and *G. hokouensis*, and for *ATP5A1* (86.8%) between

L. reevesii rubritaeniata and *P. sinensis*. The six gene homologues were all located on the short arm of chromosome 2 in the order *ACO1*/*IREBP-RPS6-DMRT1-CHD1-GHR-ATP5A1* from the centromere to the distal end (Figure 4). There was no difference in gene order found in the female and male chromosomes. This pattern was identical to those of *P. sinensis* on chromosome 6, *E. quadrivirgata* on chromosome 2p, *C. siamensis* on chromosome 3 (Kawai *et al*., 2007), *G. hokouensis* Z chromosome (Kawai *et al*., 2009), and also the ostrich Z chromosome (*Struthio camelus*) that retains the ancestral form of avian sex chromosomes (Nishida-Umehara *et al*., 2007; Tsuda *et al*., 2007) (Figure 5). These results suggested that this genetic linkage and gene order have been highly conserved since the common ancestor of Archosauromorpha and Lepidosauria first appeared around 260–290 MYA (Kumazawa and Nishida, 1999; Janke *et al*., 2001; Benton and Donoghue, 2007; Kumazawa, 2007). The gene order on the metacentric chicken Z chromosome is different from that of the ancestral avian sex chromosome, which resulted from several inversions that occurred in the lineage of the Phasianidae (Shibusawa *et al*., 2004; Itoh *et al*., 2006). The present results suggested that karyotypic evolution in reptiles and birds have occurred and highly maintain their conserved linkage homology that may have some adaptive advantage. However, further molecular cytogenetic characterization and comparative gene mapping are required for some other lacertilian species to clarify the process of karyotypic evolution and the diversity of sex chromosomal origins in Squamata.

Table 2 List of cDNA fragments of *L. reevesii rubritaeniata* homologues of six chicken Z-linked genes

Gene	Length of cDNA fragment (bp)	Accession number
ACOI/IREBP	1122[1]	AB480285, AB480286
RPS6	521	AB480287
DMRT1	628	AB480288
CHD1	961	AB480289
GHR	771	AB480290
ATP5A1	987[1]	AB480291, AB480292

[1]Total length of cDNA fragment concatenated with two PCR products.

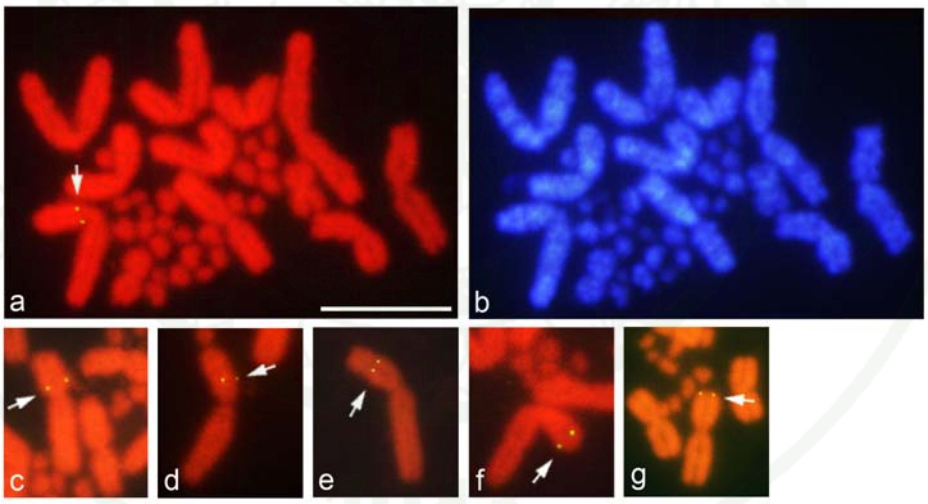

Figure 4 Chromosomal localization of six chicken Z-linked gene homologues in *L. reevesii rubritaeniata*. (a) *ACO1/IREBP*. (c) *RPS6*. (d) *DMRT1*. (e) *CHD1*. (f) *GHR*. (g) *ATP5A1*. The cDNA fragments were used for chromosome mapping as biotin-labeled probes. (b) Hoechst-stained pattern of the PI-stained metaphase spread shown in a. Arrows indicate the hybridization signals. Scale bars represent 10 μm.

Table 3 Nucleotide sequence identities of cDNA fragments of six genes among *L. reevesii rubritaeniata* (LRE), *Elaphe quadrivirgata* (EQU), *Pelodiscus sinensis* (PSI), *Crocodylus siamensis* (CSI), *Geckko hokouensis* (GHO), *Gallus gallus* (GGA) and *Homo sapiens* (HSA).

Gene	Identity (%)[1]					
	LRE-EQU[2]	LRE-PSI[2]	LRE-CSI[2]	LRE-GHO[3]	LRE-GGA[4]	LRE-HSA[5]
ACOI/IREBP[6]	83.2 (933/1121)	82.4 (924/1121)	82.5 (926/1122)	85.0 (954/1122)	79.2 (892/1126)[7]	79.2 (888/1121)
RPS6	85.7 (436/509)	86.6 (439/507)	85.5 (435/509)	90.8 (464/511)	85.2 (439/515)[7]	81.3 (412/507)
DMRT1	79.9 (509/637)[7]	77.3 (495/640)[7]	—[8]	81.0 (520/642)[7]	74.2 (475/640)[7]	69.8 (391/560)[7]
CHD1	86.3 (812/941)	85.8 (785/915)	86.7 (790/911)	88.3 (811/918)[7]	85.1 (801/941)	84.4 (794/941)
GHR	74.5 (478/642)[7]	70.5 (551/782)[7]	72.3 (553/765)[7]	77.3 (596/771)[7]	63.9 (401/628)[7]	60.7 (381/628)[7]
ATP5A1[6]	85.3 (816/957)	86.8 (828/954)	85.9 (820/955)	85.0 (804/946)	84.9 (835/983)	82.6 (808/978)

[1]The number in parenthesis indicates the number of identical bases/the number of bases in overlapped region of cDNA fragments between two species.

[2]Nucleotide sequences of the cDNA fragments of *P. sinensis*, *E. quadrivirgata* and *C. siamensis* were taken from Matsuda et al. (2005) and Kawai et al. (2007).

[3]Nucleotide sequences of the cDNA fragments of *G. hokouensis* were taken from Kawai et al. (2009).

[4]Nucleotide sequences of D16150, X81968, AF123456, XM424694, M74057 and AF332870 were used for comparison with *G. gallus*.

[5]Nucleotide sequences of NM002197, AK311861, NM021951, BC117134, NM000163 and AK289457 were used for comparison with *H. sapiens*.

[6]Concatenated nucleotide sequences of two cDNA fragments were used for alignment.

[7]The identities were calculated for the nucleotide sequences of the LRE cDNA fragments containing gaps.

[8]Not examined because of no sequence data of *C. siamensis*.

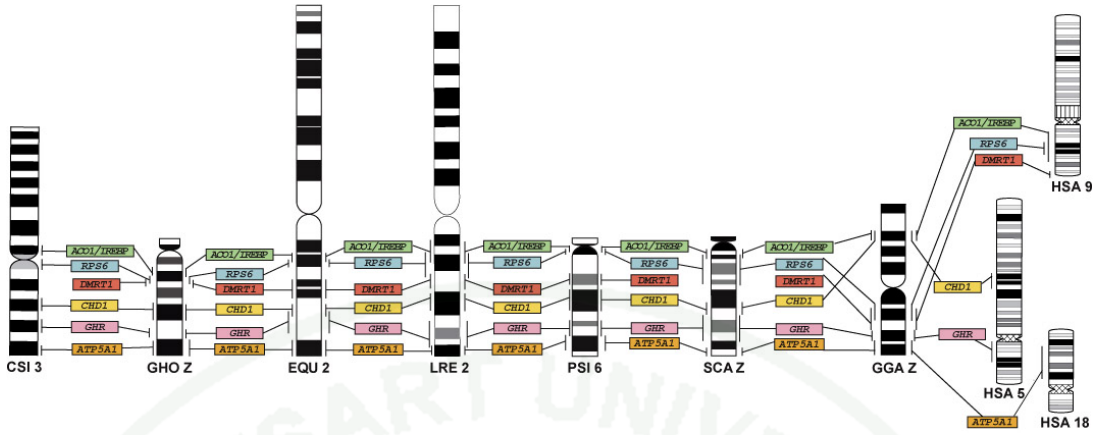

Figure 5 Comparative cytogenetic maps of six chicken Z-linked gene homologues in human (*Homo sapien*s, HSA), ostrich (*Struthio camelus*, SCA) and five reptilian species, the Chinese soft-shelled turtle (*Pelodiscus sinensis*, PSI), the butterfly lizard (*L. reevesii rubritaeniata*, LRE), the Japanese four-striped rat snake (*Elaphe quadrivirgata*, EQU), the Hokou gecko (*Gekko hokouensis*, GHO) and the Siamese crocodile (*Crocodylus siamensis*, CSI). The ideogram of *L. reevesii rubritaeniata* chromosome 2 was constructed according to the Hoechst-banded patterns. The ideogram of the G-banded chicken (*Gallus gallus*, GGA) Z chromosome was taken from the ARKdb (http://www.thearkdb.org/), human ideogram of chromosome 5, 9 and 18 at 850 band level from ISCN 1955. The mapping data in ostrich and the four reptilian species were taken from previous studies (Tsuda *et al*., 2007; Kawai *et al*., 2007, 2009). *C. siamensis* chromosome 3, *E. quadrivirgata* chromosome 2, *L. reevesii rubritaeniata* chromosome 2, the *G. gallus* Z chromosome and *H. sapiens* chromosomes 5, 9 and 18 are inverted to facilitate comparison of the order of the genes with those on the *S. camelus* Z chromosome, *P. sinensis* chromosome 6 and the *G. hokouensis* Z chromosome.

5. Chromosomal location of the 18S-28S and 5S rRNA genes and Ag-NOR banding of *L. reevesii rubritaeniata*

Fluorescence hybridization signals of the 18S-28S rRNA genes were found at secondary constriction in the subtelomeric region of the long arm of chromosome 1 of one female and one male (Figure 6a,c), and it was confined by Ag-NOR bands (Figure 6b,d). FISH enabled us to visualize all 18S-28S rRNA gene copies, whereas silver staining allowed us to detect only the transcriptionally active nucleolar organizer regions (Miller *et al*., 1976; Durica and Krider, 1977; Silva *et al*., 2008). Copy number variation of the 18S-28S rRNA genes between homologous chromosomes and/or between individuals is common in vertebrates such as mice (Kurihara *et al*., 1994), salmonid fishes (Fujiwara *et al*., 1998), chicken (Delany and Krupkin, 1999) and Siamese crocodile (Kawagoshi *et al*., 2008). One female of *L. reevesii rubritaeniata* showed a large difference in the copy number of the 18S-28S rRNA genes between homologous chromosomes, while no size difference was observed in the Ag-NOR bands (Fig. 6a, b). This result suggested that the expression of the 18S-28S rRNA genes is equalized among loci which is independent of their copy number difference. The 18S-28S rRNA gene is a cytogenetic marker to assess the karyological relationship between species. In Iguania, the 18S-28S rRNA genes are generally located on a pair of microchromosomes or chromosome 2 (Porter *et al*., 1991). By contrast, these gene cluster were localized on chromosome 1 in *L. reevesii rubritaeniata*, while *Tropidurus* (Tropiduridae), whose karyotypes are similar to *L. reevesii rubritaeniata*, the nucleolar organizer region was located on chromosome 6 (Kasahara *et al*., 1987). Cytogenetics is necessary for rRNA gene localization in other lacertilian species such as Iguania and it could reveal the process of the transposition on different chromosomes.

The 5S rRNA genes were located in the pericentromeric region of the long arm of chromosome 6 in both male and female (Figure 7d,f). This is the first report on the chromosomal location of the 5S rRNA genes in Squamata.

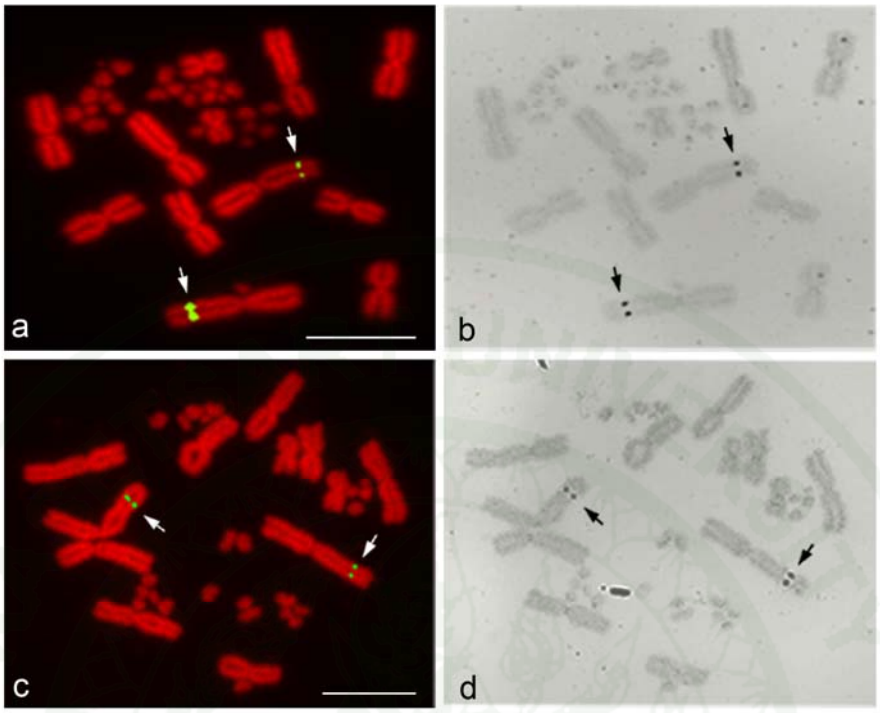

Figure 6 Chromosomal localization of the 18S-28S rRNA genes and nucleolar organizer regions (NORs) in *L. reevesii rubritaeniata*. (a, c) FISH patterns of the 18S-28S rRNA genes on PI-stained metaphase chromosome spreads of one female (a) and one male (c). (b, d) Ag-stained patterns of the metaphase spreads shown in a and c. Arrows indicate FISH signals of the 18S-28S rRNA genes (a, c) and Ag-NOR bands (b, d). Scale bars represent 10 μm.

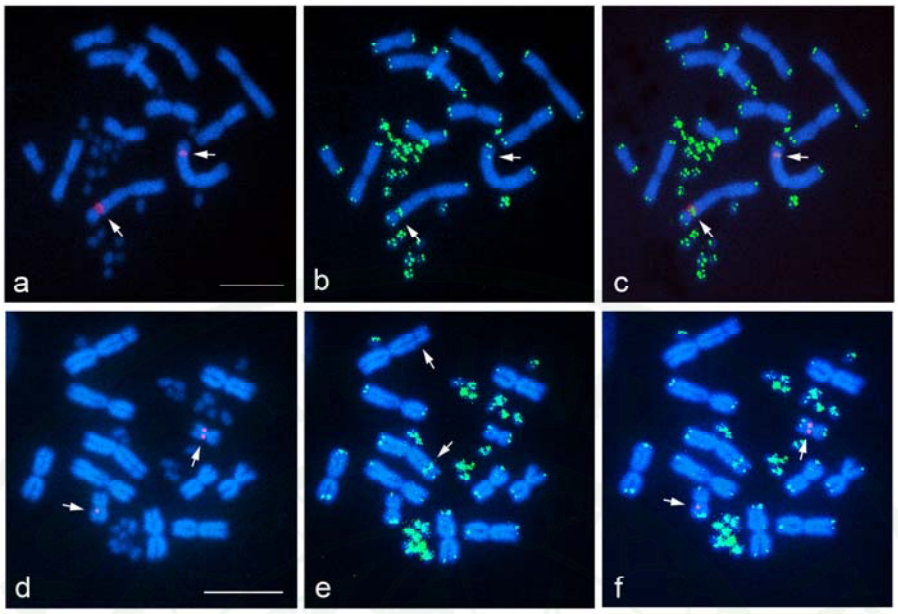

Figure 7 Chromosomal localization of the 18S-28S and 5S rRNA genes and $(TTAGGG)_{20}$ sequences in female *L. reevesii rubritaeniata*. (a–c) Hybridization patterns of the 18S-28S rRNA genes (red) (a) and $(TTAGGG)_{20}$ sequences (green) (b) on DAPI-stained chromosomes, and their co-hybridization pattern (c). (d–f) Hybridization patterns of the 5S rRNA genes (red) (d) and $(TTAGGG)_{20}$ sequences (green) (e), and their co-hybridization pattern (f). Arrows indicate FISH signals of the 18S-28S rRNA genes (a, c), the 5S rRNA genes (d, f), and interstitial telomeric sites (ITSs) (b, c, e, f). Scale bars represent 10 μm.

6. Chromosomal location of the 18S-28S and 5S rRNA genes *of L. belliana belliana* and *L. boehmei*

Fluorescence hybridization signals of the 18S-28S rRNA genes were localized at the secondary constriction in the subtelomeric region of the long arm on chromosome 1, whereas the 5S rRNA genes were also located in the pericentromeric region of the long arm of chromosome 6 in two butterfly lizard species. These features were comparable to those of *L. reevesii rubritaeniata*, indicating that the position of major and minor ribosomal RNA genes might be the unique character of *Leiolepis* species in iguanian lizards (Figures 8, 9).

7. Chromosomal location of $(TTAGGG)_{20}$ sequences of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*

Fluorescence signals of $(TTAGGG)_{20}$ sequences were observed at telomeric ends of all chromosomes in the three butterfly lizards studied (Figures 7, 8). The hybridization signals were weak on macrochromosomes; by contrast, high intensity of signals were observed on almost every microchromosomes, suggesting that the $(TTAGGG)_{20}$ sequences have been amplified site-specifically on microchromosomes. This was also observed in several avian species of Galliformes, Anseriformes and Passeriformes (Nanda *et al*., 2002; Nishida *et al*., unpublished data) but it was not reported in Squamata. ITSs have been also found in three species of Squamata, *Gonatodes taniae* of the Gekkonidae (Schmid *et al*., 1994), *Leposoma guianense* and *L. oswaldoi* of the Gymnophthalmidae (Pellegrino *et al*., 1999), in which a large copy number of $(TTAGGG)_{20}$ sequences were observed in the pericentromeric regions. In *L. reevesii rubritaeniata* and *L. boehmei*, ITSs were co-localized in the subtelomeric region of chromosome 1 with the 18S-28S rRNA loci (Figures 7c, 8i). This phenomenon has been found in several vertebrate species (Meyne *et al*., 1990; Salvadori *et al*., 1995; Reed and Phillips, 1995; Abuin *et al*., 1996; Liu and Fredga, 1999). ITSs appear as a relic of chromosome rearrangement such as fusion or inversion (Nanda and Schmid, 1994; Go *et al*., 2000); therefore, the present result provides a cytogenetic evidence in *L. reevesii rubritaeniata* and *L. boehmei* that a tandem fusion might have occurred between chromosome 1 and a microchromosome where the 18S-28S rRNA genes are located. However, ITSs was not found in that of *L. belliana belliana*, indicating that chromosomes might have few copies of TTAGGG sequences due to which they could not be detected (Fugures 8c,f). Also, it might be a gradual loss of the repeat sequences during chromosomal evolution. Further molecular cytogenetic characterizations are required on several other *Leiolepis* species to clarify the process of karyotypic evolution as well.

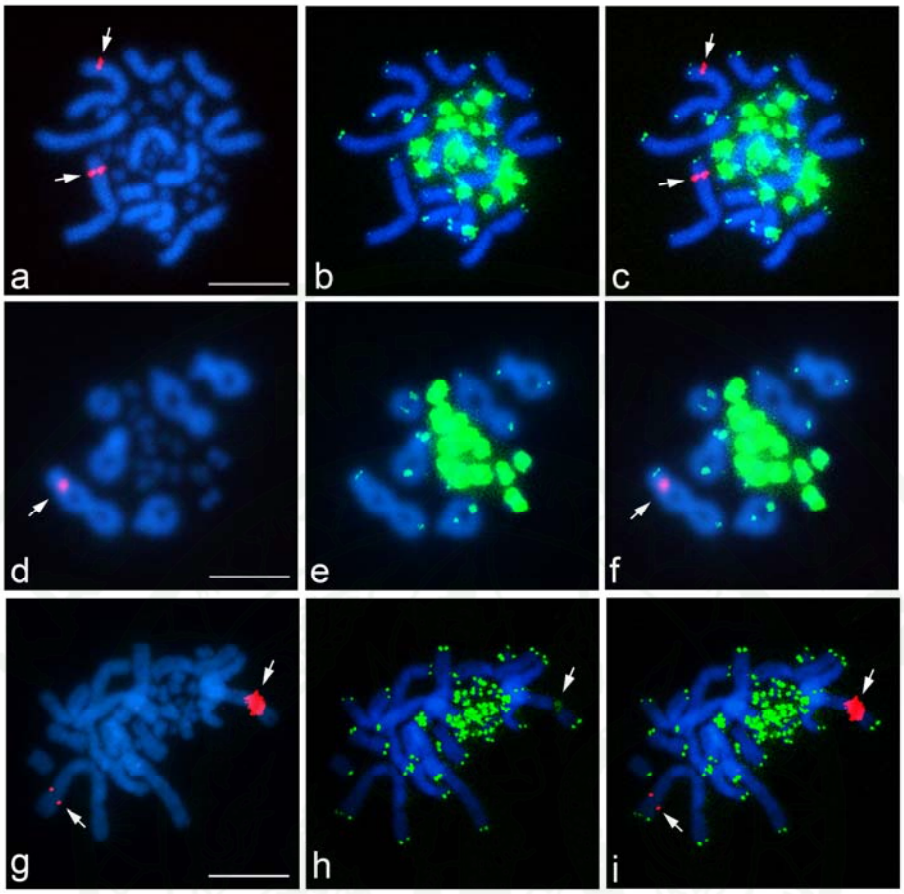

Figure 8 Chromosomal localization of the 18S-28S and (TTAGGG)*n* sequences in *L. belliana belliana* mitotic mataphase chromosome (a-c) and meiotic chromosome (d-f), and *L. boehmei* mitotic metaphase chromosome (g-i). Hybridization patterns of the 18S-28S rRNA genes (red) (a, d, g) and (TTAGGG)*n* sequences (green) (b, e, h) on DAPI-stained chromosomes, and their co-hybridization pattern (c, f, i). Arrows indicate FISH signals of the 18S-28S rRNA genes (a, c, d, f, g, i), and interstitial telomeric sites (ITSs) (h, i). Scale bars represent 10 μm.

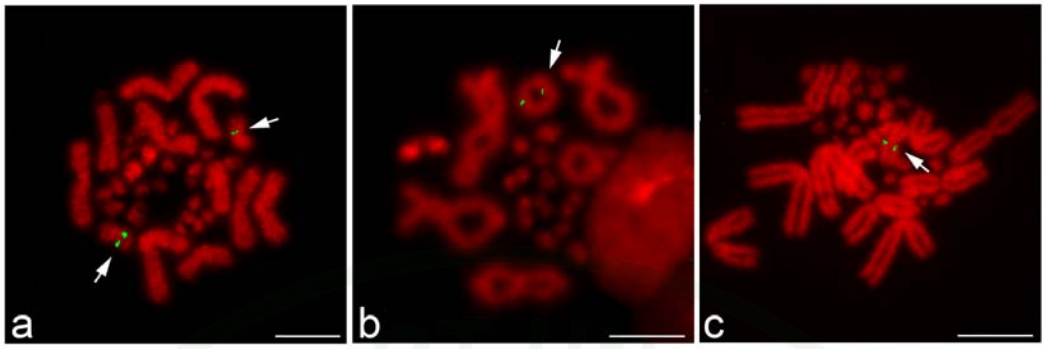

Figure 9 Chromosomal localization of the 5S rRNA genes in *L. belliana belliana* mitotic mataphase chromosome (a) and meiotic chromosome (b), and *L. boehmei* mitotic metaphase chromosome (c). Hybridization patterns of the 5S rRNA genes (red) on PI-stained chromosomes. Arrows indicate FISH signals of the 5S rRNA genes. Scale bars represent 10 μm.

8. Nuclear gene sequencing of *L. reevesii rubritaeniata, L. belliana belliana and L. boehmei*

8.1 General properties of sequences of *RAG1*, C-*mos* and combined *RAG1*/C-*mos*

The individual *RAG1* and C-*mos* data sets, and combined data set of the same species were used to determine the genetic relationship and phylogenetic position of *Leiolepis* spp. in Squamata. The *RAG1* data set included 657 aligned nucleotide positions consisting of 353 variable sites and 295 parsimony informative sites, which contained 66.78% of third codon position (Table 4). Similarly, the third codon position of C-*mos* data set has exposed 49.77% informative sites, whereas the respective numbers of aligned C-*mos* data set were 348 characters comprising 237 variable characters and 215 parsimony informative characters. These results collectively suggested that the third codon position was mainly informative characters to find phylogenetic relationship out of *RAG1* and C-*mos* data sets. To dictate the potentially misleading effects of heterogeneous base composition among taxa in phylogenetic reconstruction, the nucleotide contents of two gene data sets were subsequently analyzed as individual and as all codon position (Steel *et al*., 1993;

Tarrio *et al*., 2000; 2001). The results showed that the nucleotide frequencies were generally similar between two genes in three butterfly lizards, and there were also no statistically significant proportions differences between squamate and other reptile species (Table 5), indicating that our two data set analyses were not heterogeneity of base frequencies, and the codon bias might have not distorted phylogenetic inference. Surprisingly, the GC contents of C-*mos* gene sequences were clearly different between squamate species (average 43.72% and 19.92% for all codon and third codon position, respectively) and other reptile species (average 53.71% and 29.53% for all codon and third codon position, respectively) (Table 6), although base frequencies at third codon position were not significantly heterogeneous. The substantial base composition difference between in group taxa and out group taxa might also be responsible for incorrect rooting (Tarrio *et al*., 2000). Harris (2003) found that the high difference of GC content and codon usage between teiid lizards and other squmates, indicating that codon bias could cause the misconstruction of phylogeny. However, all topologies of MP and ML analyses of full C-*mos* data set were similar to those of MP and ML analyses of C-*mos* data set using first and second codon position. This situation was comparable to C-*mos* data set according to Townsend *et al*. (2004), which had different third codon position GC content between out group taxa (average 63.2%) and in group taxa (average 41.5%). Furthermore, the two nuclear gene data sets had similar patterns of the total number of transitions + transversions against genetic distance in individual and in all codon positions (Figure 10). The regression lines were not momentously different from straight lines, implying that saturation of third codon positions did not occur to cause a problem in two nuclear gene sequences at the level of homoplasy, and that there was phylogenetic cue for all codon positions.

The ILD test revealed that there was some incongruence between the two nuclear genes (p=0.01), suggesting an extensive heterogeneity occurred between the two data sets. The GC contents and rate of evolution might be the case. However, the combination of partial *RAG1* and C-*mos* sequences was commonly used (Townsend *et al*., 2004; Vidal and Hedges, 2004) to reconstruct reliable phylogenetic trees. Here, we also found that all topologies from *RAG1* data set were highly similar to those from the combined data set. Therefore, we combined two data sets and discussed on the results.

Table 4 Classification and accession numbers of species used in *RAG1* and C-*mos* sequence analysis[1].

Class	Order	Suborder	Infraorder	Family	Species	Accession for *RAG1* gene	Accession for C-*mos* gene
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis reevesii rubritaeniata*	AB516967*	AB516970*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis belliana belliana*	AB516968*	AB516971*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis boehmei*	AB516969*	AB516972*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Agama agama*	EU402825	AF137530
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Physignathus cocincinus*	FJ356737	AF039476
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Uromastyx acanthinura*	AY988025	AY987992
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Physignathus lesueurii*	AY662581	DQ340689
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	*Chamaeleo jacksonii*	AY988023	AF137528
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Microlophus peruvianus*	EF616443	EF615791
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Oplurus cuvieri*	AY662601	EU099681
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Chalarodon madagascariensis*	AY988018	EU099654
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Diplolaemus darwinii*	AY988019	AY987988
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Crotaphytus collaris*	FJ356749	AY987985
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Basiliscus plumifrons*	AY662599	AY987986
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Phrynosoma cornutum*	FJ356738	AY987989
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Liolaemus lineomaculatus*	FJ356740	AY367903
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Polychrus marmoratus*	FJ356748	AY987983
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Dipsosaurus dorsalis*	FJ356747	AF148705

Table 4 (Continued)

Class	Order	Suborder	Infraorder	Family	Species	Accession for *RAG1* gene	Accession for C-*mos* gene
Reptilia	Squamata	Lacertilia	Gekkota	Gekkonidae	*Gekko gecko*	AY444054	EU366455
Reptilia	Squamata	Lacertilia	Dibamia	Dibamidae	*Dibamus montanus*	AY444053	AY444027
Reptilia	Squamata	Lacertilia	Scincomorpha	Scincidae	*Scelotes anguina*	AY662635	AY217878
Reptilia	Squamata	Lacertilia	Scincomorpha	Teiidae	*Aspidoscelis tigris*	EU402829	AF039481
Reptilia	Squamata	Lacertilia	Diploglossa	Anniellidae	*Anniella pulchra*	AY662605	AY487350
Reptilia	Squamata	Lacertilia	Diploglossa	Anguidae	*Ophisaurus gracilis*	AY444056	AY444030
Reptilia	Squamata	Lacertilia	Diploglossa	Xenosauridae	*Xenosaurus grandis*	AY662607	AY662567
Reptilia	Squamata	Lacertilia	Platynota	Varanidae	*Varanus salvator*	EU402828	AF435017
Reptilia	Squamata	Lacertilia	Platynota	Helodermatidae	*Heloderma suspectum*	AY662606	AY487348
Reptilia	Squamata	Lacertilia	Platynota	Lanthanotidae	*Lanthanotus borneensis*	AY662609	AY662564
Reptilia	Squamata	Amphisbaenia		Blanidae	*Blanus strauchi*	AY444050	AY444024
Reptilia	Squamata	Amphisbaenia		Bipedidae	*Bipes biporus*	AY662616	AF039482
Reptilia	Squamata	Amphisbaenia		Amphisbaenidae	*Geocalamus acutus*	AY444043	FJ441784
Reptilia	Squamata	Amphisbaenia		Rhineuridae	*Rhineura floridana*	AY662618	AY444022
Reptilia	Squamata	Amphisbaenia		Trogonophidae	*Trogonophis wiegmanni*	AY662617	FJ441727
Reptilia	Squamata	Serpentes		Pythonidae	*Python reticulatus*	EU624119	AF544675
Reptilia	Squamata	Serpentes		Viperidae	*Daboia russellii*	EU402843	AF471156
Reptilia	Squamata	Serpentes		Acrochordidae	*Acrochordus granulatus*	EU402831	AF544706
Reptilia	Squamata	Serpentes		Cylindrophiidae	*Cylindrophis ruffus*	AY662613	AF471133

Table 4 (Continued)

Class	Order	Suborder	Infraorder	Family	Species	Accession for *RAG1* gene	Accession for C-*mos* gene
Reptilia	Squamata	Serpentes		Loxocemidae	*Loxocemus bicolor*	EU402854	AY099969
Reptilia	Squamata	Serpentes		Xenopeltidae	*Xenopeltis unicolor*	EU402870	DQ465561
Reptilia	Squamata	Serpentes		Boidae	*Charina trivirgata*	EU402852	AY099974
Reptilia	Squamata	Serpentes		Colubridae	*Xenodermus javanicus*	EU402869	AF544711
Reptilia	Squamata	Serpentes		Elapidae	*Naja kaouthia*	EU402857	AY058938
Reptilia	Squamata	Serpentes		Anomalepididae	*Liotyphlops albirostris*	EU402853	AF544727
Reptilia	Squamata	Serpentes		Typhlopidae	*Ramphotyphlops braminus*	AY444062	AF544717
Reptilia	Squamata	Serpentes		Leptotyphlopidae	*Leptotyphlops humilis*	EU402851	AY099979
Reptilia	Squamata	Serpentes		Aniliidae	*Anilius scytale*	EU402834	AF544722
Reptilia	Squamata	Serpentes		Bolyeriidae	*Casarea dussumieri*	EU402840	AF471114
Reptilia	Squamata	Serpentes		Tropidophiidae	*Ungaliophis continentalis*	EU402867	AY099970
Reptilia	Rhynchocephalia	Sphenodontida		Sphenodontidae	*Sphenodon punctatus*	AY662576	AF039483
Reptilia	Crocodylia	Eusuchia		Crocodylidae	*Crocodylus porosus*	EU375509	FJ011695
Reptilia	Crocodylia	Eusuchia		Crocodylidae	*Alligator sinensis*	AY239171	AY447979
Reptilia	Crocodylia	Eusuchia		Crocodylidae	*Tomistoma schlegelii*	AY239176	EF414017
Reptilia	Testudines	Pleurodira		Pelomedusidae	*Pelomedusa subrufa*	AY988102	FJ230876
Aves	Passeriformes			Callaeatidae	*Creadion carunculatus*	AY443317	DQ469305

[1] New sequences from our study indicated by *

Table 5 Properties of character variation for *RAG1*, C-*mos* and combined *RAG1*/ C-*mos* sequence data sets.

Data set	All aligned sequence	Parsimony-informative sites	Variable sites	Nucleotide bias				χ^2	d.f.	*p*-value	Best model[1]	I^2	G^3
				%A	%C	%G	%T						
RAG1	657	295	353	28.99	19.95	24.18	26.88	51.86	159	1.0000	TrN+I+G	0.4088	1.7124
1st position	219	63	89	29.33	16.23	36.93	17.51	13.53	159	1.0000			
2nd position	219	35	54	31.22	21.64	16.74	30.40	8.54	159	1.0000			
3rd position	219	197	210	26.41	21.98	18.88	32.72	125.49	159	0.9768			
C-*mos*	348	215	197	27.40	20.65	23.70	28.25	124.37	159	0.9805	HKY+I+G	0.2910	3.4515
1st position	116	62	71	29.42	19.52	32.08	18.98	58.74	159	1.0000			
2nd position	116	46	55	29.23	20.01	19.46	31.30	22.76	159	1.0000			
3rd position	116	107	111	23.55	22.41	19.57	34.48	153.96	159	0.5979			
Combine	1005	510	590								TrN+I+G	0.3724	2.2296

[1]Best models were selected with Modeltest version 3.6 (Posada and Crandall, 1998).

[2]I : Proportion of invariable site

[3]G : Gamma shape parameter

Table 6 Comparison of the base contents within *RAG1* and C-*mos* data sets.

Taxonomic organism	Percentage of bases with presented *RAG1* sequences					Percentage of bases with presented C-*mos* sequences				
	A	C	G	T	GC	A	C	G	T	GC
L. reevesii rubritaeniata	29.68	18.57	24.20	27.55	42.77	27.30	20.98	22.14	29.60	43.12
L. belliana belliana	29.68	19.03	24.05	27.25	43.08	27.01	20.69	22.13	30.17	42.82
L. boehmei	29.68	18.87	23.74	27.70	42.61	27.01	20.98	22.41	29.60	43.39
Agamidae	28.93	19.03	24.48	27.55	43.51	27.42	20.84	22.19	29.56	43.03
Chamaeleonidae	28.77	19.03	24.35	27.85	43.38	27.30	22.41	22.41	27.84	44.82
Iguanidae	29.49	20.10	23.80	26.62	43.90	27.66	19.19	24.28	28.87	43.47
Iguania	29.23	19.63	24.09	27.05	43.72	27.55	20.01	23.36	29.08	43.37
Gekkota	29.38	19.48	23.29	27.85	42.77	28.45	20.12	23.28	28.16	43.40
Dibamia	28.92	22.68	23.29	25.11	45.97	26.30	19.83	25.14	28.74	44.97
Scincomorpha	29.38	19.71	25.04	25.88	44.75	29.22	20.13	22.01	28.63	42.14
Diploglossa	27.30	21.31	25.82	25.57	47.13	27.74	20.62	23.80	27.84	44.42
Platynota	27.96	20.60	25.62	25.82	46.22	27.39	20.74	24.57	27.30	45.31
Lacertilia	28.89	20.02	24.45	26.63	44.47	27.66	20.16	23.50	28.68	43.66
Serpentes	29.27	19.42	23.28	28.04	42.70	28.24	19.48	22.40	29.87	41.88

Table 6 (Continued)

Taxonomic organism	Percentage of bases with presented *RAG1* sequences					Percentage of bases with presented C-*mos* sequences				
	A	**C**	**G**	**T**	**GC**	**A**	**C**	**G**	**T**	**GC**
Amphibaenia	29.28	20.40	24.20	26.12	44.60	28.84	21.44	22.86	26.86	44.30
Sphenodon punctatus	29.38	21.01	23.74	25.88	44.75	23.06	23.92	27.09	25.94	51.01
Crocodylus porosus	27.40	21.01	25.42	26.18	46.43	22.70	25.00	28.45	23.85	53.45
Alligator sinensis	28.46	20.55	24.81	26.18	45.36	23.56	25.00	27.59	23.85	52.59
Tomistoma sinensis	27.85	20.40	25.42	26.33	45.82	22.99	26.15	28.16	22.70	54.31
Pelomedusa subrufa	28.62	21.16	24.81	25.42	45.97	22.13	27.01	28.45	22.41	55.46
Creadion carunculatus	29.22	19.48	26.79	24.51	46.27	23.56	24.14	31.32	20.98	55.46

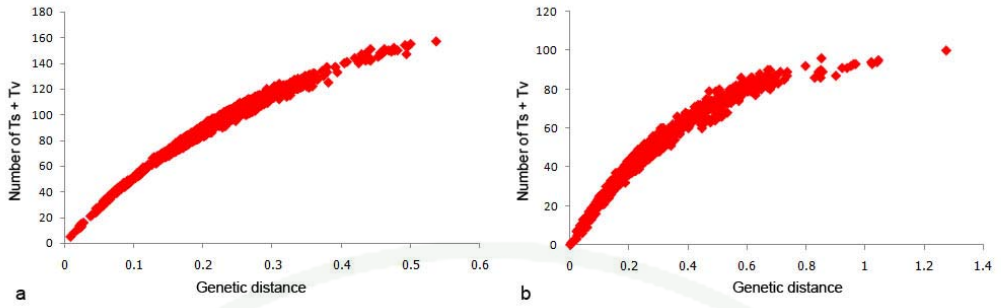

Figure 10 The relationship between the total number of transitions (Ts) + transversions (Tv) and corrected distance for all pairwise comparisons in (**a**) *RAG1* sequence data set and (**b**) C-*mos* sequence data set.

8.2 Phylogenetic analyses of *RAG1*, C-*mos* and combined *RAG1*/C-*mos*

The cladistic analyses were reconstructed based on *RAG1* and C-*mos* genes as separate and combined data sets using BI, ML, MP and NJ. Squamata was distinctly presented as monophyletic group (Figures 11-22) but the phylogenetic pattern chiefly within the basal splits differed among several analysis methods. Specifically, the BI phylogram was well similar to ML phylogram, and close to the previous molecular phylogenetic tree of Squamata *RAG1* and C-*mos* gene tree (Vidal and Hedges, 2003), *RAG1*, C-*mos* and *ND2* gene tree (Townsend *et al*., 2004), mitochondrial nucleotide sequence (Kumazawa, 2007) and *TSHZ1* and *RAG1* gene tree (Schulte and Cartwright, 2009). However, all methods illustrated a high agreement concerning the relationships within the infraorders and families of Squamata. The grouping of Gekkota, Dibamia and Scincomorpha well supported basal position of Squamata by all analyses. The large infraorder Iguania comprising two groups of Iguanidae and Acrodonta (Agamidae and Chamaeleonidae), was tremendously formed a single clade with BI posterior probability (99%), which was also a sister relationship with Anguimorpha (Diploglossa and Platynota). The other significant clusters were Serpentes and Amphibaenia, which was strongly sustained with support value of 99% and 79%, respectively, in BI analysis.

Agamidae was categorized into two subfamilies, Agaminae and Leiolepidinae, which were classified as *Leiolepis* and *Uromastyx* (Uets, 2009). However, the position of *Leiolepis*, *Uromastyx* and Chamaeleonidae, were diversely grouped in our phylogram. *Uromastyx* and Chamaeleonidae were sister taxon in *RAG1* and combined *RAG1*/C-*mos* BI analyses, whereas the latter taxa was monophyletic in C-*mos* analysis. These inconclusive results were comparable to the individual and combined *RAG1*/C-*mos* gene tree and the *ND2* gene tree (Townsend *et al*., 2004) and combined *TSHZ1*-*RAG1* gene tree (Schulte and Cartwright, 2009), suggesting that the phylogenetic topology was influenced by many parameters. Therefore, out groups, genes and taxon sampling might be explored as the relative effect (Albert *et al*., 2009). On the other hand, the morphological characters, the albumin immunological distance (Joger, 1991), lizard skull character (Stayton, 2005) and osteological and soft anatomical data (Lee, 2005), strongly supported Agamidae as monophyletic group. Schulte *et al*. (1998) suggested that the phylogenetic relationship of Agamidae and Leiolepidinae were metataxon, which monophyly was not found but not statistically rejected. Thus, more molecular and morphological markers, and taxa sampling are necessarily further studied to examine the relationship of Acrodonta.

In *Leiolepis*, all method statistical analyses strongly supported (100%) that *L. reevesii rubritaeniata* was more adjacent to *L. belliana belliana* than *L. boehmei* in *RAG1* and combined *RAG1*/C-*mos* analyses (Figure 11-14, 19-22). On the contrary, the phylogenetic tree of the individual C-*mos* gene data set showed *L. reevesii rubritaeniata* was adjacent to *L. boehmei* rather than *L. belliana belliana*. The fragments of the C-*mos* gene have been performed to assess relationship across squamate species (Saint *et al*, 1998; Donnellan *et al*., 1999; Harris *et al*, 1999 and 2001); however, most relationships between families were not quite robust. They might be caused rapid cladogenesis or artifact of limited sampling. Nevertheless, contrary to the chromosome number whose *L. reevesii rubritaeniata* and *L. belliana belliana* were 36, containing 12 bi-armed macrochromosomes (NF=24) and 24 microchromosomes, while *L. boehmei* was 34 containing 12 bi-armed macrochromosomes (NF=24) and 22 microchromosomes (Aranyavalai, 2003;

Aranyavalai *et al*., 2004; Srikulnath *et al*., 2009). In addition, Aranyavalai (2003) asserted that *L. boehmei* exhibited 29 of 31 characters, which were significantly morphologically different (body color, pattern and shape) from other congeneric species in Thailand. These results lead us to predict that *L. reevesii rubritaeniata* was closely related to *L. belliana belliana* rather than *L. boehmei*. However, the addition of taxa improved the accuracy of relationship rather than the addition of characters (Graybeal, 1998). Therefore, other molecular and morphological studies with additional taxa for genus *Leiolepis* are also desired to precisely delineate phylogenetic relationship and hierarchy.

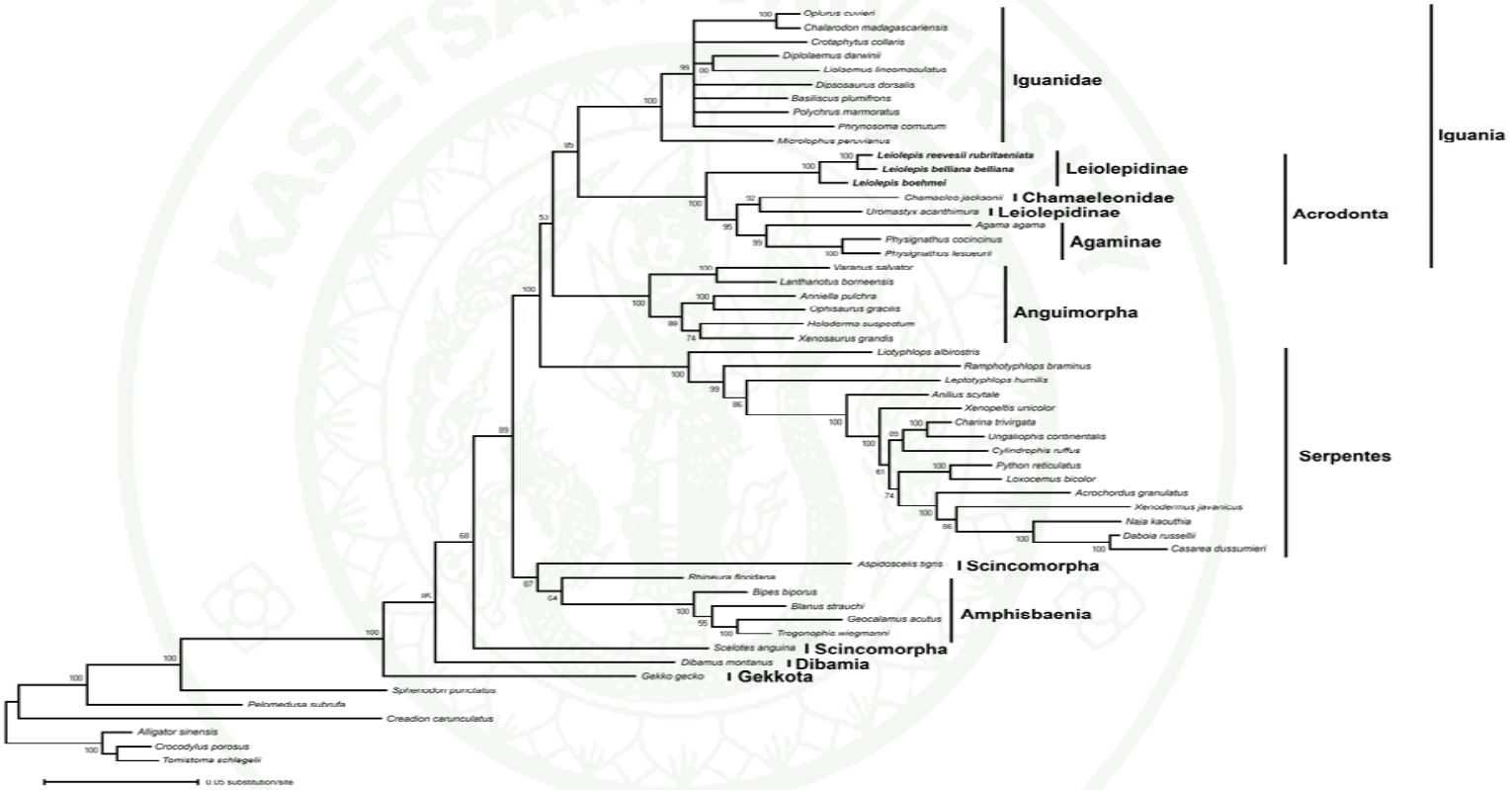

Figure 11 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the *RAG1* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Hasegawa-Kishino-Yano, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

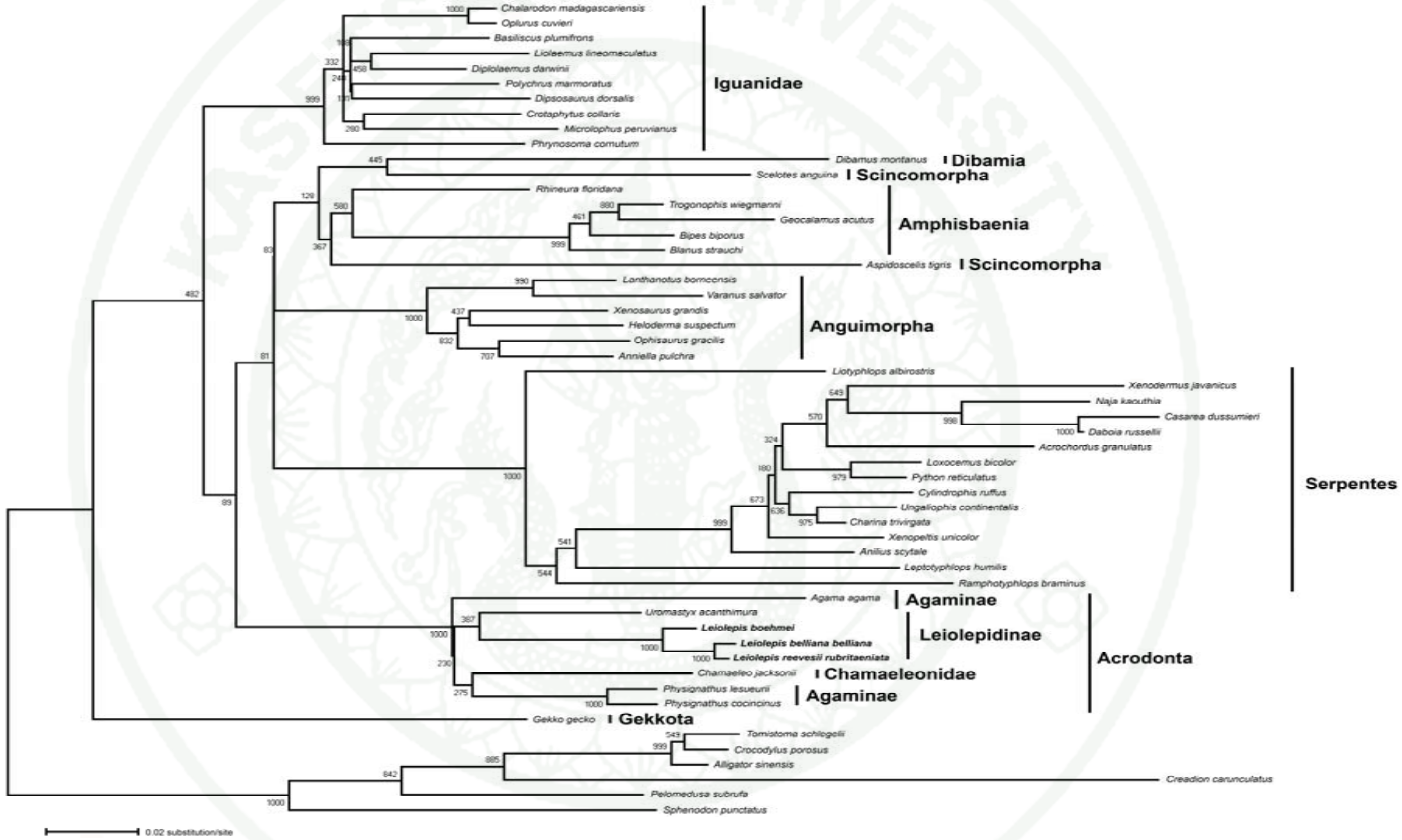

Figure 12 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the *RAG1* sequence data set based on Hasegawa-Kishino-Yano, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

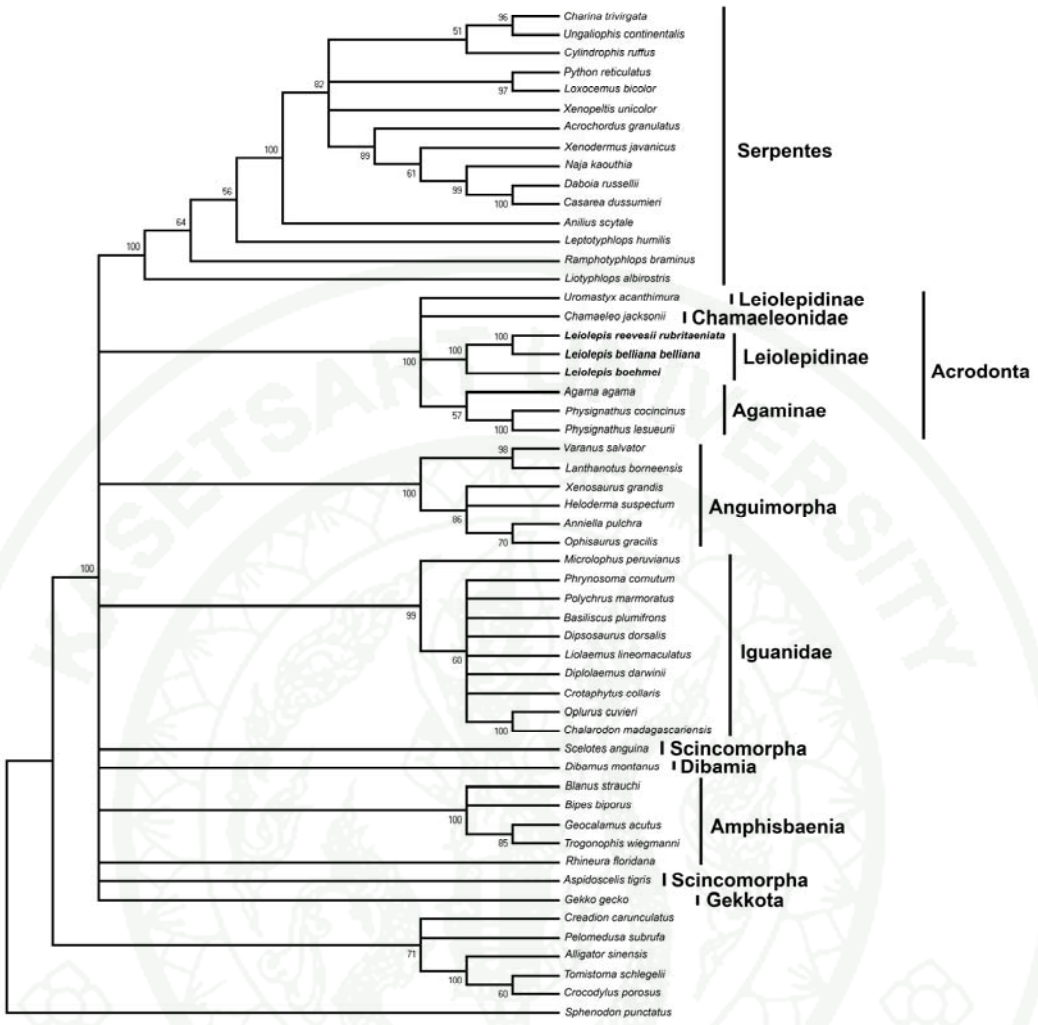

Figure 13 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the *RAG1* sequence data set. The bootstrap values were shown on the corresponding branches.

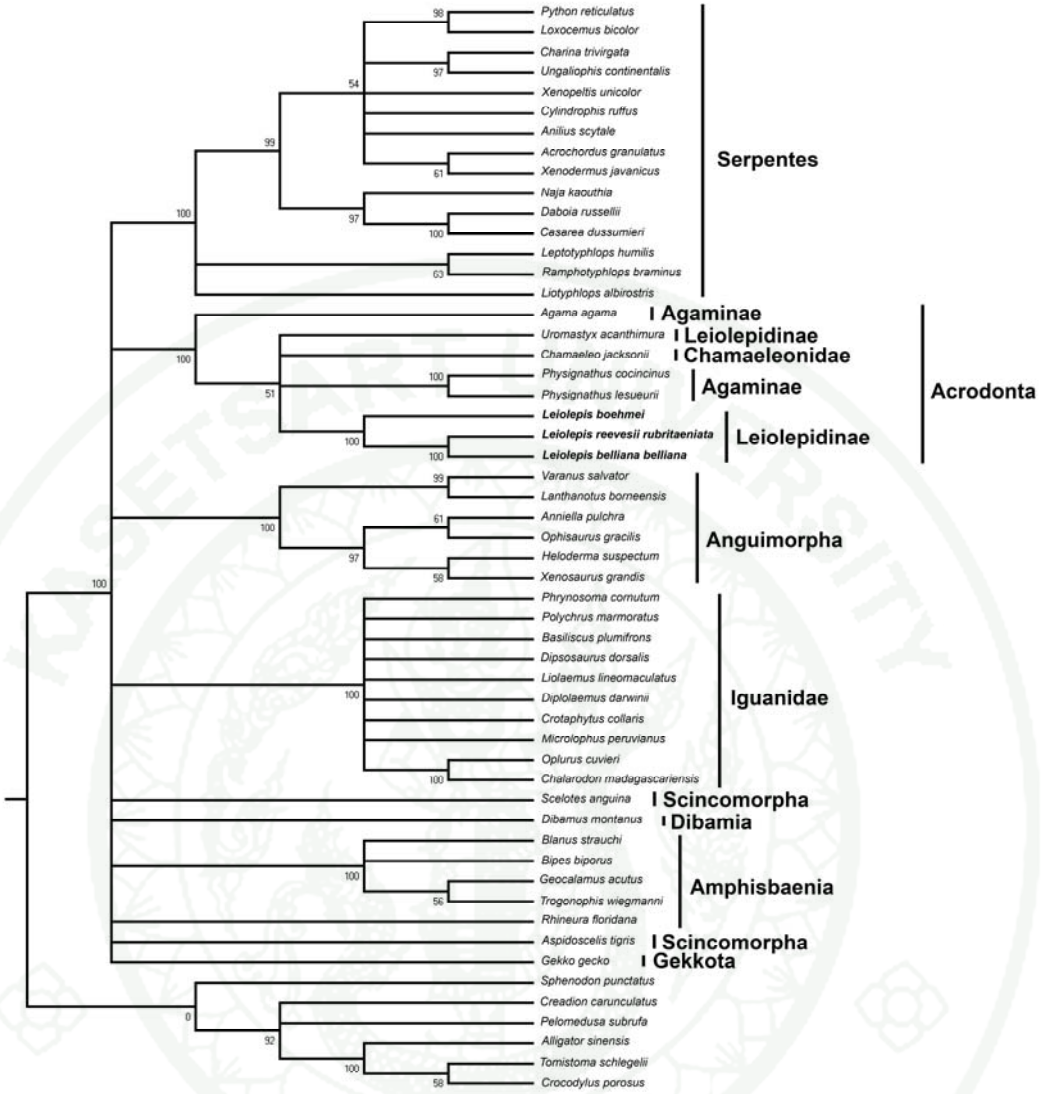

Figure 14 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the *RAG1* sequence data set based on Hasegawa-Kishino-Yano, AIC model. The bootstrap values were shown on the corresponding branches.

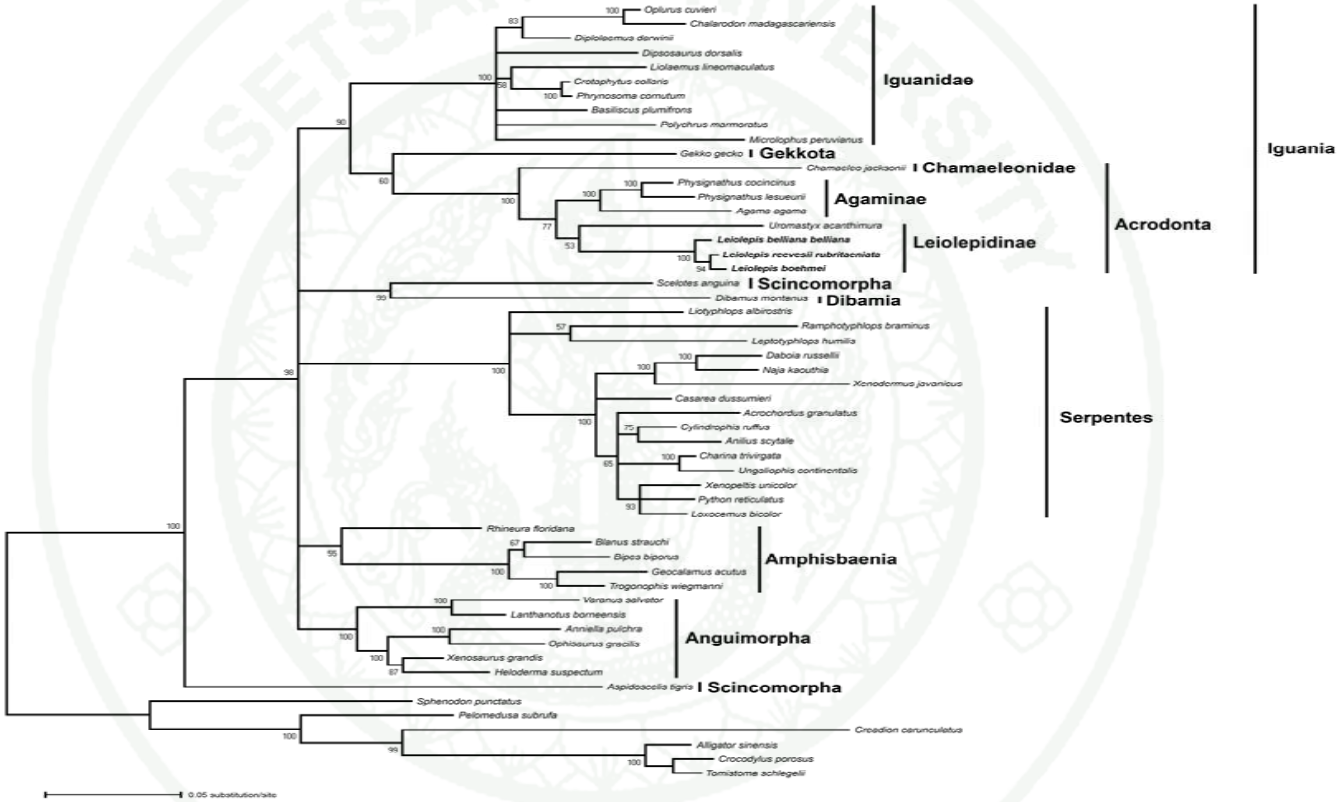

Figure 15 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the C-*mos* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Tamura-Nei, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

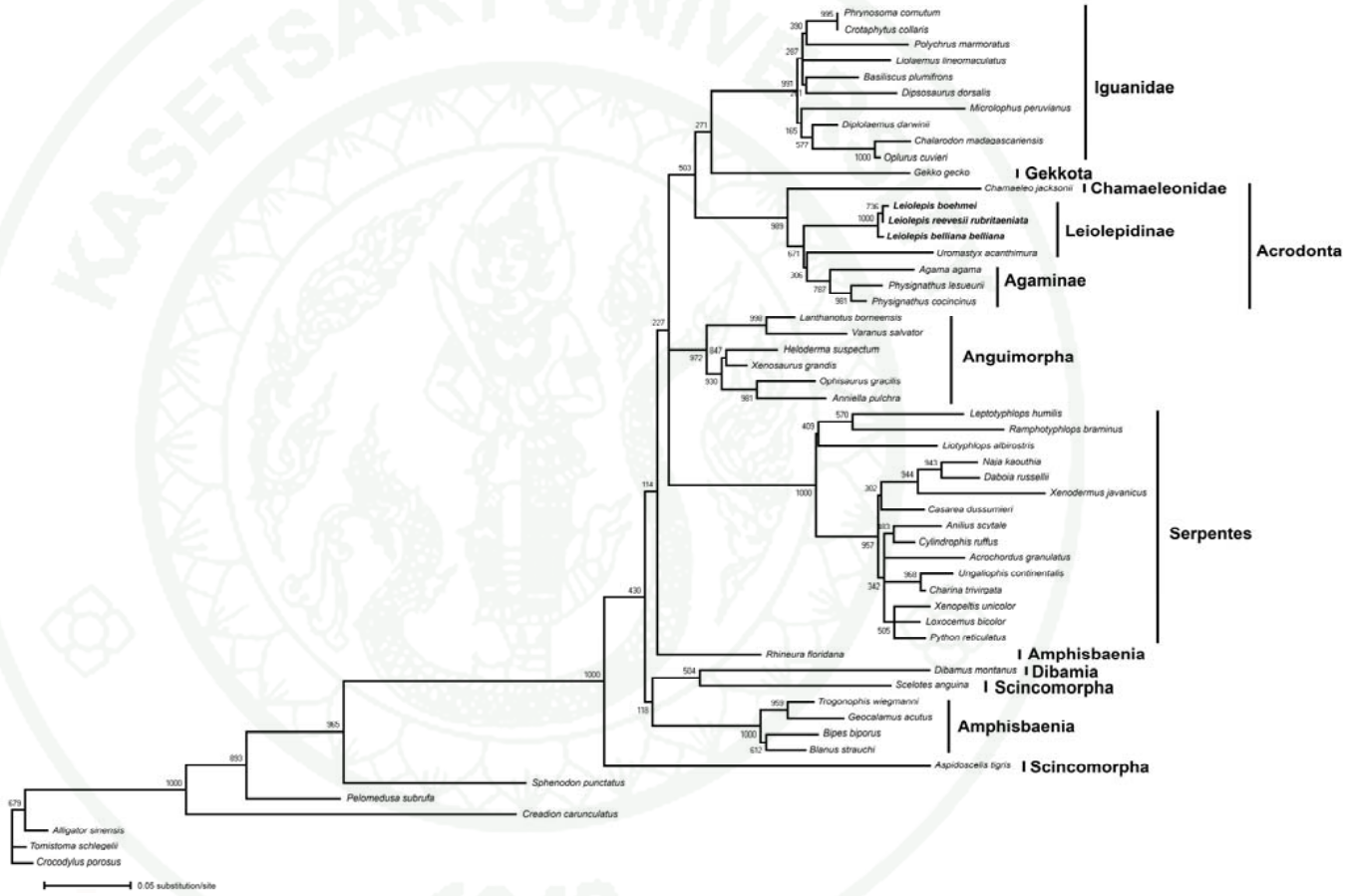

Figure 16 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the C-*mos* sequence data set based on Tamura-Nei, AIC model was shown. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

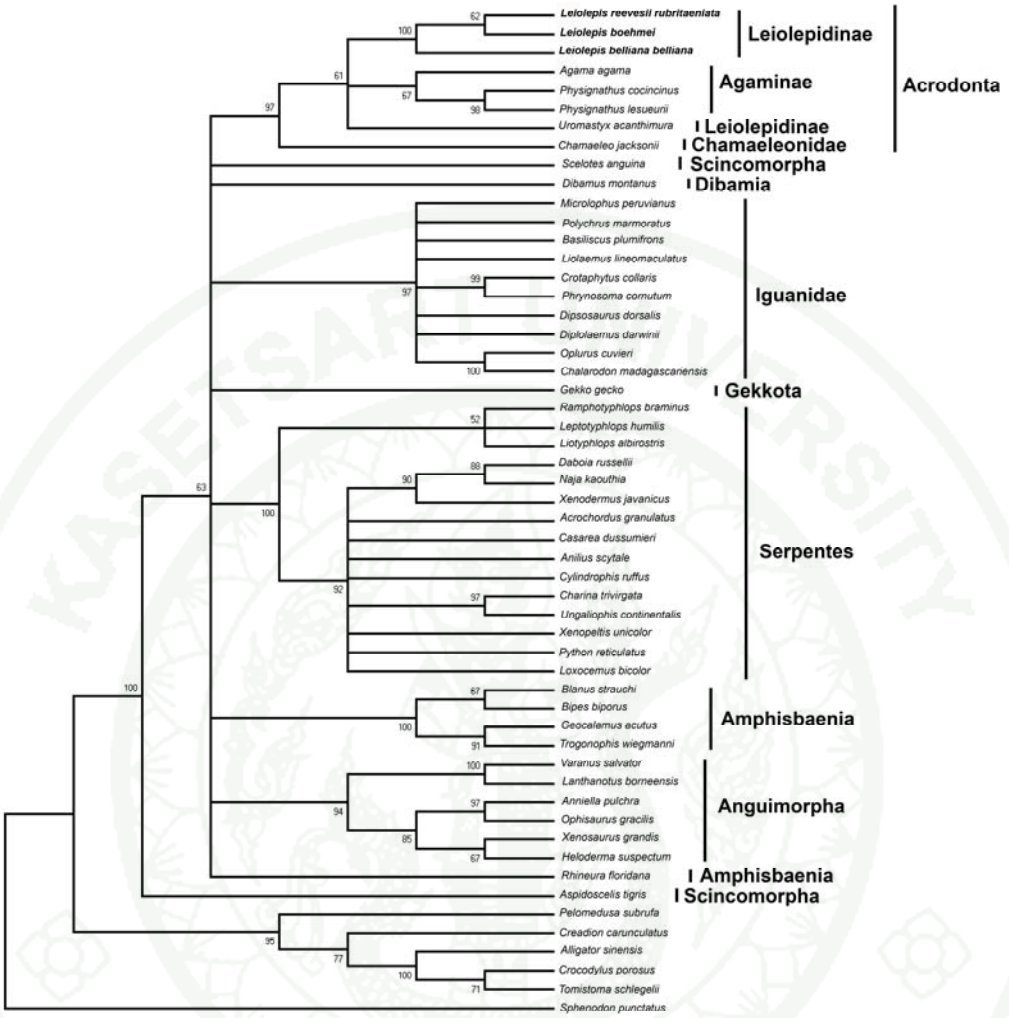

Figure 17 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the C-*mos* sequence data set. The bootstrap values were shown on the corresponding branches.

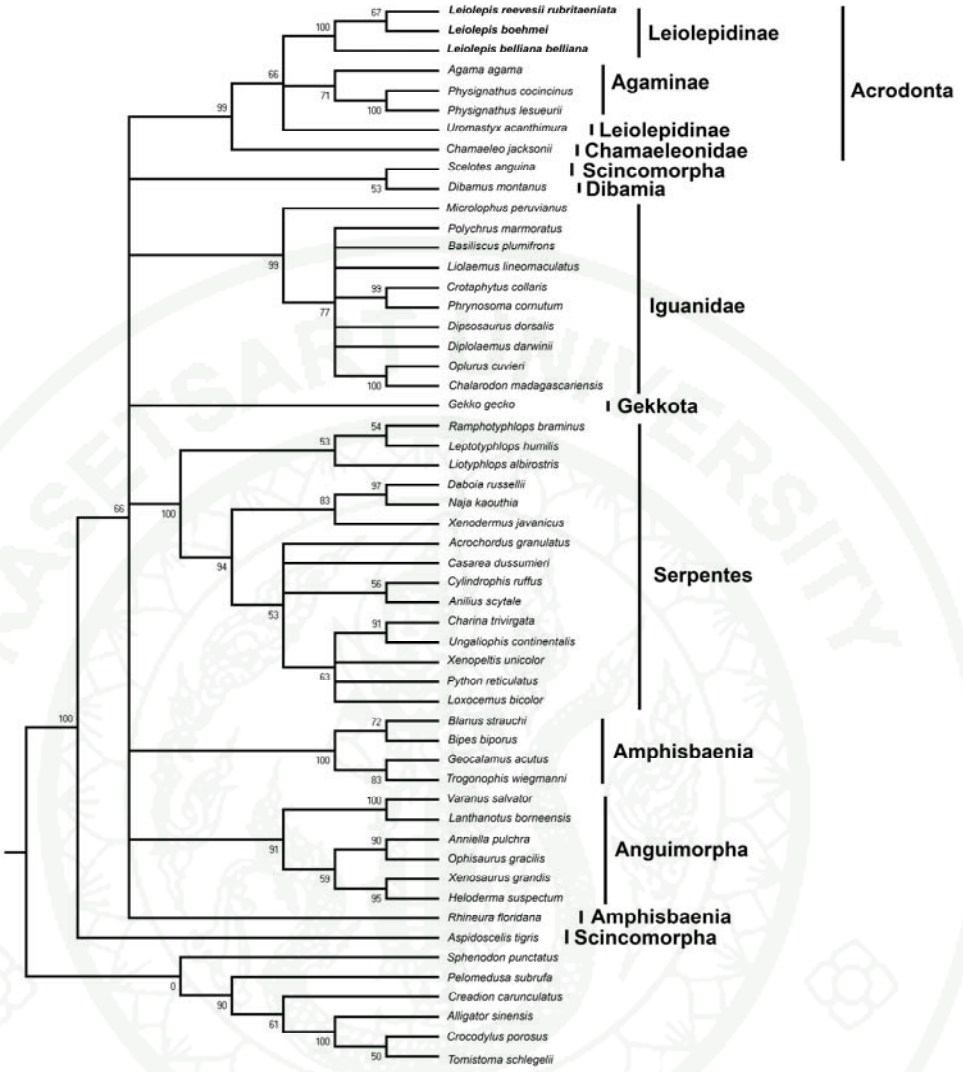

Figure 18 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the C-*mos* sequence data set based on Tamura-Nei, AIC model. The bootstrap values were shown on the corresponding branches.

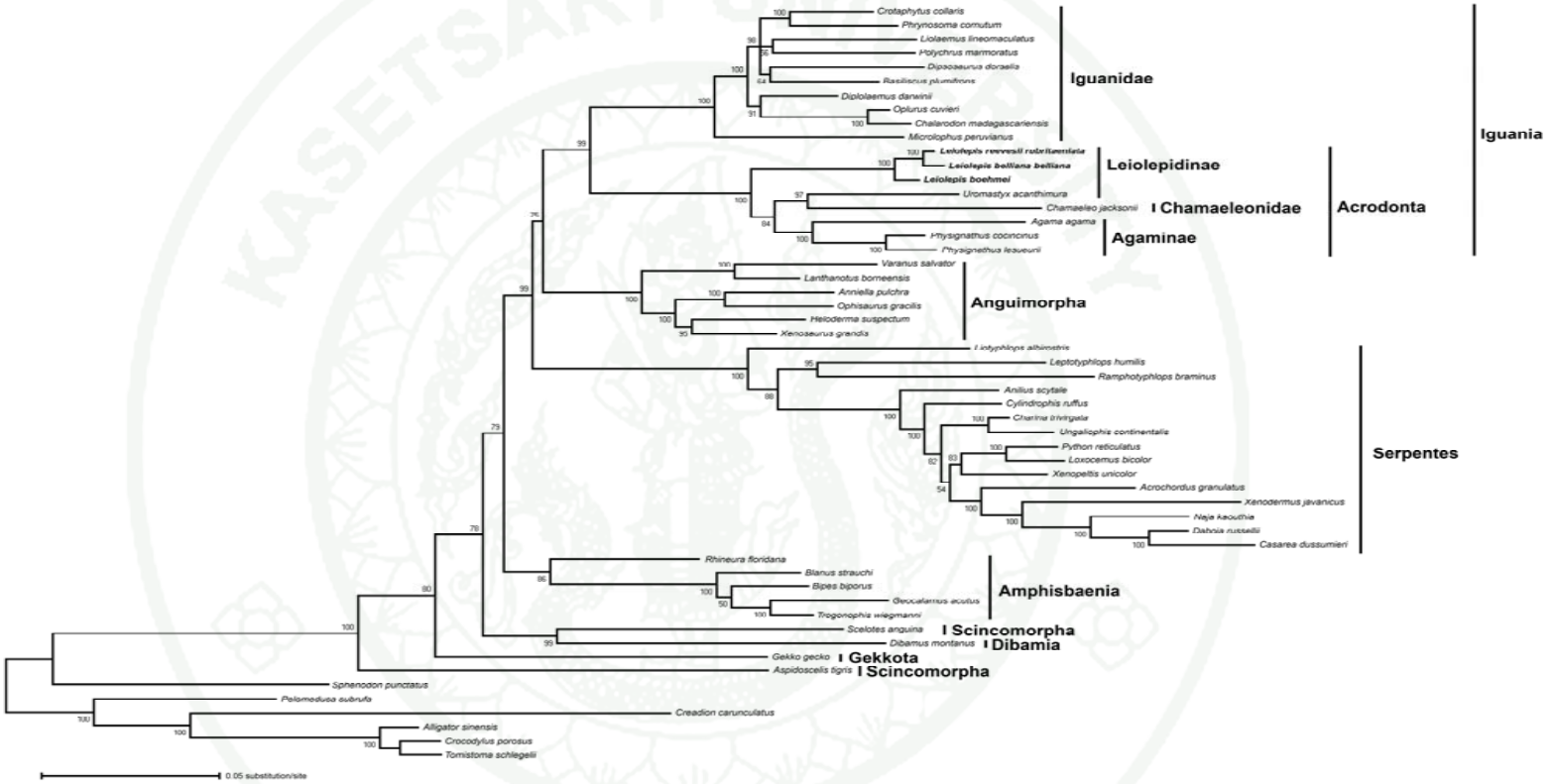

Figure 19 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the combined *RAG1*/ C-*mos* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Tamura-Nei, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

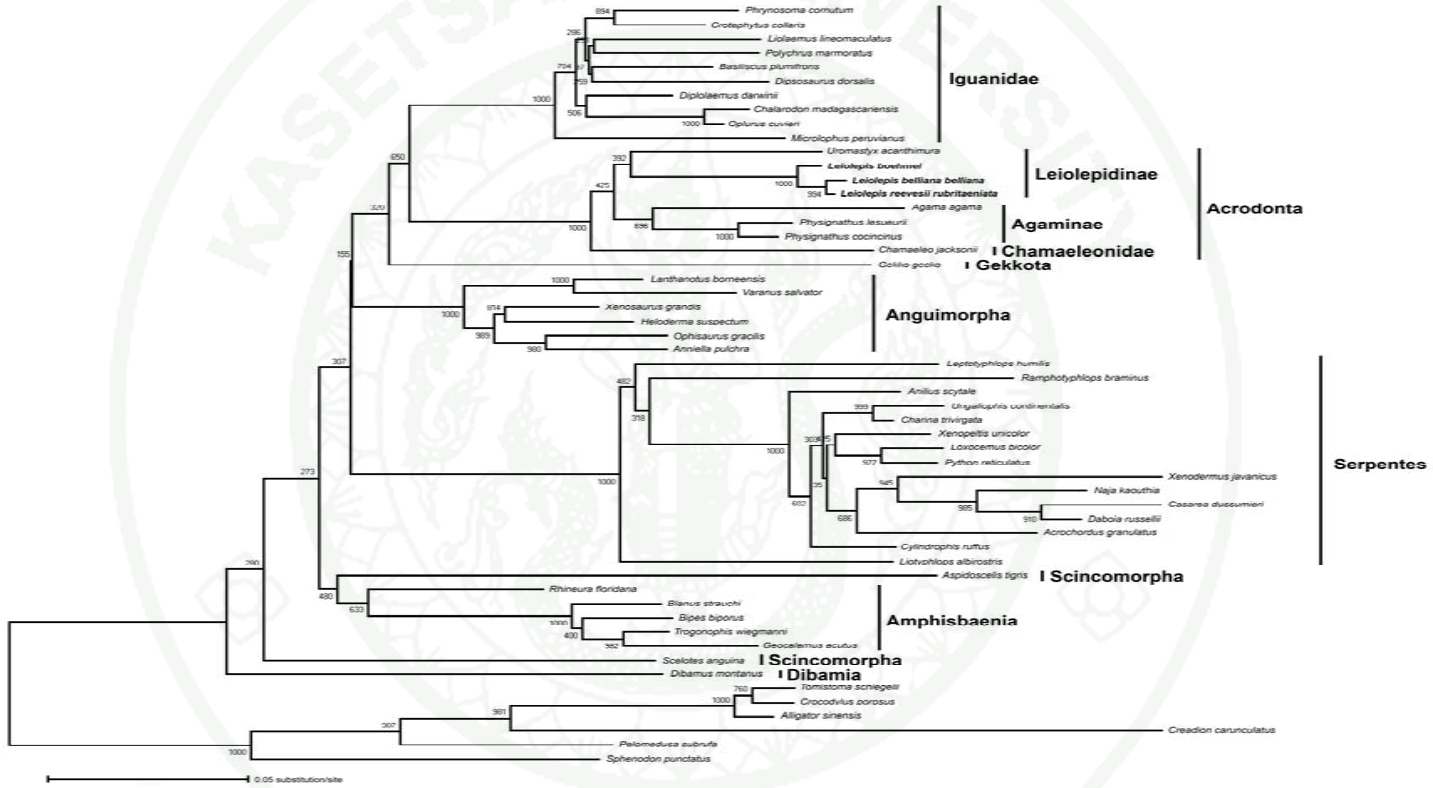

Figure 20 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the combined *RAG1*/ C-*mos* sequence data set based on Tamura-Nei, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

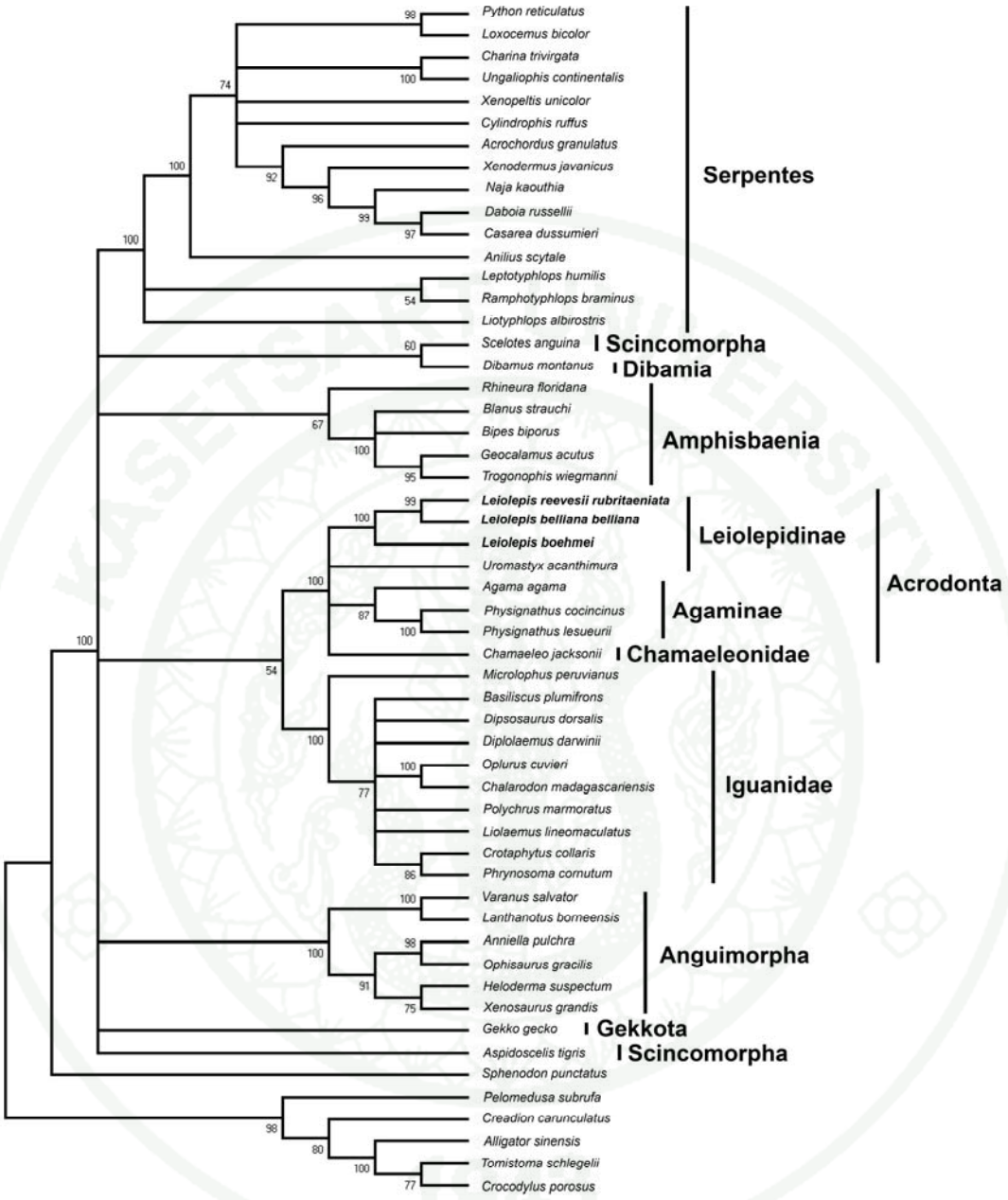

Figure 21 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the combined *RAG1*/ C-*mos* sequence data set. The bootstrap values were shown on the corresponding branches

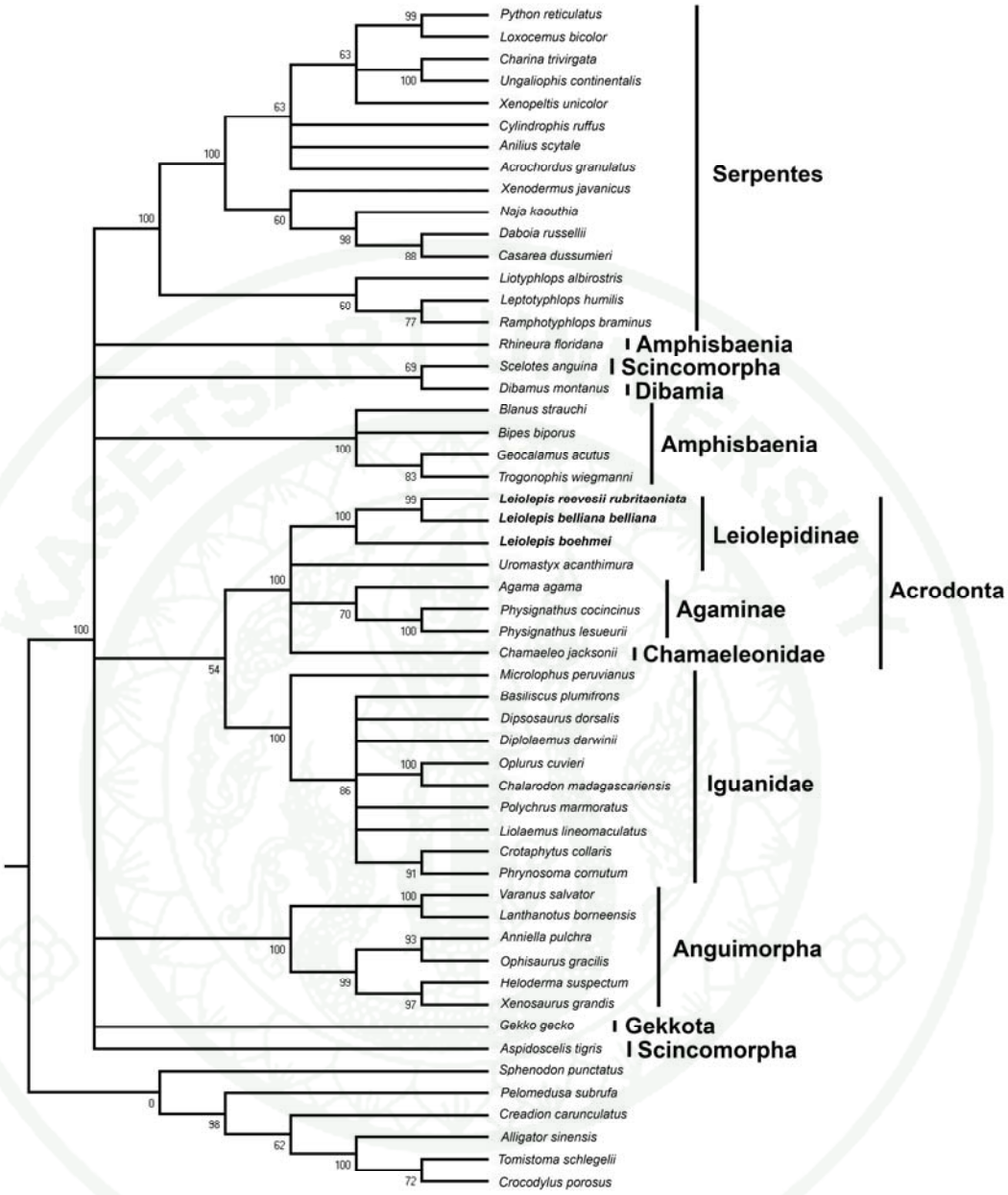

Figure 22 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Iguania and other squamate groups constructed using the combined *RAG1*/ C-*mos* sequence data set based on Tamura-Nei, AIC model. The bootstrap values were shown on the corresponding branches.

8.3 General properties of sequences of *α-Enol*, *GAPD* and combined *α-Enol*/*GAPD*

The individual *α-Enol* and *GAPD* data sets, and combined data set of the same species were also used to confirmingly determine the genetic relationship of *Leiolepis* spp. (Table 7). The *α-Enol* and *GAPD* data sets included 206 and 252 aligned nucleotide positions consisting of 127 and 212 variable sites and 68 and 82 parsimony informative sites, respectively (Table 8). In the same analyses with *RAG1* and C-*mos* data set properties, *α-Enol* and *GAPD* data sets were tested for the character of the nucleotide frequencies, the saturation data analyses and ILD test. The results showed that the nucleotide frequencies among two genes were generally similar to three butterfly lizards, and there were also no statistically significant differences in species of Agamidae (Table 9), indicating that our two data set analyses were not heterogeneity of base frequencies, and the codon bias might have not distorted phylogenetic inference either. In addition, the total number of transitions + transversions against genetic distance of the two nuclear gene data sets has revealed the resembling patterns. The regression lines were not distinctively different from straight lines, implying that saturation did not occur to cause a problem in two nuclear gene sequences at the level of homoplasy, and that there was a phylogenetic signal (Figure 23).

The ILD test revealed that there was some incongruence between the two nuclear genes (p=0.01), suggesting an extensive heterogeneity occurred between the two data sets. Since the results of data set analysis showed that the sequences of the two genes were intron, which suggested that this could be due to the rate of evolution. However, we found that all topologies from the combined data set which were corresponded to lizard taxonomy were comparable to the mixing topology of *α-Enol* plus *GAPD* data set. Therefore, we combined two data sets and discussed on the results.

Table 7 Classification and accession numbers of species used in *α-Enol* and *GAPD* sequence analysis[1].

Infraorder	Family	Subfamily	Species	Accession for *α-Enol* gene	Accession for *GADPH* gene
Iguania	Agamidae	Leiolepidinae	*Leiolepis reevesii rubritaeniata*	AB531438*	AB531439*
Iguania	Agamidae	Leiolepidinae	*Leiolepis belliana belliana*	AB531440*	AB531442*
Iguania	Agamidae	Leiolepidinae	*Leiolepis boehmei*	AB531441*	AB531443*
Iguania	Agamidae	Agaminae	*Amphibolurus muricatus*	FJ200046	FJ200070
Iguania	Agamidae	Agaminae	*Amphibolurus norrisi*	FJ200045	FJ200069
Iguania	Agamidae	Agaminae	*Amphibolurus nobbi*	FJ200043	FJ200067
Iguania	Agamidae	Agaminae	*Diporiphora bilineata*	FJ200044	FJ200068
Iguania	Agamidae	Agaminae	*Tympanocryptis pinguicolla*	FJ200042	FJ200066
Iguania	Agamidae	Agaminae	*Pogona vitticeps*	FJ200041	FJ200065
Iguania	Agamidae	Agaminae	*Pogona barbata*	FJ200040	FJ200064
Iguania	Agamidae	Agaminae	*Ctenophorus fordi*	FJ200039	FJ200063
Iguania	Agamidae	Agaminae	*Ctenophorus pictus*	FJ200036	FJ200060
Iguania	Agamidae	Agaminae	*Ctenophorus adelaidensis*	FJ200033	FJ200056
Scincomorpha	Scincidae		*Mabuya bistriata*	DQ239349	DQ239079

[1]New sequences from our study indicated by *

Table 8 Properties of character variation for α-*Enol* and *GAPD* and combined *α-Enol*/ *GAPD* data sets.

Data set	All aligned sequence	Parsimony-informative sites	Variable sites	Nucleotide bias				χ^2	d.f.	*p*-value	Best model[1]	I[2]	G[3]
				%A	%C	%G	%T						
α-Enol	206	68	127	26.29	23.22	24.53	25.96	9.38	39	0.9999	TrN+G	0.0000	3.0497
GADPH	252	82	212	24.65	18.15	23.20	34.00	11.05	39	0.9999	HKY	0.0000	0.0000
Combine	458	150	339								HKY+G	0.0000	2.6718

[1]Best models were selected with Modeltest version 3.7 (Posada and Crandall, 1998).

[2]I : Proportion of invariable site

[3]G : Gamma shape parameter

Table 9 Comparison of the base contents within α-*Enol* and *GAPD* data sets.

Taxonomic organism	Percentage of bases with presented *α-Enol* sequence					Percentage of bases with presented *GADPH* sequence				
	A	C	G	T	GC	A	C	G	T	GC
Leiolepis reevesii rubritaeniata	27.08	23.96	25.00	23.96	48.96	26.67	19.56	22.22	31.56	41.78
Leiolepis belliana belliana	27.60	23.44	25.00	23.96	48.44	27.11	19.11	21.78	32.00	40.89
Leiolepis boehmei	25.52	24.48	26.04	23.96	50.52	25.22	19.03	23.01	32.74	42.04
Amphibolurus muricatus	25.79	24.21	24.21	25.79	48.42	25.00	16.07	24.55	34.38	40.62
Amphibolurus norrisi	25.26	24.74	24.21	25.79	48.95	24.11	16.52	24.55	34.82	41.07
Amphibolurus nobbi	27.37	24.21	24.21	24.21	48.42	24.03	18.88	22.32	34.76	41.20
Diporiphora bilineata	27.37	24.21	24.74	23.68	48.95	25.86	16.81	22.85	34.48	39.66
Tympanocryptis pinguicolla	24.87	23.81	26.46	24.87	50.27	25.00	17.24	21.98	35.78	39.22
Pogona vitticeps	25.93	23.81	22.75	27.51	46.56	23.83	19.57	23.83	32.77	43.40
Pogona barbata	25.93	23.81	22.75	27.51	46.56	22.54	22.54	20.90	34.02	43.44
Ctenophorus fordi	26.84	22.63	24.74	25.79	47.37	24.45	17.47	24.02	34.06	41.49
Ctenophorus pictus	27.51	21.69	23.28	27.51	44.97	24.45	16.59	23.58	35.37	40.17
Ctenophorus adelaidensis	26.98	20.64	24.87	27.51	45.51	25.00	16.67	23.68	34.65	40.35
Mabuya bistriata	24.10	19.49	25.13	31.28	44.62	22.22	17.70	25.51	34.57	43.21

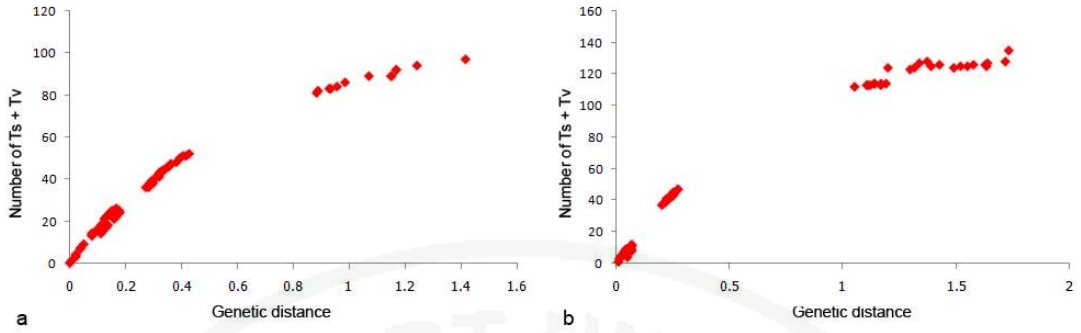

Figure 23 The relationship between the total number of transitions (Ts) + transversions (Tv) and corrected distance for all pairwise comparisons in (**a**) *α-Enol* sequence data set and (**b**) *GAPD* sequence data set.

8.4 Phylogenetic analyses of *α-Enol*, *GAPD* and combined *α-Enol*/*GAPD*

The phylogenetic analyses of the individual *α-Enol* and *GAPD* data sets, and combined data set were reconstructed using BI, ML, MP and NJ. The phylogram showed some currently available lizard in Agaminae and *Mabuya bistriata* (Scincidae, Scincomorpha) as an out group. The phylogram of all analysis methods was clearly showed that Agaminae was grouped with Leiolepidinae as family Agamidae, even though there were no sequences analysis of the *Uromastyx* sp. which is a sister group of the genus *Leiolepis* in subfamily Leiolepidinae. However, the unusual sister group of *Pogona vitticeps* and *Amphibolurus nobbi* was sustained in all method analysis of *GAPD* data set which were different from the phylogram of *α-Enol* and combined *α-Enol*/*GAPD* data set, and were distinguishable with lizard taxonomy, indicating that *GAPD* gene might not be suitable to determine the phylogenetic relationship in Agaminae. In *Leiolepis*, statistical analyses of all method strongly supported (100%) that *L. reevesii rubritaeniata* was more adjacent to *L. belliana belliana* than *L. boehmei* in *GAPD* and combined *α-Enol* / *GAPD* analyses (Figures 28-35). These findings were also comparable to *RAG1* and combined *RAG1*/C-*mos* analyses, and concatenate protein coding gene phylogram (Figure 45-48). By contrast, the phylogenetic tree of the individual *α-Enol* gene data set showed *L. belliana belliana* was adjacent to *L. boehmei* rather than *L. reevesii rubritaeniata*

(Figures 24-27), suggesting that *α-Enol* gene might not be appropriate to determine the genetic relationship in genus *Leiolepis* spp. The *α-Enol* and *GAPD* gene appear to be applicable in each taxonomic group. However, the combined *α-Enol*/*GAPD* data set showed the mixing topology of *α-Enol* plus *GAPD* data set which was harmoniously compatible to determine the phylogenetic relationship in Agamidae. The addition of taxa is required to identify the most optimal strategy systematic and to address these dilemmas.

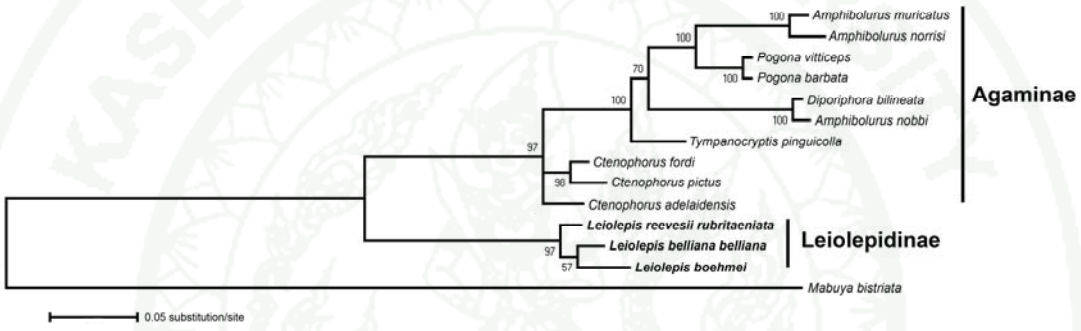

Figure 24 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *α-Enol* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Tamura-Nei, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

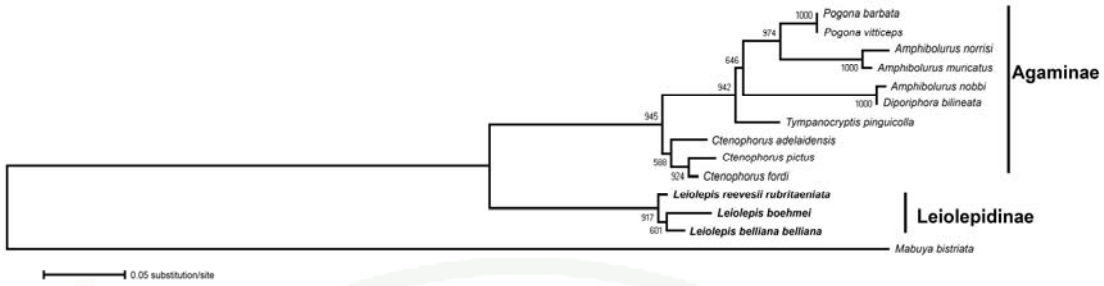

Figure 25 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *α-Enol* sequence data set based on Tamura-Nei, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

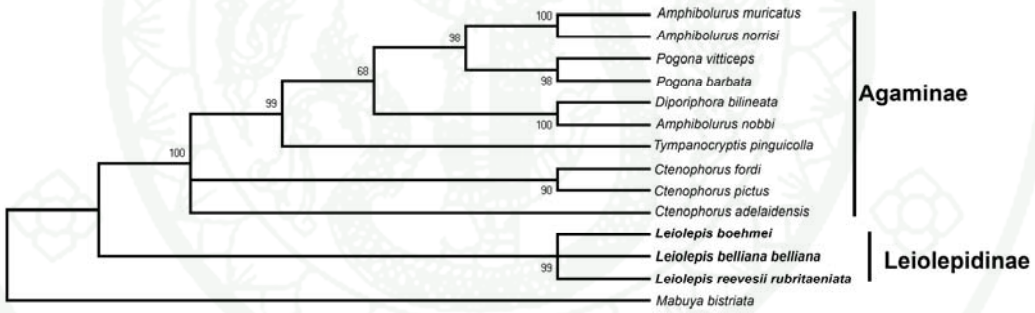

Figure 26 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *α-Enol* data set. The bootstrap values were shown on the corresponding branches.

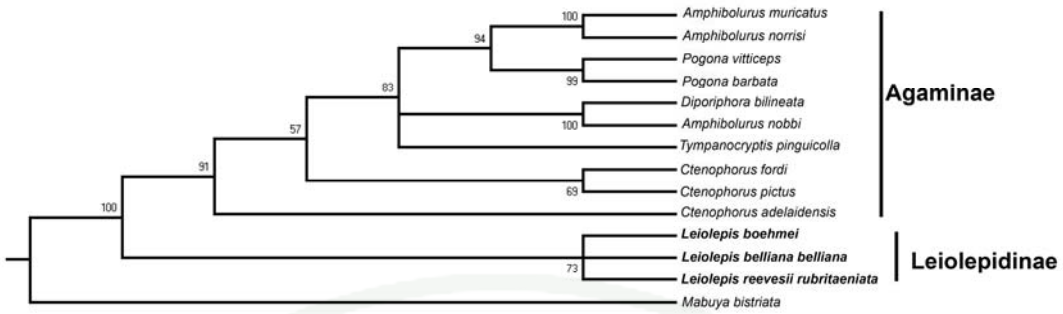

Figure 27 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *α-Enol* sequence data set based on Tamura-Nei, AIC model. The bootstrap values were shown on the corresponding branches.

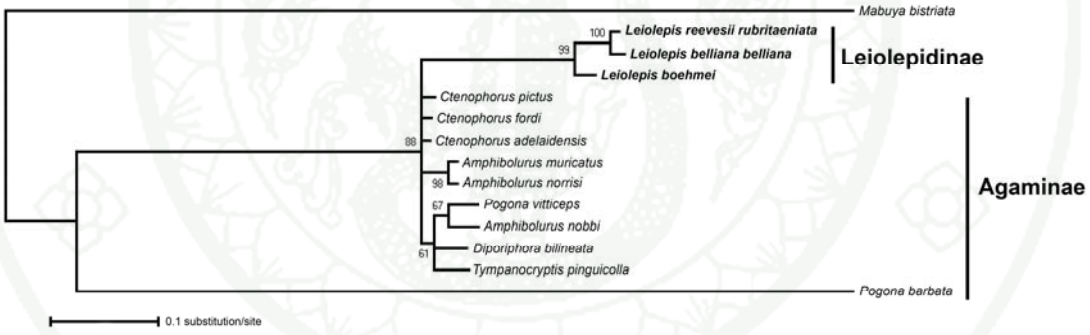

Figure 28 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *GAPD* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Hasegawa-Kishino-Yano, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

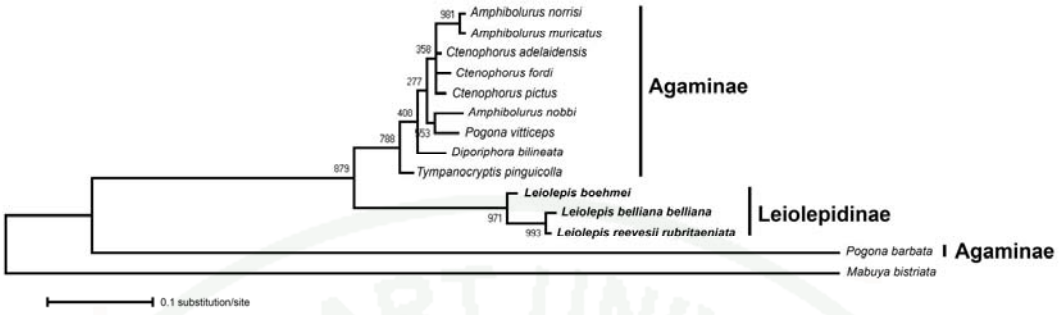

Figure 29 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *GAPD* sequence data set based on Hasegawa-Kishino-Yano, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

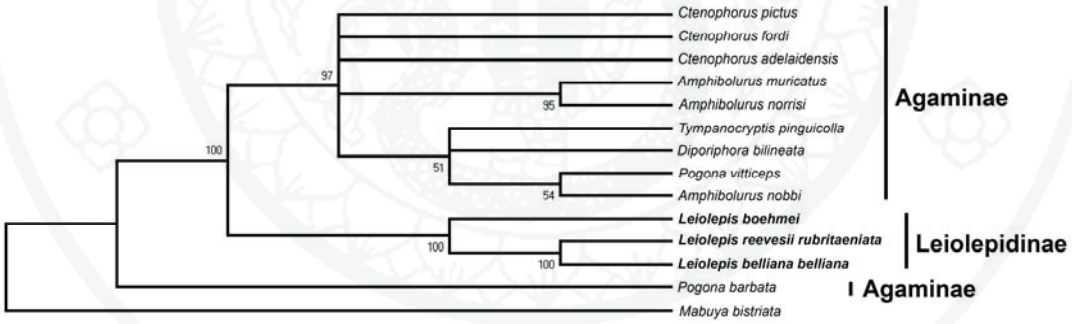

Figure 30 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *GAPD* data set. The bootstrap values were shown on the corresponding branches.

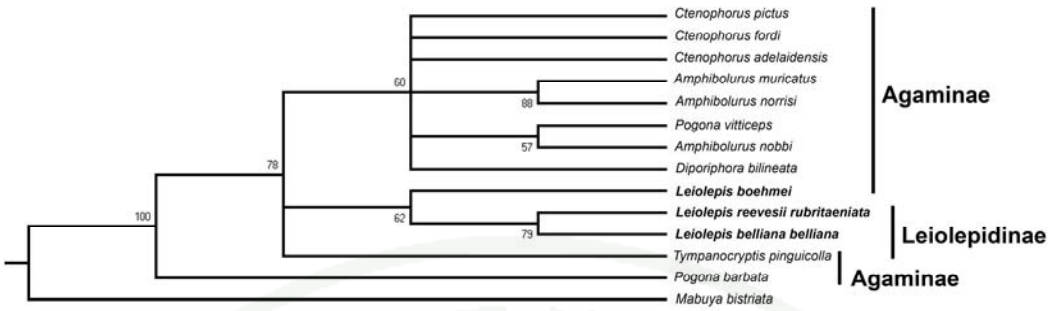

Figure 31 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the *GAPD* sequence data set based on Hasegawa-Kishino-Yano, AIC model. The bootstrap values were shown on the corresponding branches.

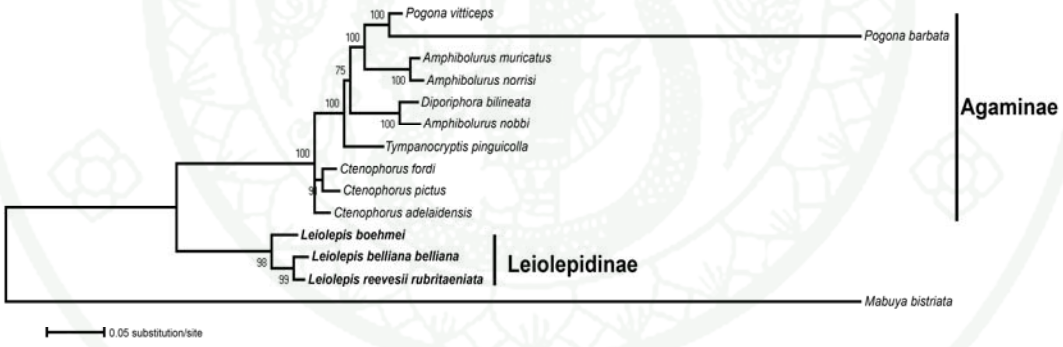

Figure 32 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the combined *α-Enol*/ *GAPD* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on Hasegawa-Kishino-Yano, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

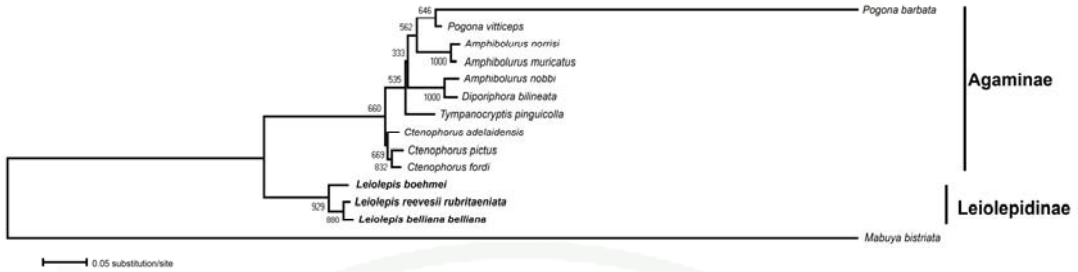

Figure 33 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the combined *α-Enol*/ *GAPD* sequence data set based on Hasegawa-Kishino-Yano, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

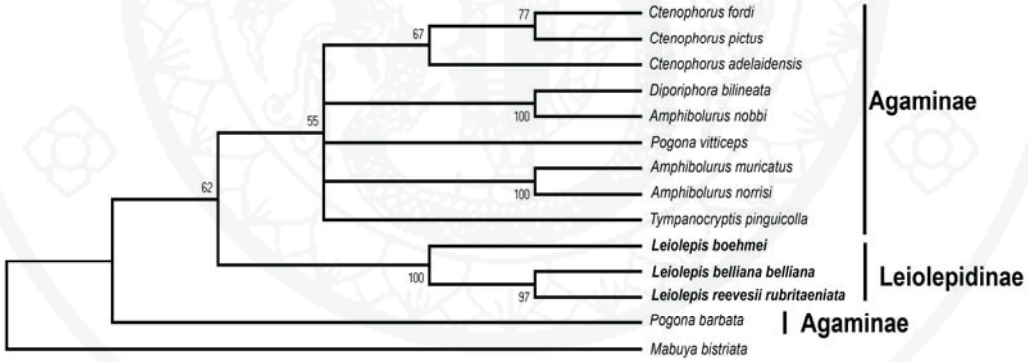

Figure 34 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the combined *α-Enol*/ *GAPD* data set. The bootstrap values were shown on the corresponding branches.

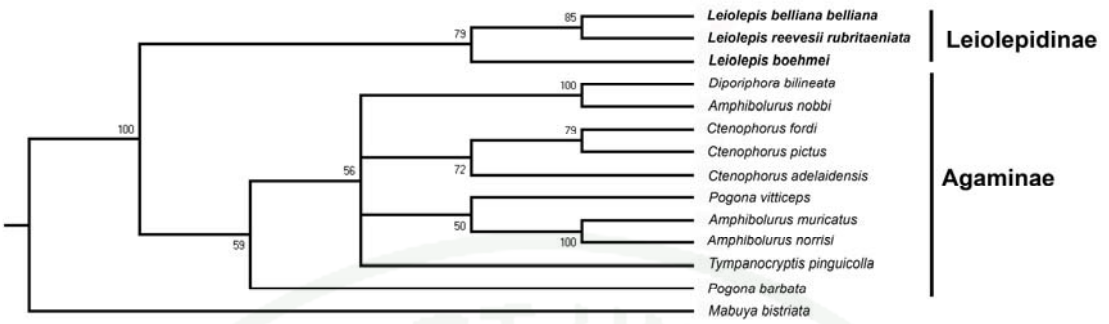

Figure 35 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the combined *α-Enol*/ *GAPD* sequence data set based on Hasegawa-Kishino-Yano, AIC model. The bootstrap values were shown on the corresponding branches.

9. Complete mitochondrial genome sequencing of *L. reevesii rubritaeniata, L. belliana belliana and L. boehmei*

9.1 Genome organization

The complete mitochondrial genome sequences of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei* were reported for the first time and deposited at DDBJ, AB537553, AB537554, AB537555, respectively. The total mitochondrial genome sequences of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei* were 16,053 bp, 16,109 bp and 16,106 bp in length, respectively, and comprised 13 protein coding genes, two ribosomal RNA genes, 22 tRNA genes and a non-coding control region (Figure 36, Tables 10-12). There were 28 genes encoded in the majority-strand (H-strand) and 9 genes in the minority-strand (L-strand), whereas the noncoding control region was surrounded by $tRNA^{Pro}$ and $tRNA^{Phe}$ genes in the three mitochondrial genomes of butterfly lizards. The relative position and orientation of all the genes, and control region were similar to those of most other vertebrates (Anderson *et al*., 1981; Kartavtsev *et al*., 2007).

Comparing the genome arrangements in known iguanian lizard mitochondrial genomes, five types of genome organization were summarized and shown in Figure 36. Various genome arrangements have occurred in mitochondrial genome of acrodont lizards [Agamidae (Agaminae and Leiolepidinae) and Chamaeleonidae]. Type I represented gene organizations of all lizard in Chamaeleonidae and *Xenagama taylori* (Agaminae). They had similar organization to that of typical vertebrate except the position of control region located between $tRNA^{Thr}$ and $tRNA^{Pro}$. The duplication of control region has occurred in *Pogona vitticepes* and *Chlamydosaurus kingii* (type II). They were located between the genes for ND5 and ND6, and between $tRNA^{Pro}$ and $tRNA^{Phe}$. Moreover, the identifiable origin of light-stranded replication (O_L) was absent as well. These characters were also found in most Australasian agamid lizard. However, these occurrences disappeared in *Calotes vesicolor* (Agaminae) and some Asian agamid lizard (type III) where $tRNA^{Pro}$ was exclusively found in the heavy-stranded position, and all butterfly lizards (Leiolepidinae) (type IV), indicating that the paralogous control region have evolved in an ancestral lineage of Australasian agamids (Amer and Kumazawa, 2005). By contrast, the order of 37 genes and the non-coding control region was conserved among all iguanid lizards (Iguanidae), which corresponds to the so-called typical vertebrate gene organization (Boore, 1999; Okajima and Kumazawa, 2009) as type V. Most of the divergence was IQM tRNA gene cluster which rearranged into QIM. This feature shared in all acrodont lizards (Type I – IV) and differed from iguanid lizard and other vertebrate species (Macey *et al*., 2000). The overall nucleotide composition of the H-strand was 34.3% A, 28.0% C, 13.2% G and 24.6% T for *L. reevesii rubritaeniata*, 34.4% A, 28.2% C, 13.2% G and 24.2% T for *L. belliana belliana*, and 35.2% A, 27.9% C, 13.2% G and 23.7% T for *L. boehmei.* The overall A-T content was 58.9%, 58.6% and 58.9%, respectively, which corresponded well with the values found in other vertebrate species (Asakawa *et al*., 1991; Janke and Arnason, 1997; Janke *et al*., 2001).

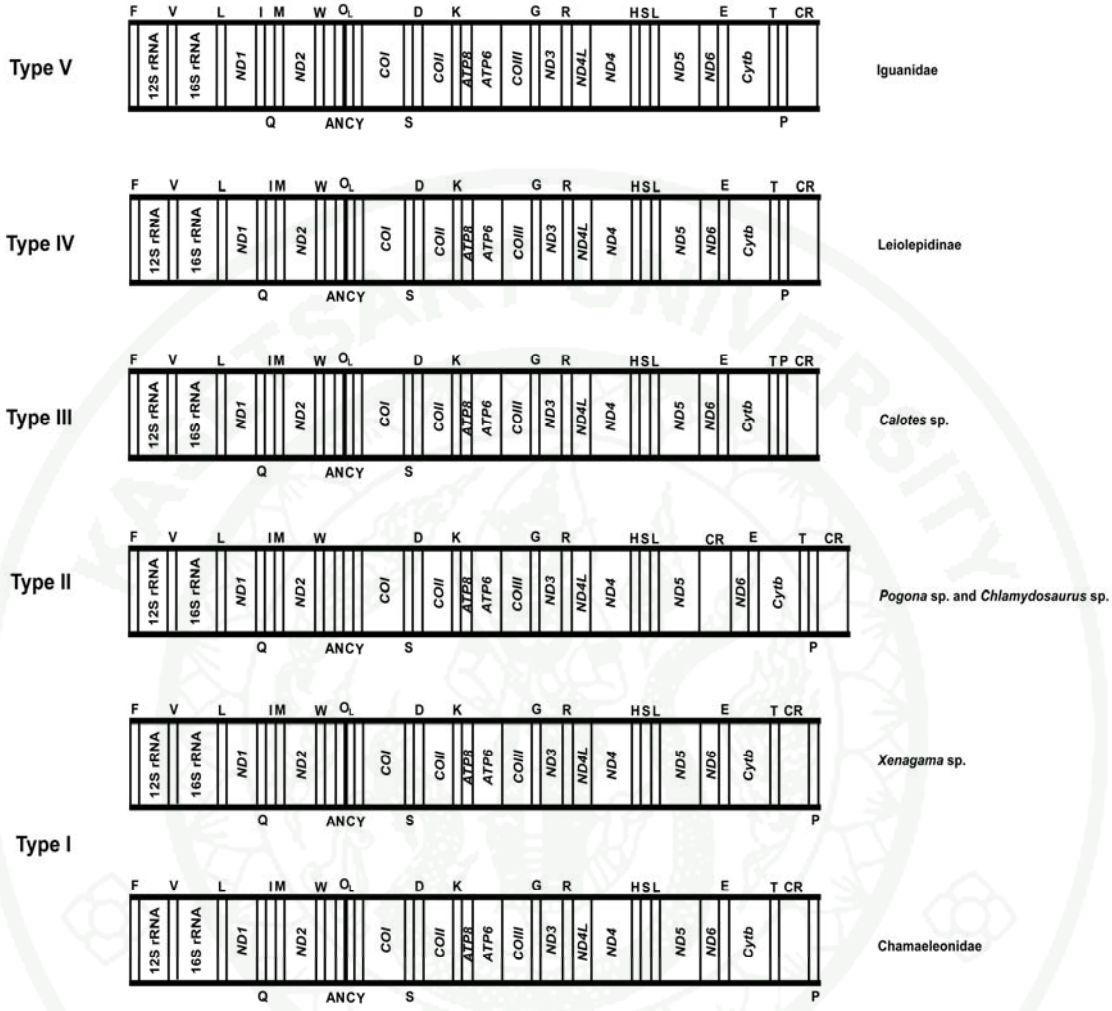

Figure 36 Comparison of mitochondrial gene organizations of acrodont lizards and iguanid lizards.

Table 10 Genome organization and features in the mitochondrial genome of *L. reevesii rubritaeniata*.

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Phe	F	1	70	70				UUC	GAA		H
12S rRNA		71	932	862							H
tRNA Val	V	933	998	66				GUA	TAC		H
16S rRNA		999	2545	1547							H
tRNA Leu	L	2546	2619	74				UUA	TAA		H
ND1		2621	3595	975	324	ATA	TAG			+1	H
tRNA Gln	Q	3592	3663	72				CAA	TTG	-4	L
tRNA Ile	I	3677	3746	70				AUC	GAT	+13	H
tRNA Met	M	3748	3812	65				AUG	CAT	+1	H
ND2		3814	4846	1033	344	CTG[1]	T++[2]			+1	H
tRNA Trp	W	4847	4916	70				UGA	TCA		H
tRNA Ala	A	4920	4988	69				GCA	TGC	+3	L
tRNA Asn	N	4991	5060	70				AAC	GTT	+2	L
L-strand rep		5063	5089	27						+2	
tRNA Cys	C	5093	5149	57				UGC	GCA	+3	L
tRNA Tyr	Y	5150	5221	72				UAC	GTA		L
COI		5222	6766	1545	514	ATG	AGG				H
tRNA Ser	S	6758	6829	72				UCA	TGA	-9	L

Table 10 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Asp	D	6841	6910	70				GAC	GTC	+11	H
COII		6911	7591	681	226	ATG	TAA				H
tRNA Lys	K	7600	7668	69				AAA	TTT	+8	H
ATPase8		7669	7830	162	53	TTG[1]	TAA				H
ATPase6		7824	8506	683	227	ATG	TA+[2]			-7	H
COIII		8506	9289	784	261	ATG	T++[2]			-1	H
tRNA Gly	G	9290	9358	69				GGA	TCC		H
ND3		9359	9701	343	114	ATA	T++[2]				H
tRNA Arg	R	9703	9769	67				CGA	TCG	+1	H
ND4L		9770	10066	297	98	ATA	TAA				H
ND4		10060	11424	1365	454	ATG	TAA			-7	H
tRNA His	H	11432	11505	74				CAC	GTG	+7	H
tRNA Leu	L	11566	11636	71				CUA	TAG	+1	H
ND5		11637	13433	1797	598	GTG	AGA				H
ND6		13413	13931	519	172	GTG	TAA			-21	L
tRNA Glu	E	13933	14001	69				GAA	TTC	+1	L
Cytb		14003	15131	1129	376	ATG	T++[2]			+1	H
tRNA Thr	T	15132	15196	65				ACA	TGT		H

Table 10 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Pro	P	15171	15236	66				CCA	TGG	-26	L
control region		15237	16052	814							

[1]translation except for position 1..3, amino acid: Met.

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

Table 11 Genome organization and features in the mitochondrial genome of *L. belliana belliana*.

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Phe	F	1	70	70				UUC	GAA		H
12S rRNA		71	989	919							H
tRNA Val	V	990	1055	66				GUA	TAC		H
16S rRNA		1056	2602	1547							H
tRNA Leu	L	2603	2676	74				UUA	TAA		H
ND1		2677	3651	975	324	ATA	TAG				H
tRNA Gln	Q	3648	3719	72				CAA	TTG	-4	L
tRNA Ile	I	3728	3797	70				AUC	GAT	+8	H
tRNA Met	M	3799	3863	65				AUG	CAT	+1	H
ND2		3865	4897	1033	344	TTG[1]	T++[2]			+1	H
tRNA Trp	W	4898	4967	70				TGA	TCA		H
tRNA Ala	A	4971	5039	69				GCA	TGC	+3	L
tRNA Asn	N	5041	5112	72				AAC	GTT	+1	L
L-strand rep[3]		5115	5140	26						+2	H
tRNA Cys	C	5144	5200	57				UGC	GCA	+3	L
tRNA Tyr	Y	5200	5271	72				UAC	GTA	-1	L
COI		5272	6819	1548	515	ATG	AGG				H
tRNA Ser	S	6812	6881	70				UCA	TGA	-8	L

Table 11 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		codon	anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Asp	D	6892	6961	70				GAC	GTC	+10	H
COII		6962	7642	681	226	ATG	TAA				H
tRNA Lys	K	7647	7715	69				AAA	TTT	+4	H
ATPase8		7716	7877	162	53	CTG[1]	TAA				H
ATPase6		7871	8553	683	227	ATG	TA+[2]			-7	H
COIII		8553	9336	784	261	ATG	T++[2]			-1	H
tRNA Gly	G	9337	9405	69				GGA	TCC		H
ND3		9406	9748	343	114	ATT	T++[2]				H
tRNA Arg	R	9750	9816	67				CGA	TCG	+1	H
ND4L		9817	10113	297	98	ATG	TAA				H
ND4		10107	11471	1365	454	ATG	TAA			-7	H
tRNA His	H	11479	11551	73				CAC	GTG	+7	H
tRNA Ser	S	11552	11609	58				AGY	ACT		H
tRNA Leu	L	11611	11681	71				CTA	TAG	+1	H
ND5		11682	13478	1797	598	GTG	AGA				H
ND6		13458	13979	520	173	ATG				-21	L
tRNA Glu	E	13981	14050	70				GAA	TTC	+1	L
Cytb		14053	15196	1144	381	ATG	T++[2]			+2	H
tRNA Thr	T	15197	15261	65				ACA	TGT		H

Table 11 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		codon	anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Pro	P	15237	15302	66				CCA	TGG	-25	L
control region		15303	16109	805							

[1]translation except for position 1..3, amino acid: Met.

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

Table 12 Genome organization and features in the mitochondrial genome of *L. boehmei*.

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Phe	F	1	70	70				UUC	GAA		H
12S rRNA		71	939	869							H
tRNA Val	V	941	1006	66				GTA	TAC	+1	H
16S rRNA		1007	2573	1567							H
tRNA Leu	L	2574	2647	74				UUA	TAA		H
ND1		2649	3623	975	324	ATA	TAG			+1	H
tRNA Gln	Q	3620	3693	74				CAA	TTG	-4	L
tRNA Ile	I	3711	3781	71				AUC	GAT	+17	H
tRNA Met	M	3783	3847	65				AUG	CAT	+1	H
ND2		3849	4881	1033	344	TTG[1]	T++[2]			+1	H
tRNA Trp	W	4883	4952	70				UGA	TCA	+1	H
tRNA Ala	A	4956	5024	69				GCA	TGC	+3	L
tRNA Asn	N	5032	5103	72				AAC	GTT	+7	L
L-strand rep[3]		5106	5131	26						+2	H
tRNA Cys	C	5136	5190	55				UGC	GCA	+4	L
tRNA Tyr	Y	5192	5263	72				UAC	GTA	+1	L
COI		5264	6811	1548	515	ATG	AGG				H
tRNA Ser	S	6804	6873	70				UCA	TGA	-8	L

Table 12 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Asp	D	6880	6949	70				GAC	GTC	+6	H
COII		6950	7630	681	226	ATG	TAA				H
tRNA Lys	K	7649	7718	70				AAA	TTT	+18	H
ATPase8		7719	7880	162	53	TTG	TAA				H
ATPase6		7874	8556	683	227	ATG	TA+[2]			-7	H
COIII		8556	9339	784	261	ATG	T++[2]			-1	H
tRNA Gly	G	9340	9408	69				GGA	TCC		H
ND3		9409	9751	343	114	ATA	T++[2]				H
tRNA Arg	R	9753	9820	68				CGA	TCG	+1	H
ND4L		9821	10117	297	98	ATA	TAA				H
ND4		10111	11475	1365	454	ATG	TAA			-7	H
tRNA His	H	11483	11556	74				CAC	GTG	+7	H
tRNA Ser	S	11557	11615	59				AGY	ACT		H
tRNA Leu	L	11617	11687	71				CTA	TAG	+1	H
ND5		11688	13484	1797	598	GTG	AGA				H
ND6		13467	13982	516	171	ATG	TAG			-18	L
tRNA Glu	E	13983	14051	69				GAA	TTC		L
Cytb		14053	15187	1135	378	ATG	T++			+1	H
tRNA Thr	T	15188	15252	65				ACA	TGT		H

Table 12 (Continued)

Gene/element	Amino acid	Position number		Size (bp)	Size of amino acid (residues)	Codon		Codon	Anticodon	Spacer/Overlap	Strand
		From	To			start	stop				
tRNA Pro	P	15228	15293	66				CCA	TGG	-25	L
control region		15294	16106	813							

[1]translation except for position 1..3, amino acid: Met.

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

A characteristic stem-and-loop feature for the putative origin of the light-stranded replication found in the typical vertebrate location between $tRNA^{Asn}$ and $tRNA^{Cys}$ genes was present in the mitochondrial genome of three butterfly lizards (Figure 37). *L. belliana belliana* showed 8 bp stem, which was identical with *L. belliana* (Macey *et al*., 1997). Furthermore, *L. reevesii rubritaeniata* and *L. boehmei* illustrated 10 and 7 bp stem, respectively. This feature was also found in *L. guentherpetersi* with 9 bp stem (Macey *et al*., 2000); however, the O_L stem-and-loop feature has not been found in genus *Uromasti*x (Macey *et al*., 1997), which was classified into the same subfamily (Leiolepidinae) with the butterfly lizard, suggesting that the putative origin of the light-stranded replication might be exclusively identified in genus *Leiolepis*. In Acrodonta, the O_L stem-and-loop feature has been also revealed in Chamaeleonidae (Macey *et al*., 1997, 2008; Kumazawa, 2007). Nevertheless, the origin for the light-stranded replication structure was absent in *P. vitticepes*, *Physignathus cocincinus* and most Australasian agamids, implying that the loss of O_L stem-and-loop feature might have occurred in multiple lineages of acrodont lizards (Macey *et al*., 2000; Amer and Kumazawa, 2005). Surprisingly, the O_L stem-and-loop structure observed in the iguanid and anguid 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' sequence required for *in vitro* replication in humans (Hixson *et al*., 1986). On the contrary, the heavy-stranded sequence required for *in vitro* replication in humans could not identify in three butterfly lizard species, even though the point of light-stranded elongation in mouse were determined. By contrast, Chamaeleonidae has been found only the sequence that resembled the heavy-stranded sequence 3'-GBCCB-5'. These results collectively suggested that these acrodont lizard sequences did not form a functional O_L (Macey *et al*., 1997).

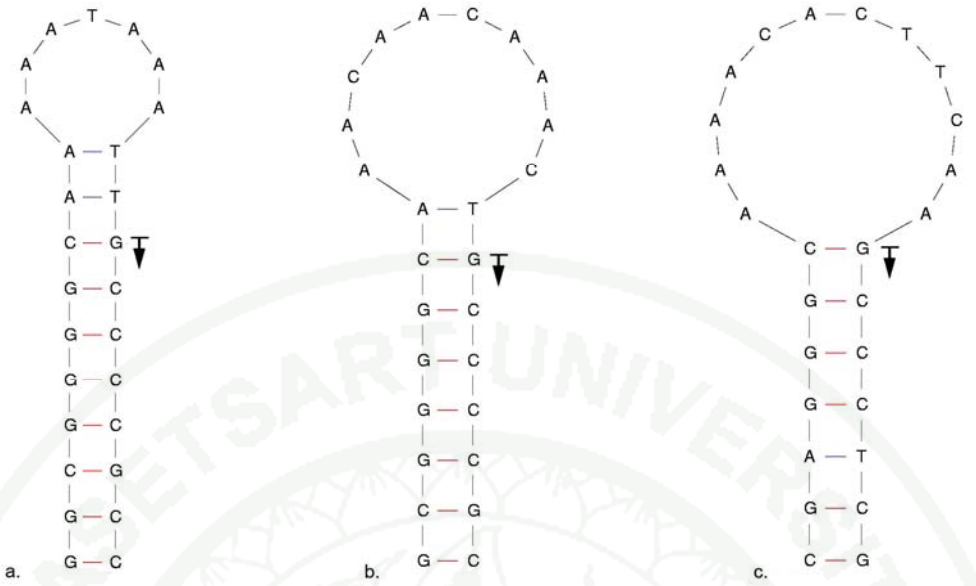

Figure 37 A characteristic stem-and-loop feature for the putative origin of the light-stranded replication in three butterfly lizards. (**a**) *L. reevesii rubritaeniata*. (**b**) *L. belliana belliana*. (**c**) *L. boehmei*. Arrows indicate the 3'-GCC-5' heavy stranded template sequence identified as the point of light-strand elongation in mouse (Brennicke and Clayton, 1981).

9.2 Protein-coding sequences

The boundaries between protein-coding genes of mitochondrial genomes of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei* were determined by aligning their sequences and by identifying translocation initiation and termination codons with those of known other agamid lizards. The 13 protein-coding genes in three butterfly lizards were 10,794 bp, 10,812 bp and 10,803 bp, respectively. *ND6* gene encoded in the L-strand, and the other 12 genes encoded in the H-strand. The longest one was the *ND5* gene (1,797 bp in the three butterfly lizards), whereas the shortest one was *ATPase 8* (162 bp in the three butterfly lizards). The length of protein-coding sequences *COI*, *ND6* and *Cytb* were different among the three butterfly lizards, while the rest of protein-coding sequences were the same in length (Tables 13-15). Some overlapping was found in the three butterfly lizards among the

13 protein-coding genes as shown in Tables 10-12 such as *ATPase8*-*ATPase6* and *ND4L*-*ND4*. 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 three butterfly lizards was ATG (six, eight and seven of thirteen protein-coding genes, respectively) (Tables 10-12). *ND1*, *ND3* and *ND4L* were initiated by ATA codon, and *ND5* and *ND6* were started by GTG codon for *L*. *reevesii rubritaeniata*. *ND1*, *ND3* and *ND5* were initiated by ATA, ATT and GTG codon, respectively, for *L*. *belliana belliana*. *ND1*, *ND3* and *ND4L* were initiated by ATA codon, and *ND5* were started by GTG codon for *L*. *boehmei*. The ATA, ATT and GTG codon were determined as start codon among mitochondrial protein-coding genes of vertebrates. However, there were two genes (*ND2* and *ATPase 8*) in three butterfly lizards which appeared to be the unusual start codon. CTG, TTG and TTG were identified as initiation codon of *ND2* gene, whereas TTG, CTG and TTG were identified for initiation codon of *ATPase8* gene among the three butterfly lizards, respectively. TTG has been introduced as start codon (Wolstenholme, 1992; Seutin *et al*., 1994), plausibly being recognized by the f-met-charged tRNA when they occurred in the initiation position. For CTG, it has been also found in Turnip-tailed Agama (*Xenagama taylori*) (Macey *et al*., 2006), suggesting that it might follow the same process of the start codon that of TTG. Interestingly, there were seven protein-coding genes (*ND1*, *COI*, *COII*, *ATPase 6*, *COIII*, *ND4* and *ND5*) with the same initiation codon, and six protein-coding genes (*ND2*, *ATPase 8*, *ND3*, *ND4L* and *Cytb*) with the disparate start codon among the three butterfly lizards. Four protein-coding genes in the three butterfly lizards mitochondrial genome end with complete stop codons, TAA (*COII*, *ATPase8*, *ND4L* and *ND4*), TAG (*ND1*), AGG (*COI*) and AGA (*ND5*), and the other seven protein-coding genes end with incomplete stop codons, T (*ND2*, *COIII*, *ND3* and *Cytb*) and TA (*ATPase6*), which appeared to be created by post transcriptional polyadenylation (Ojala *et al*., 1981). For *ND6* gene, the termination codon was determined in the three butterfly lizards as TAA, T and TAG, respectively.

The nucleotide usage of the three butterfly lizards protein-coding genes are shown in Tables 13-15. The nucleotide content was generally similar among all genes except for *ND6* (Figures 38-40). 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. There were also statistically significant proportions differences (χ^2 =403.6127, 708.8618 and 461.2915, respectively, d.f. = 5, p<0.01 for the three butterfly lizards). For the nucleotide content of the first, second and third position codons of protein-coding genes on H-strand, the relative order of nucleotide composition was A>C>T>G with the first and third codon position, whereas that of the second codon position was T>C>A>G. However, the content of A-T : C-G in the first, second and third position codons of protein-coding genes were 54.15% : 45.85%, 59.22 % : 40.78% and 62.84% : 37.16%, respectively for *L. reevesii rubritaeniata*, 54.11% : 45.89%, 58.87 % : 41.13% and 62.77% : 37.23%, respectively, for *L. belliana belliana* and 54.16% : 45.84%, 59.58 % : 40.42% and 63.55% : 36.45%, respectively, for *L. boehmei*, which were also not statistically significant proportions difference (χ^2 = 1.5611, 1.5405 and 1.8255, d.f. = 2, p = 0.4582, 0.4629 and 0.4014, respectively, for the three butterfly lizards) (Tables 16-18). These findings were also consistent with A-T content-rich of the mitochondrial genome.

Table 13 Nucleotide composition (% of total number) for protein-coding genes found in the mitochondrial genome of *L. reevesii rubritaeniata*.

Gene	Nucleotide, all positions				Total number		
	A	C	G	T	AT	CG	
ND1	32.60	30.30	12.10	25.00	57.60	42.40	975
ND2	35.40	31.50	10.20	22.90	58.40	41.60	1033
COI	28.80	27.20	16.80	27.20	56.10	43.90	1545
COII	33.50	26.90	14.50	25.10	58.60	41.40	681
ATPase8	42.00	25.90	8.00	24.10	66.00	34.00	162
ATPase6	32.70	31.20	10.10	26.10	58.70	41.30	683
COIII	32.90	27.70	13.60	25.80	58.70	41.30	784

Table 13 (Continued)

Gene	Nucleotide, all positions						Total number
	A	C	G	T	AT	CG	
ND3	32.10	28.60	11.40	28.00	60.10	39.90	343
ND4L	30.30	29.00	10.10	30.60	60.90	39.10	297
ND4	34.80	28.40	11.60	25.30	60.10	39.90	1365
ND5	36.10	30.10	10.30	23.50	59.60	40.40	1797
Cytb	30.40	28.90	12.20	28.50	58.90	41.10	1129
ND6	15.00	9.10	31.20	44.70	59.70	40.30	519
Average[1]	33.09	29.03	12.23	25.65	58.75	41.25	899.50

[1]Average are given for 12 genes (ND6 excluded).

Table 14 Nucleotide composition (% of total number) for protein-coding genes found in the mitochondrial genome of *L. belliana belliana*.

Gene	Nucleotide, all positions						Total number
	A	C	G	T	AT	CG	
ND1	32.90	29.70	11.90	25.40	58.40	41.60	975
ND2	35.70	30.60	10.80	22.80	58.60	41.40	1033
COI	29.80	27.40	16.30	26.50	56.30	43.70	1548
COII	32.90	29.50	14.40	23.20	56.10	43.90	681
ATPase8	43.20	29.00	7.40	20.40	63.60	36.40	162
ATPase6	33.10	31.90	8.90	26.10	59.20	40.80	683
COIII	32.30	28.60	13.30	25.90	58.20	41.80	784
ND3	29.70	30.60	11.70	28.00	57.70	42.30	343
ND4L	31.00	31.30	10.80	26.90	57.90	42.10	297
ND4	34.90	28.80	11.10	25.30	60.10	39.90	1365
ND5	36.90	29.30	10.10	23.70	60.60	39.40	1797
Cytb	29.80	29.10	12.80	28.20	58.00	42.00	1144
ND6	13.30	10.40	32.30	44.00	57.30	42.70	520
Average[1]	33.28	29.33	12.09	25.31	58.58	41.42	901.00

[1]Average are given for 12 genes (ND6 excluded).

Table 15 Nucleotide composition (% of total number) for protein-coding genes found in the mitochondrial genome of *L. boehmei*.

Gene	Nucleotide, all positions						Total number
	A	C	G	T	AT	CG	
ND1	34.60	28.60	11.90	24.90	59.50	40.50	975
ND2	37.20	30.90	9.80	22.20	59.30	40.70	1033
COI	30.90	26.20	16.30	26.70	57.60	42.40	1548
COII	33.60	28.90	15.10	22.30	55.90	44.10	681
ATPase8	43.20	25.90	8.60	22.20	65.40	34.60	162
ATPase6	32.20	32.80	10.50	24.50	56.70	43.30	683
COIII	33.80	27.00	13.50	25.60	59.40	40.60	784
ND3	32.40	30.60	10.80	26.20	58.60	41.40	343
ND4L	32.70	32.00	9.80	25.60	58.20	41.80	297
ND4	35.70	29.00	10.80	24.50	60.20	39.80	1365
ND5	36.40	28.50	11.10	23.90	60.40	39.60	1797
Cytb	32.00	28.40	11.80	27.80	59.80	40.20	1135
ND6	11.60	9.10	32.80	46.50	58.10	41.90	516
Average[1]	34.21	28.78	12.13	24.88	59.09	40.91	900.25

[1]Average are given for 12 genes (ND6 excluded).

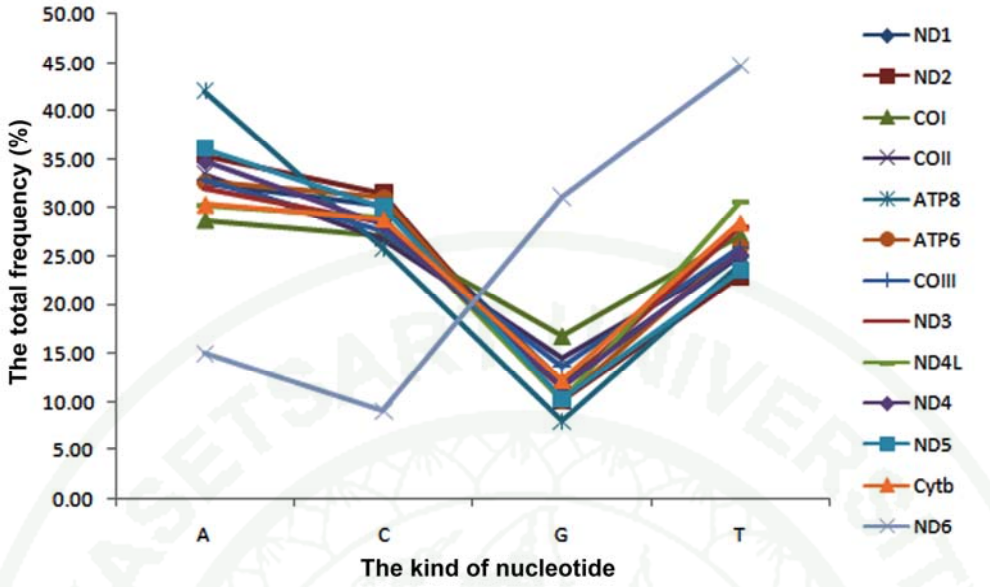

Figure 38 The relationship between the total frequency (%) and the kind of nucleotide in *L. reevesii rubritaeniata*.

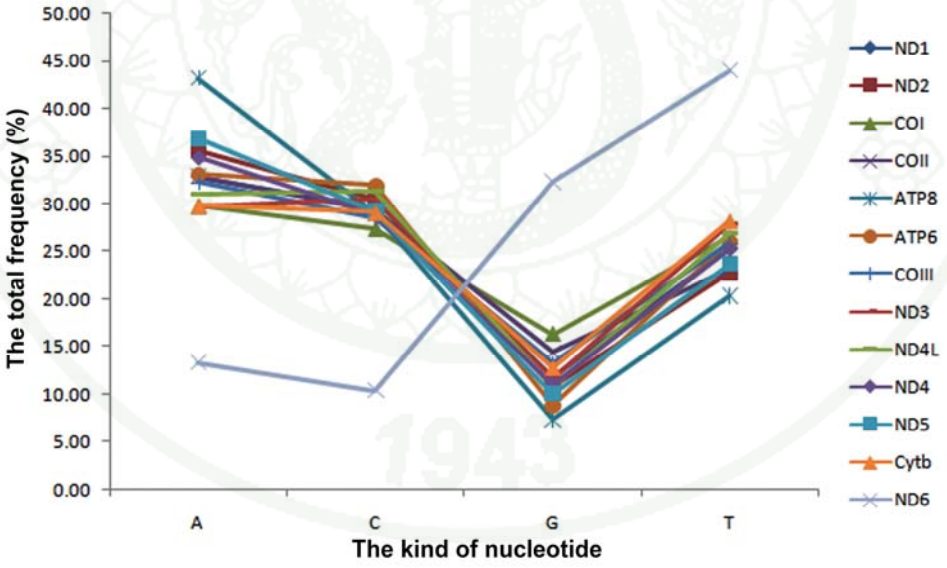

Figure 39 The relationship between the total frequency (%) and the kind of nucleotide in *L. belliana belliana*.

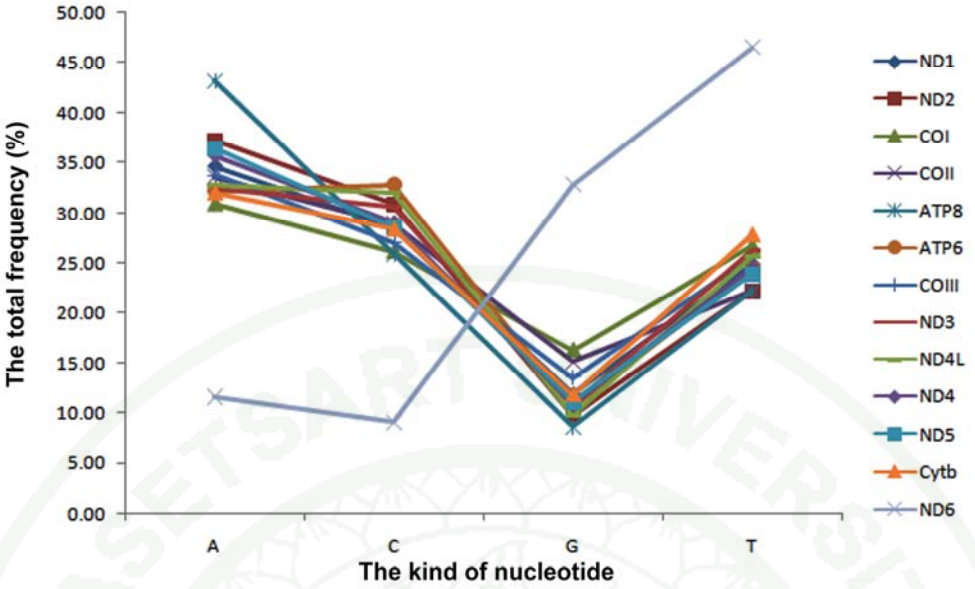

Figure 40 The relationship between the total frequency (%) and the kind of nucleotide in *L. boehmei*.

Table 16 Nucleotide composition (% of total number) for protein-coding genes, tRNA, rRNA and the control region found in the mitochondrial genome of *L. reevesii rubritaeniata*.

Gene, codon position	Nucleotide frequency						Total number
	A	C	G	T	AT	CG	
Protein-coding[1]							
1	34.24	26.24	19.61	19.91	54.15	45.85	3601
2	19.77	29.22	11.57	39.45	59.22	40.78	3597
3	45.27	31.62	5.51	17.60	62.84	37.16	3596
Total	33.09	29.03	12.23	25.65	58.75	41.25	10794
tRNAs	34.70	24.30	15.60	25.30	60.00	40.00	1508
rRNAs	37.50	25.40	17.40	19.70	57.20	42.80	2409
control region	33.50	25.50	11.40	29.60	63.10	36.90	815

[1]Combined data for 12 protein-coding genes

Table 17 Nucleotide composition (% of total number) for protein-coding genes, tRNA, rRNA and the control region found in the mitochondrial genome of *L. belliana belliana*.

Gene, codon position	Nucleotide frequency						Total number
	A	C	G	T	AT	CG	
Protein-coding[1]							
1	34.07	26.17	19.71	20.04	54.11	45.89	3607
2	20.01	29.36	11.77	38.86	58.87	41.13	3603
3	45.75	32.45	4.78	17.02	62.77	37.23	3602
Total	33.28	29.33	12.09	25.31	58.58	41.42	10812
tRNAs	35.00	24.30	15.90	24.80	59.80	40.20	1507
rRNAs	37.70	25.40	17.50	19.40	57.10	42.90	2466
control region	32.80	25.00	11.60	30.50	63.30	36.70	807

[1]Combined data for 12 protein-coding genes

Table 18 Nucleotide composition (% of total number) for protein-coding genes, tRNA, rRNA and the control region found in the mitochondrial genome of *L. boehmei*.

Gene, codon position	Nucleotide frequency						Total number
	A	C	G	T	AT	CG	
Protein-coding[1]							
1	34.57	26.00	19.84	19.59	54.16	45.84	3604
2	20.25	29.00	11.42	39.33	59.58	40.42	3600
3	47.82	31.34	5.11	15.73	63.55	36.45	3599
Total	34.21	28.78	12.13	24.88	59.09	40.91	10803
tRNAs	35.30	24.50	15.30	24.90	60.20	39.80	1509
rRNAs	37.50	25.90	17.70	19.00	56.50	43.50	2436
control region	34.60	24.80	11.90	28.70	63.20	36.80	813

[1]Combined data for 12 protein-coding genes

The nucleotides of mitochondrial genome were not randomly distributed, and such nucleotide bias was often related to the unequal of synonymous codons as usual. The vertebrate mitochondrial genome was influenced toward nucleotide A and T. The A-T content of protein-coding genes on H-strand was 58.75%, 58.58% and 59.09% for the three butterfly lizards, respectively. Similarly, the protein-coding genes on L-strand consisted of A-T composition 59.70%, 57.30% and 58.10% for the three butterfly lizards, respectively (Table 19, Appendix Figures 1-15, Appendix Tables 5-7). This affirmative action in nucleotide composition toward A-T affected both the codon usage pattern and amino acid composition of proteins. In these three *Leiolepis* species mitochondrial genomes, the most frequently used codon was CTA (Leu). On the other hand, the least utilized codon was TCG (Ser) for *L. reevesii rubritaeniata*, CCG (Pro) for *L. belliana belliana*, and CCG (Pro) and CGG (Arg) for *L. boehmei*. For the third codon position, nucleotide A was the most often used, and C was the least frequently exert as the relative order of nucleotide composition: A > C > T > G, excluding the amino acid of glycine (A > C > G > T), which was not discrepancy with the relative order of nucleotide composition of the mitochondrial genome. These findings reinforced the deduction that the favorite nucleotide usage at the third codon position of protein-coding genes deliberated the overall nucleotide content of *Leiolepis* sp. mitochondrial genome.

There were six AT-rich codons (with AT, TT and AA in a triplet) which represented amino acid Phe, Ile, Met, Tyr, Asn and Lys, whereas four GC-rich codons represented amino acid Pro, Ala, Arg and Gly. Specifically, the protein-coding genes of the three butterfly lizard mitochondrial genomes were biased toward using amino acids encoded by nucleotide A-, C- and T-rich codons. A-rich codons (with ≥ 2 As in a triplet) composed of Glu (1.96 % GAA), Lys (0.26 % AAG and 2.58 % AAA), Met (5.56 % ATA), Asn (1.24 % AAT and 3.05 % AAC), Gln (2.59 % CAA) and Thr (4.57 % ACA), and account for 21.82 % of the total amino acid composition (Table 19). C- and T-rich codons represented 18.13 % and 17.01 % of the total amino acid composition, respectively. By contrast, the frequency of G-rich codons was 6.95 %. This codon bias against G was even more evident when only the third codon positions were contemplated in both four and two fold degenerate codon families. When the

AT-rich group was juxtaposed, the proportion was always decreased if the third position was substituted by G such as Met (5.56% ATA and 1.03 % ATG). These results lead us to predict that the third codon positions mostly reflect mutational bias against G. In nematode, the three *Toxocara* spp. mitochondrial genomes were clarified that the mutational influence was commonly opposed by C at the third codon positions (Li *et al*., 2008). The greater translational efficiency has also been considered to be a potential cause underlying observed codon usage bias (Ikemura, 1982).

Table 19 Codon pattern composition (% of total number) for all protein-coding genes found in the mitochondrial genome of the three butterfly lizards.

Amino acid	Codon	Codon composition in total LRE	Codon composition in total LBE	Codon composition in total LBO
Ala	GCG	0.21	0.29	0.29
Ala	GCA	3.07	3.20	2.96
Ala	GCT	1.28	0.88	1.07
Ala	GCC	2.83	3.01	3.25
Cys	TGT	0.29	0.48	0.40
Cys	TGC	0.56	0.45	0.45
Asp	GAT	0.40	0.43	0.48
Asp	GAC	1.20	1.31	1.15
Glu	GAG	0.35	0.37	0.43
Glu	GAA	1.92	2.00	1.97
Phe	TTT	2.51	2.42	2.32
Phe	TTC	2.67	2.50	2.96
Gly	GGG	0.91	0.83	0.91
Gly	GGA	2.64	2.69	3.04
Gly	GGT	0.67	0.61	0.56
Gly	GGC	1.25	1.28	1.01
His	CAT	1.01	0.96	0.69

Table 19 (Continued)

Amino acid	Codon	Codon composition in total LRE	Codon composition in total LBE	Codon composition in total LBO
His	CAC	1.71	1.70	2.19
Ile	ATT	3.66	3.52	3.12
Ile	ATC	4.46	4.79	4.77
Lys	AAG	0.27	0.19	0.32
Lys	AAA	2.59	2.58	2.56
Leu	TTG	0.61	0.59	0.61
Leu	TTA	2.96	2.72	2.64
Leu	CTG	1.23	0.93	1.01
Leu	CTA	7.04	7.35	7.39
Leu	CTT	1.89	1.92	1.68
Leu	CTC	2.13	2.29	2.16
Met	ATG	1.17	0.75	1.17
Met	ATA	5.42	5.51	5.76
Asn	AAT	1.25	1.20	1.28
Asn	AAC	2.94	3.01	3.20
Pro	CCG	0.32	0.08	0.11
Pro	CCA	3.31	3.28	3.92
Pro	CCT	0.69	0.96	0.72
Pro	CCC	1.52	1.33	0.91
Gln	CAG	0.21	0.29	0.13
Gln	CAA	2.62	2.61	2.56
Arg	CGG	0.13	0.11	0.11
Arg	CGA	1.07	1.20	1.20
Arg	CGT	0.29	0.32	0.21
Arg	CGC	0.29	0.37	0.27
Ser	AGT	0.51	0.35	0.32
Ser	AGC	0.88	0.96	0.83

Table 19 (Continued)

Amino acid	Codon	Codon composition in total LRE	Codon composition in total LBE	Codon composition in total LBO
Ser	TCG	0.08	0.21	0.13
Ser	TCA	2.88	2.80	3.15
Ser	TCT	1.07	1.17	0.75
Ser	TCC	1.47	1.39	1.33
Thr	ACG	0.16	0.16	0.13
Thr	ACA	4.54	4.31	4.85
Thr	ACT	1.73	1.60	1.81
Thr	ACC	3.60	4.02	3.23
Val	GTG	0.37	0.56	0.27
Val	GTA	1.71	1.57	1.89
Val	GTT	0.77	0.69	0.99
Val	GTC	1.12	0.96	0.80
Trp	TGG	0.27	0.35	0.35
Trp	TGA	2.35	2.50	2.32
Tyr	TAT	0.99	1.01	1.12
Tyr	TAC	1.95	2.10	1.79

[1] LRE: *L. reevesii rubritaeniata*.

[2] LBE: *L. belliana belliana*.

[3] LBO: *L. boehmei*.

9.3 Ribosomal RNA genes

Two ribosomal RNA (12S rRNA and 16S rRNA) genes were encoded in the three butterfly lizards. 12S rRNA and 16S rRNA genes were located between $tRNA^{Phe}$ and $tRNA^{Leu}$ and separated by $tRNA^{Val}$. They were 862 and 1,547 bp for *L. reevesii rubritaeniata*, 919 and 1,547 bp for *L. belliana belliana*, and 869 and 1,567 bp for *L. boehmei*. Interestingly, the acceptable distinction in length of 12S rRNA gene was lavished by the sequence alignment among the three butterfly lizards and acrodont lizard species. The results showed that approximately 47 bp were removed in that of *L. reevesii rubritaeniata* and *L. boehmei* (Figure 41). In order to examine the appearance of the deletion in 12S rRNA gene for *L. reevesii rubritaeniata* and *L. boehmei*, the PCR amplifications were conducted using the primer LCRf (5'-CATG CATGAA CATTAAGCACC-3') and primer L12Sr4 (5'-GTTTTACATGTCGGTGTT GGCTG-3') which encompassed a region between control region and 12S rRNA gene (Figure 42). The PCR products were about 1,000 - 1,100 bp (Figure 43). The amplified products for *L. reevesii rubritaeniata* and *L. boehmei*; however, appeared to be smaller than those of *L. belliana belliana*. Additionally, the amplicons of the three butterfly lizards were sequenced to authenticate that the deletion existed in 12S rRNA genes in *L. reevesii rubritaeniata* and *L. boehmei*. These results indicated that the deletion in 12S rRNA gene might occur in the lineage of *Leiolepis* sp. before the divergence of *L. reevesii rubritaeniata* and *L. boehmei*. Further studies are necessary for more *Leiolepis* species to elucidate deletion character and evolution. Moreover, the nucleotide contents of the three butterfly lizard ribosomal RNA genes, where A > C > T > G were similar with the protein-coding genes on H-strand (Tables 16-18).

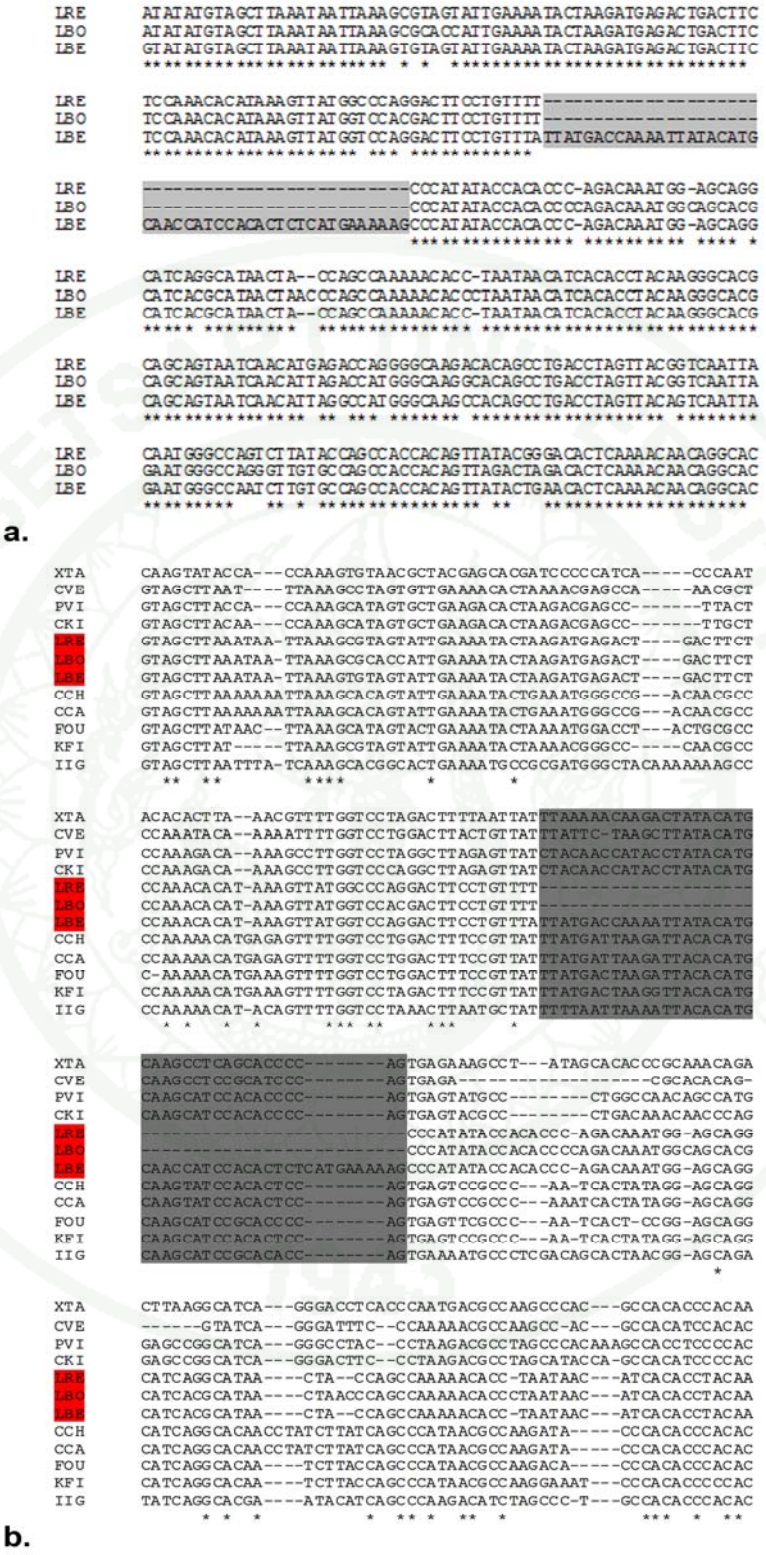

Figure 41 Nucleotide sequence alignment of the region $tRNA^{Phe}$-12S rRNA gene of the three butterfly lizards (a) and acrodont lizard species (b).

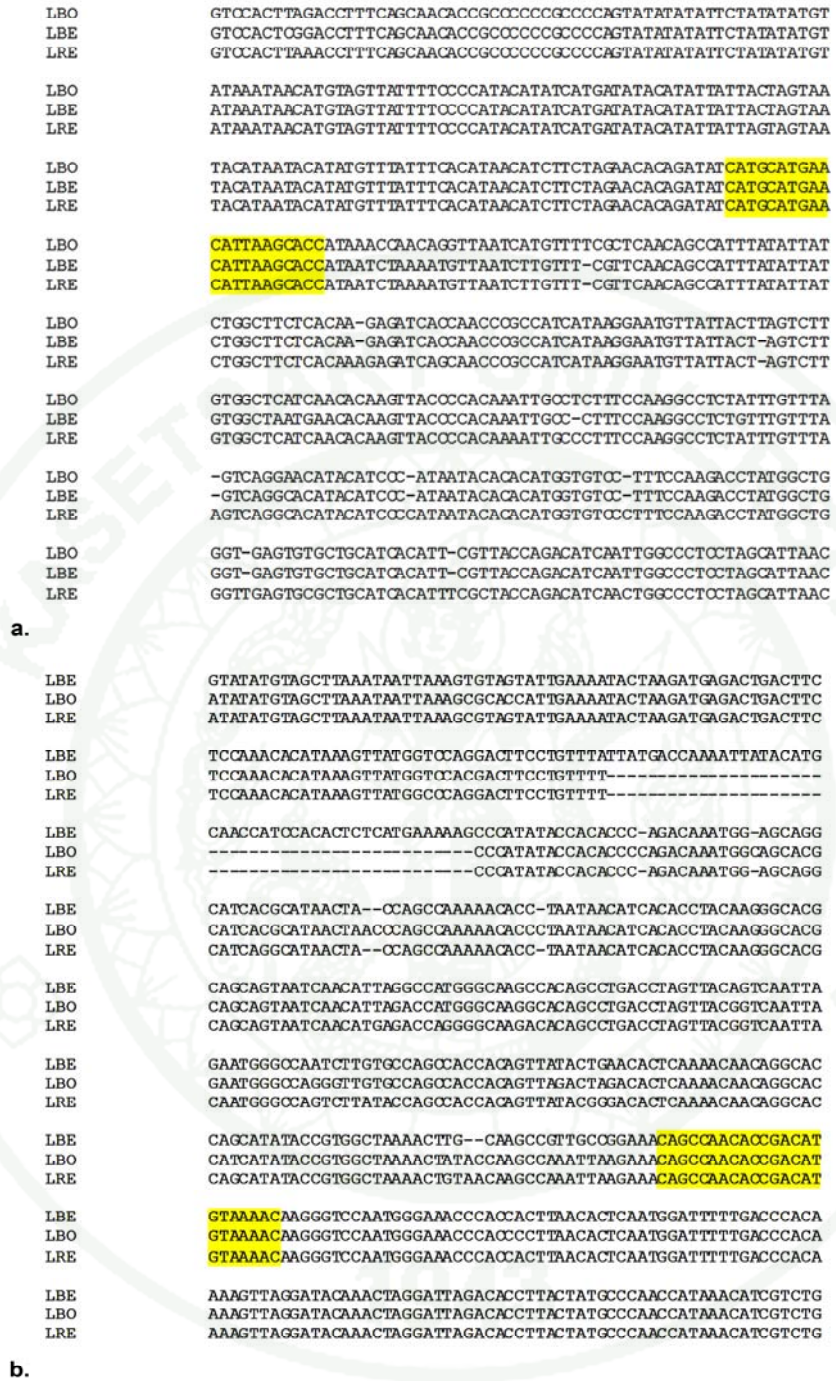

Figure 42 Nucleotide sequence alignment of the region $tRNA^{Phe}$-12S rRNA gene of the three butterfly lizards and the position of primer LCRf (a) and L12Sr4 (b).

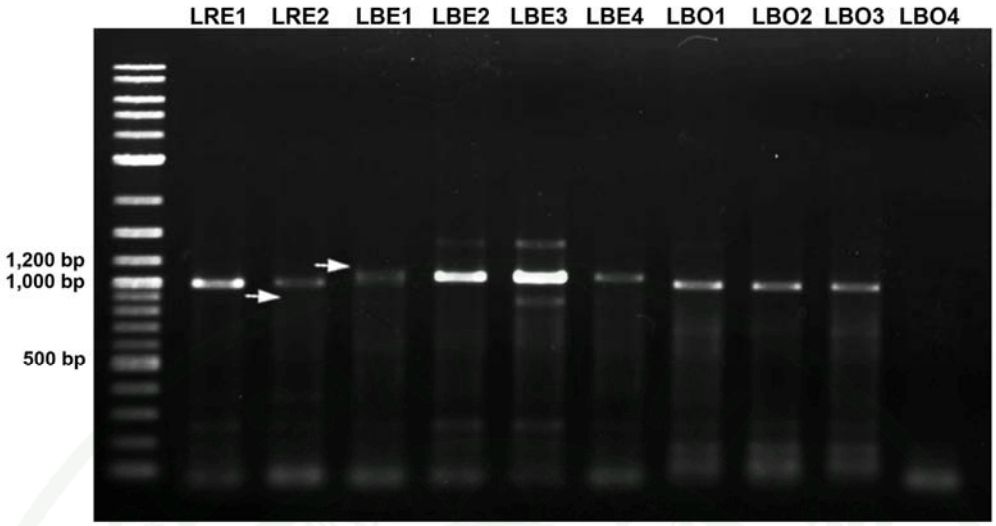

Figure 43 A PCR-mediated assay in *L. reevesii rubritaeniata* (LRE), *L. belliana belliana* (LBE) and *L. boehmei* (LBO) for examing the existence of the deletion in 12S rRNA genes. The PCR amplification could not perform in LBO4. Arrows indicate the distinctive DNA band in size.

9.4 Transfer RNA genes

Twenty-two tRNA sequences ranging from 57-74 bp were determined in the three butterfly lizard mitochondrial genomes (Tables 10-12). 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. The putative clover-leaf secondary structures in which G-U wobble pairing and occasional mismatches allowed were comparable to other squamate tRNAs. Generally, the secondary structure of tRNA constituted the highest variability in its DHU and TΨC loops, and being more conservative in the anticodon and acceptor stems. However, the $tRNA^{Cys}$ gene of the three butterfly lizards emerged to lack the DHU stem, which were also found in many other acrodont lizards as an exclusive feature (Macey *et al*., 2000). Likewise, the $tRNA^{Ser}$ (AGY) of the three butterfly lizards were appeared to be lost the DHU stem. This feature has been found in *Crocodylus siamensis* as well (Ji *et al*., 2008; Srikulnath *et al*., unpublished data).

9.5 Non-coding control region

The major non-coding control region of the three butterfly lizard mitochondrial genomes was located between $tRNA^{Pro}$ and $tRNA^{Phe}$. Their sizes were 815 bp, 807 bp and 813 bp, containing 63.10%, 63.30% and 63.20% as A-T content for *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*, respectively (Tables 16-18). Thus, they appeared to be the AT-rich region. However, the control region relative order of nucleotide contents that were A > T > C > G were incomparable to the average mitochondrial genome content, suggesting that the assortment of nucleotide content was altered by the simple sequence repeats (ATATATATATT CTATATATG TATAAA and CAATTTTTTCAAAAAAATCAACTC) which have been identified. In mammals, the control region was characterized to be the origin of H-stranded replication and initiation of both heavy- and light-stranded transcriptions, and conserved sequence blocks (CSBs) I-III were identified as conserved sequence element in the control region of mitochondrial genome (Clayton, 1992). Nevertheless, only CSBI motif was found in the three butterfly lizard control regions. Contrastingly, CSBI-III have been revealed in some other lacertilian species for instance *P. vitticepes*, *Lacerta viridis viridis* mitochondrial genome (Amer and Kumazawa, 2005; B□hme *et al*., 2007). Hence, uncommon CSB in control region might occur in the lineage of genus *Leiolepis*. Further studies are necessary for more *Leiolepis* and *Uromastyx* species as the same subfamily to explicate the CSB and other features in the control region.

9.6 General properties of sequences of concatenate protein coding gene

The concatenate protein coding gene data set were used to determine the genetic relationship and phylogenetic position of *Leiolepis* spp. in Squamata (Table 20). The concatenate protein coding gene data set included 10,381 aligned nucleotide positions consisting of 7,767 variable sites and 7,134 parsimony informative sites, which contained 32.76%, 34.12% and 33.12% for the first, second and third codon position, respectively (Table 21). These results collectively suggested that all codon position were equally informative characters to determine the phylogenetic

relationship of concatenate protein coding gene data set. The substantially delusive effects of heterogeneous base composition among taxa in phylogenetic reconstruction were subsequently analyzed as individual and as all codon positions. The findings demonstrated that even though the nucleotide frequencies were statistically consequential proportions disparity for individual and all codon position, all topologies of all analyses of concatenate protein coding gene data set were correspondingly similar to those of the previous molecular phylogenetic tree of squamate species (Böhme *et al*., 2007; Kumazawa, 2007) (Table 22). However, the patterns of the total number of transitions + transversion against genetic distance for concatenate protein coding gene data set were consonantly similar in individual and in all codon positions. The regression lines were actually consistent with the straight lines, indicating that saturation of individual and all codon positions did not occur to cause a problem in concatenate protein coding gene sequences at the level of homoplasy, and that there was phylogenetic signal for all codon positions (Figure 44).

Table 20 Classification and accession numbers of species used in concatenate protein coding gene sequence analysis[1].

Class	Order	Suborder	Infraorder	Family	Species	Accession number
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis reevesii rubritaeniata*	AB537553
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis belliana belliana*	AB537554
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Leiolepis boehmei*	AB537555
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Pogona vitticepes*	AB166795
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Calotes versicolor*	AB183287
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Chlamydosaurus kingii*	EF090421
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	*Xenagama taylori*	DQ008215
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Polychrus marmoratus*	AB266749
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Chalarodon madagascariensis*	AB266748
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Leiocephalus personatus*	AB266739
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Gambelia wislizenii*	AB218884
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Basiliscus vittatus*	AB218883
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Oplurus grandidieri*	AB218720
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Anolis carolinensis*	EU747728
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Sceloporus occidentalis*	AB079242

Table 20 (Continued)

Class	Order	Suborder	Infraorder	Family	Species	Accession number
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Iguana iguana*	AJ278511
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	*Furcifer oustaleti*	AB185326
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	*Kinyongia fischeri*	EF222188
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	*Chamaeleo calcaricarens*	EF222195
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	*Chamaeleo chamaeleon*	EF222198
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	*Sceloporus occidentalis*	AB079242
Reptilia	Squamata	Lacertilia	Gekkota	Gekkonidae	*Heteronotia binoei*	EF626816
Reptilia	Squamata	Lacertilia	Gekkota	Gekkonidae	*Gekko gecko*	AY282753
Reptilia	Squamata	Lacertilia	Scincomorpha	Lacertidae	*Lacerta viridis viridis*	AM176577
Reptilia	Squamata	Lacertilia	Scincomorpha	Xantusiidae	*Lepidophyma flavimaculatum*	AB162908
Reptilia	Squamata	Lacertilia	Diploglossa	Anguidae	*Anguis fragilis*	EU443256
Reptilia	Squamata	Lacertilia	Diploglossa	Anguidae	*Abronia graminea*	AB080273
Reptilia	Squamata	Lacertilia	Platynota	Helodermatidae	*Heloderma suspectum*	AB167711
Reptilia	Squamata	Lacertilia	Platynota	Varanidae	*Varanus salvator*	EU747731
Reptilia	Squamata	Amphisbaenia		Bipedidae	*Bipes canaliculatus*	AY605482

Table 20 (Continued)

Class	Order	Suborder	Infraorder	Family	Species	Accession number
Reptilia	Squamata	Amphisbaenia		Amphisbaenidae	*Blanus cinereus*	EU443257
Reptilia	Squamata	Serpentes		Colubridae	*Elaphe poryphyracea*	GQ181130
Reptilia	Squamata	Serpentes		Elapidae	*Naja naja*	DQ343648
Reptilia	Testudines	Cryptodira		Trionychidae	*Pelodiscus sinensis*	AY687385
Reptilia	Crocodylia	Eusuchia		Crocodylidae	*Crocodylus Siamensis*	EF581859
Aves	Galliformes			Phasianidae	*Gallus gallus*	AP003580

[1]New sequences from our study indicated by *

Table 21 Properties of character variation for concatenate protein coding gene sequence data set.

Data set	All aligned sequence	Parsimony-informative sites	Variable sites	Nucleotide bias				χ^2	d.f.	*p*-value	Best model[1]	I^2	G^3
				%A	%C	%G	%T						
Total	10,381	7,134	7,767	31.77	29.29	12.57	26.36	1912.08	102	0.0000	GTR+I+G	0.1937	0.5557
1st position	3,462	2,337	2,537	31.43	29.61	13.11	25.85	605.95	102	0.0000			
2nd position	3,460	2,434	2,653	31.76	29.73	11.43	27.08	692.70	102	0.0000			
3rd position	3,459	2,363	2,577	32.13	28.53	13.18	26.16	707.15	102	0.0000			

[1]Best models were selected with Modeltest version 3.6 (Posada and Crandall, 1998).

[2]I : Proportion of invariable site

[3]G : Gamma shape parameter

Table 22 Comparison of the base contents within concatenate protein coding gene data set.

Taxonomic organism	Percentage of bases with presented concatenate protein coding gene data set.				
	A	**C**	**G**	**T**	**GC**
Leiolepis reevesii rubritaeniata	32.80	28.87	12.56	25.77	41.42
Leiolepis belliana belliana	33.01	29.26	12.35	25.39	41.61
Leiolepis boehmei	33.90	28.74	12.42	24.95	41.16
Pogona vitticepes	32.21	31.06	12.09	24.63	43.15
Calotes versicolor	31.53	28.61	12.72	27.14	41.33
Chlamydosaurus kingii	34.06	30.00	11.13	24.81	41.14
Xenagama taylori	34.39	28.18	11.79	25.64	39.97
Polychrus marmoratus	29.66	30.53	11.91	27.89	42.45
Chalarodon madagascariensis	31.53	27.97	12.59	27.91	40.56
Leiocephalus personatus	31.84	26.11	13.15	28.90	39.27
Gambelia wislizenii	31.65	29.01	12.73	26.61	41.74
Basiliscus vittatus	31.39	28.77	12.97	26.87	41.74
Oplurus grandidieri	33.72	26.29	11.95	28.04	38.25
Anolis carolinensis	29.85	25.58	14.09	30.48	39.67
Furcifer oustaleti	35.27	26.30	11.19	27.23	37.49
Iguana iguana	30.29	33.68	12.72	23.31	46.40
Kinyongia fischeri	32.48	30.29	12.35	24.89	42.64
Chamaeleo calcaricarens	34.92	27.18	11.65	26.26	38.82
Chamaeleo chamaeleon	35.49	26.73	11.22	26.57	37.94
Sceloporus occidentalis	31.40	29.44	13.61	25.55	43.05
Heteronotia binoei	30.65	32.33	12.40	24.63	44.73
Gekko gecko	31.01	28.40	12.89	27.71	41.29
Lacerta viridis viridis	29.78	28.28	12.53	29.41	40.80
Lepidophyma flavimaculatum	28.34	31.54	13.82	26.31	45.35
Anguis fragilis	28.34	31.67	14.38	25.60	46.06
Abronia graminea	31.74	27.54	12.55	28.17	40.09

Table 22 (Continued)

Taxonomic organism	Percentage of bases with presented concatenate protein coding gene data set.				
	A	**C**	**G**	**T**	**GC**
Heloderma suspectum	31.50	24.86	12.17	31.48	37.03
Varanus salvator	29.49	33.27	12.06	25.18	45.33
Bipes canaliculatus	29.09	33.89	14.81	22.22	48.69
Blanus cinereus	31.19	31.20	13.00	24.62	44.20
Elaphe poryphyracea	33.77	28.69	12.11	25.43	40.80
Naja naja	32.57	29.30	12.58	25.55	41.88
Pelodiscus sinensis	34.42	26.18	11.03	28.37	37.21
Crocodylus Siamensis	29.87	30.77	14.10	25.26	44.87
Gallus gallus	28.93	34.66	12.46	23.95	47.12

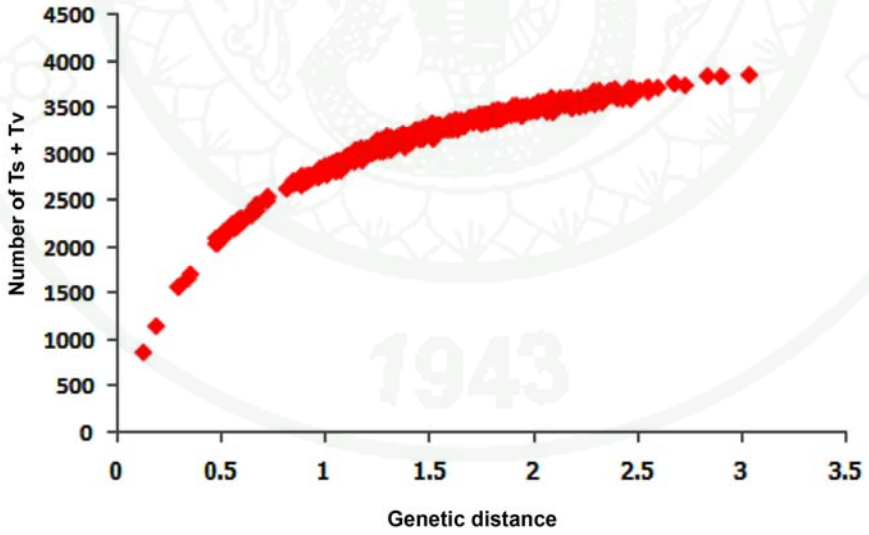

Figure 44 The relationship between the total number of transitions (Ts) + transversions (Tv) and corrected distance for all pairwise comparisons in concatenate protein coding gene sequence data set.

9.7 Phylogenetic analyses of concatenate protein coding sequence

The phylogenetic analyses of concatenate protein coding gene were reconstructed using BI, ML, MP and NJ. The phylogram showed some currently available squamate complete mitochondrial genomes and that of out group, *Crocodylus siamensis*, *Pelodiscus sinensis* and *Gallus gallus* without *Sphenodon punctatus* because of the missing information for ND5 gene in Genbank. Squamata, however, was distinctly present as monophyletic group. The phylogenetic pattern within the basal splits was different among several analysis methods. By contrast, all methods illustrated a high agreement concerning the relationships within the infraorders and families of Squamata. Nevertheless, The unusual sister group of Acrodonta and Serpentes was strongly sustained with support value of 100% in all analysis method which were different from the phylogram of combined *RAG1*/C-*mos* data set as mentioned above (Figures 45-48). This topology was also comparable to the previous molecular phylogenetic tree which was reconstructed by mitochondrial gene (Townsend *et al*., 2004; Böhme *et al*., 2007; Albert *et al*., 2009), suggesting that both acrodont lizards and snake exhibited relatively long branches in mitochondrial-based phylogenies, and their sister group relationship could be due to a long branch attraction artifact which might affect the main drawback to accurately reconstruct phylogenetic tree (Felsenstein, 1978; Townsend *et al*., 2004). Therefore, it would be desirable to find snake and acrodont lizards exhibiting slower mitochondrial evolutionary rates for incorporating them into the analysis (Albert *et al*., 2009). Furthermore, Gekkota was grouped with Amphisbaenia as a sister group which was similar to the previous molecular phylogenetic tree using mitochondrial nucleotide sequence (Zhou *et al*., 2006). However, it was contradicted with the position of Gekkota which was the basal position of squamate reptile for the combined *RAG1*/C-*mos* phylogram as mentioned above and other molecular phylogenetic tree (Townsend *et al*., 2004; Böhme *et al*., 2007), or Amphisbaenia was grouped with Acrodonta + Serpentes (Douglas *et al*., 2006). Moreover, Albert *et al*. (2009) asserted that the non-parametric tests approximately unbiased (AU) and Kishino-Hasegawa (KH) speculated that the alternative hypotheses placing Amphisbaenia as a sister group of either Gekkota or Acrodonta + Serpentes, which were not significantly different from

that of the unconstrained hypothesis. The phylogentic topology was also differed from taxon sampling as the relative effect.

In Acrodonta, the phylogram of all analysis methods clearly showed that Agaminae was grouped with Leiolepidinae as family Agamidae, and Agamidae was positioned to a sister group with Chamaeleonidae. However, the missing information of complete mitochondrial sequence of *Uromastyx* sp. might affect the cladistic analysis. Such phylogenetic relationships of the acrodont lizards have been reported on several molecular phylogram which included *Uromastyx* sp. in analysis (Townsend *et al*., 2004; Schulte and Cartwright, 2009). Our *RAG1*, C-*mos* and combined *RAG1*/C-*mos* phylogram showed varied topologies in Acrodonta as well. Nevertheless, the complete mitochondrial genome organization showed the various pattern of gene rearrangement of acrodont lizards that were consistent with concatenate protein coding gene phylogram. Thus, complete mitochondrial genome of *Uromastyx* sp. is necessarily further studied to examine the relationship of Acrodonta. For the clade of *Leiolepis* spp., all method of statistical analyses strongly supported (100%) that *L. reevesii rubritaeniata* was more adjacent to *L. belliana belliana* than *L. boehmei*. This finding was agreeable with *RAG1* and combined *RAG1*/C-*mos* data set, and *GAPD* and combined *α-Enol* / *GAPD* data set as molecular phylogenetic analyses, and was also consistent with morphological and cytogenetic information (Aranyavalai, 2003; Aranyavalai *et al*., 2004; Srikulnath *et al*., 2009 and unpublished data). Accordingly, the occurrences of sequence deletion in 12S rRNA gene lead us to predict that *L. belliana belliana* might be the most primitive followed by *L. reevesii rubritaeniata* while *L. boehmei* is likely the most recent. Other molecular and morphological studies with additional taxa in genus *Leiolepis* are also needed to clarify the phylogenetic relationship and evolution.

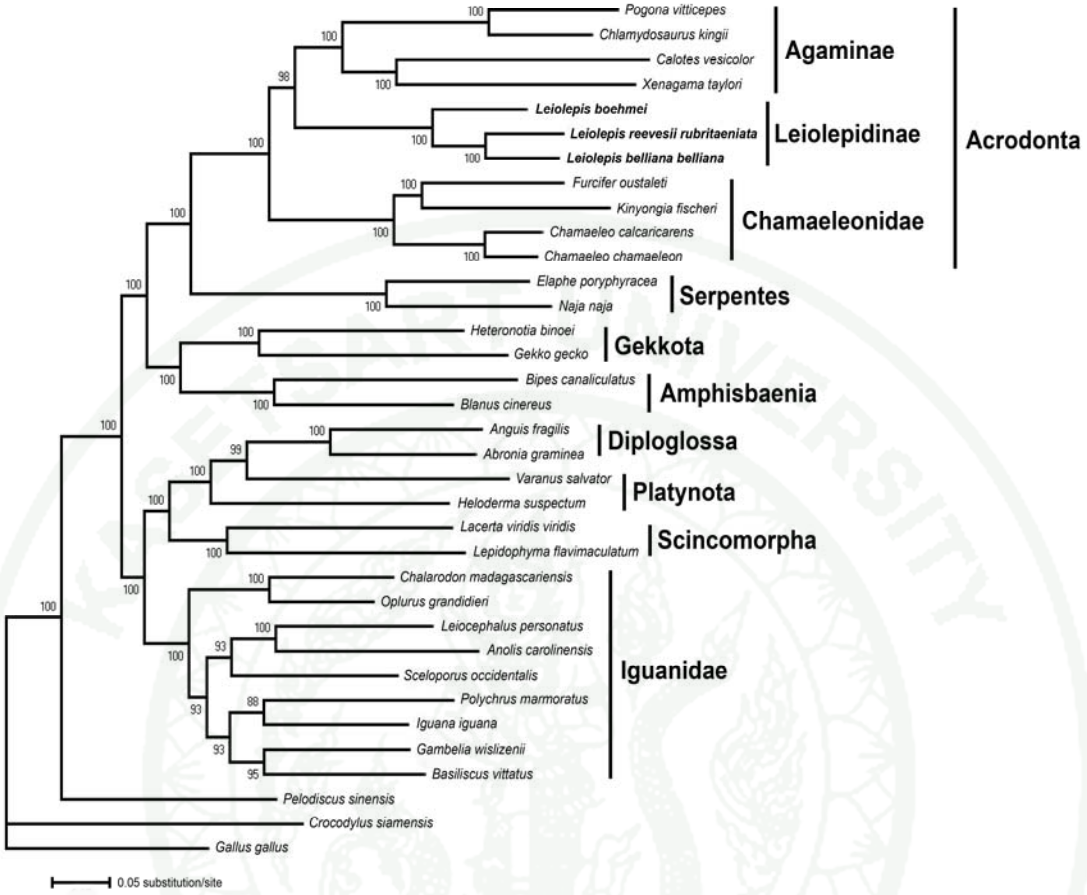

Figure 45 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the concatenate protein coding gene sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Baysian inference based on General time-reversible, AIC model was shown. Branch lengths were mean estimates. The posterior probability values were shown on the corresponding branches when ≥ 50%.

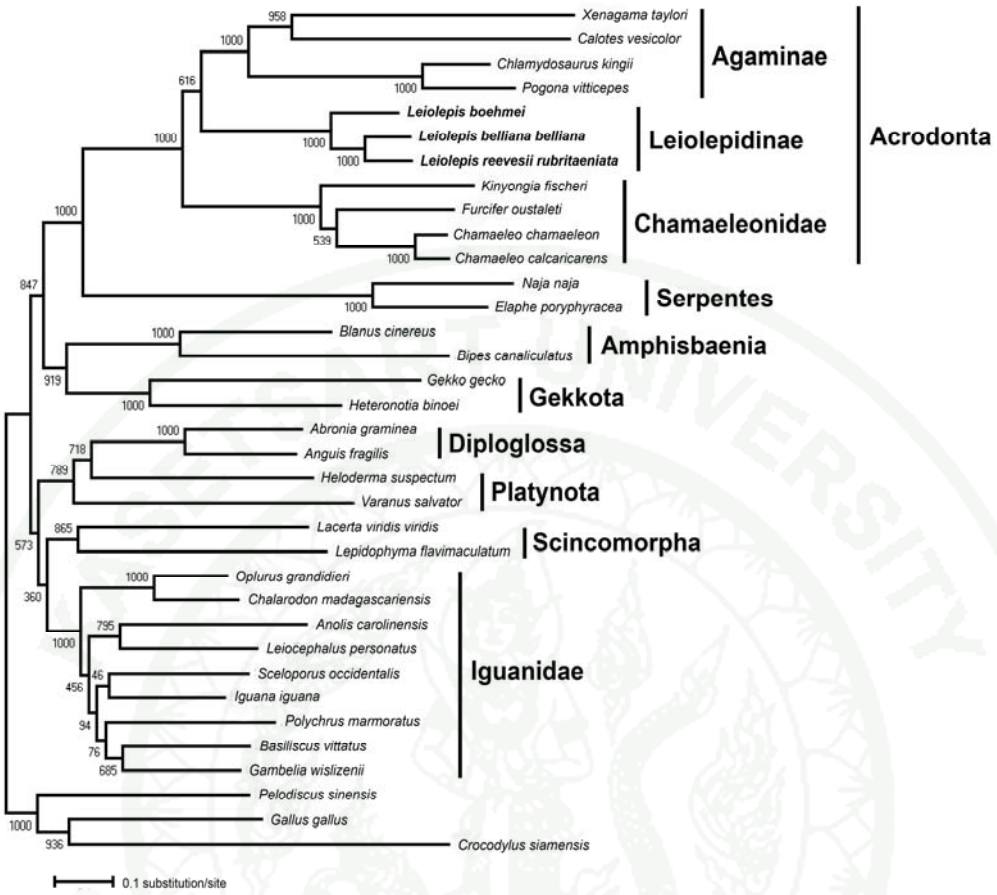

Figure 46 An ML phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the concatenate protein coding gene sequence data set based on General time-reversible, AIC model. Branch lengths were mean estimates. The bootstrap values were shown on the corresponding branches.

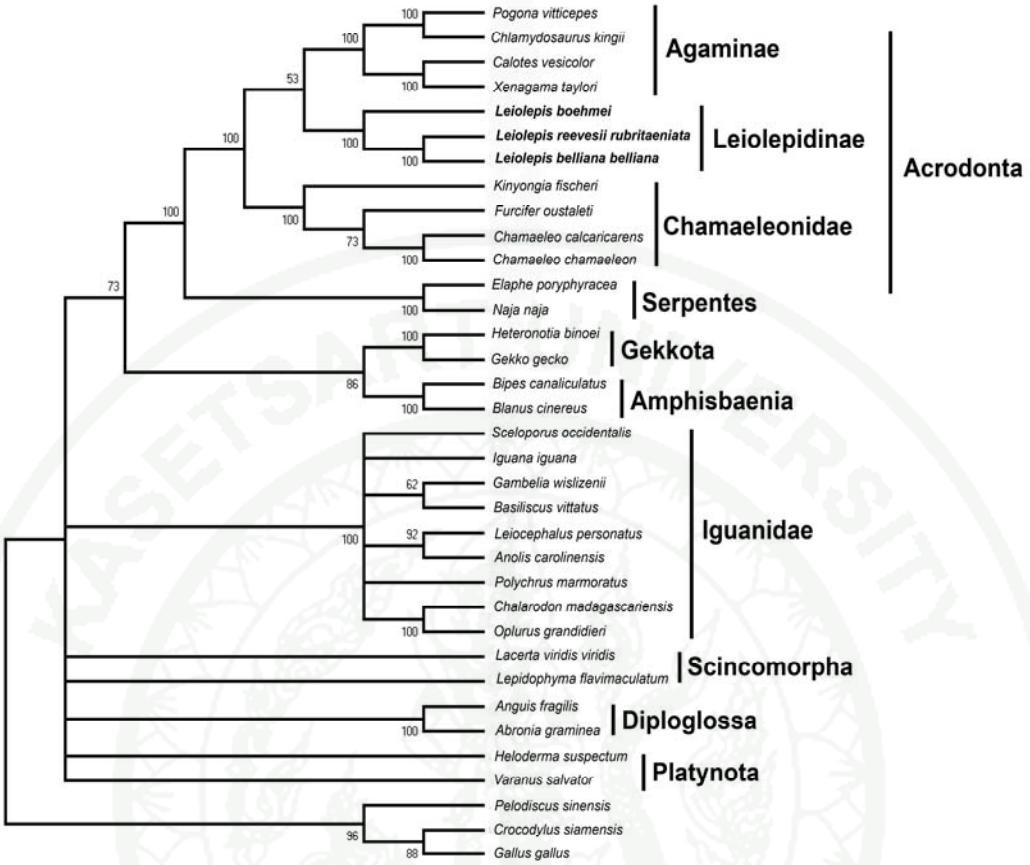

Figure 47 An MP cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the concatenate protein coding gene sequence data set. The bootstrap values were shown on the corresponding branches.

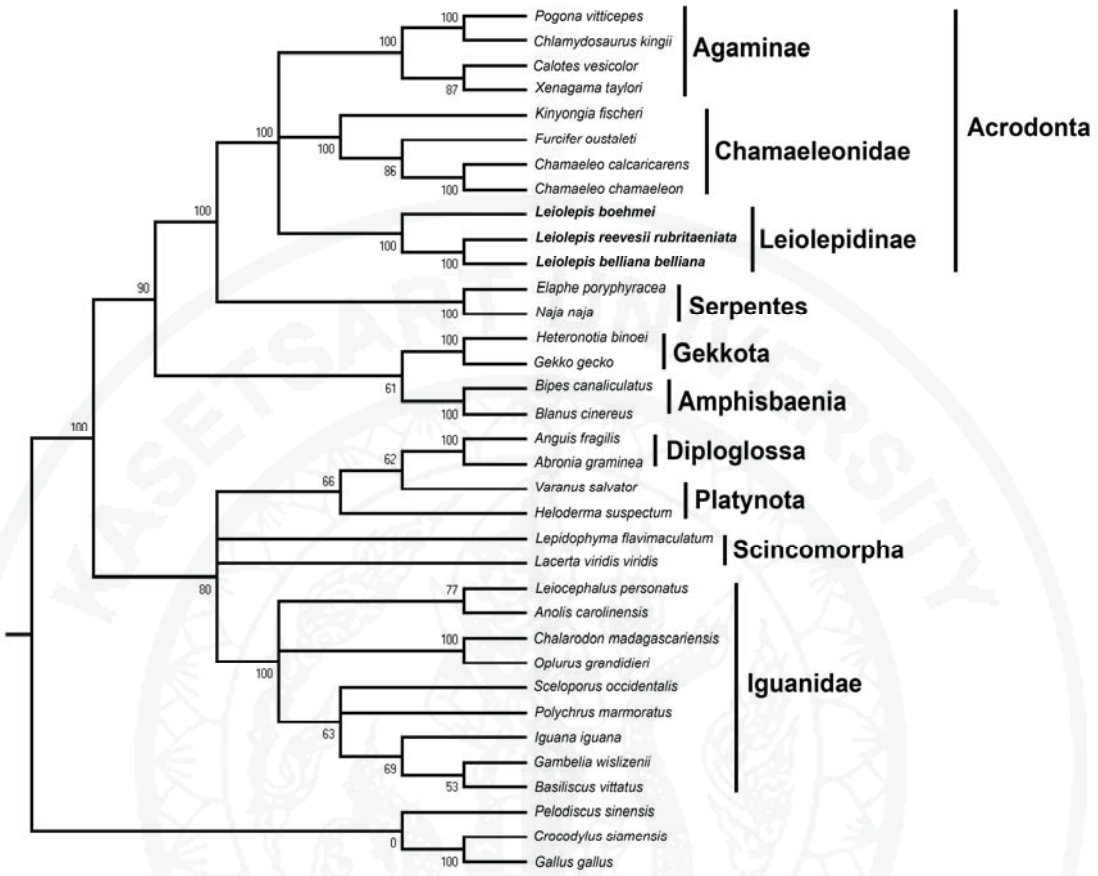

Figure 48 An NJ cladogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of Leiolepidinae and other Agaminae species constructed using the the concatenate protein coding gene sequence data set based on General time-reversible, AIC model. The bootstrap values were shown on the corresponding branches.

10. DNA marker from the butterfly lizard mitochondrial genome for *Leiolepis* species discriminating

Four efficiently discriminated DNA markers were designed from the three complete mitochondrial genomes of butterfly lizards to identify *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*. The first DNA marker was designed from 16S rRNA and *ND1* gene. At the PCR condition, semi-duplex PCR comprising primer BL16Sf, BLnd1r1 and BLnd1r2 was obtained (Figure 49). The species-spcecific amplicons of approximate 650 and 750 bp could be observed only in *L. belliana belliana* and *L. boehmei*, respectively. The second DNA marker amplified from the mitochondrial DNA compassed *ND2* and *COI* genes. PCR amplifications were conducted using three primers LeiolepisND2f, BLND2COIr1 and BLND2COIr2. Approximately 1,200 and 1,000 bp of PCR product were found from *L. reevesii rubritaeniata* and *L. boehmei*, respectively (Figure 50). The distinctive sites of the *COI* gene sequence among the three butterfly lizards were selected to construct the third DNA marker. The PCR reactions of COI primers containing BLCOIf, BLCOIr1 and BLCOIr2, were carried out. The PCR product sizes of *L. belliana belliana* and *L. boehmei* were about 300 and 800 bp, respectively (Figure 51). However, one specimen of *L. reevesii rubritaeniata* was observed with the fainted DNA bands having the same size as those of *L. belliana belliana*, suggesting that this primer set could not completely discriminate the DNA from *L. reevesii rubritaeniata* and *L. belliana belliana*.

One pair of primer, LeiolepisND5f-LeiolepisND5r, was conveyed to amplify in PCR conditions as the fourth DNA marker. Approximately 1.1 kb amplicons could be amplified from *L. reevesii rubritaeniata* and *L. boehmei* (Figure 52).

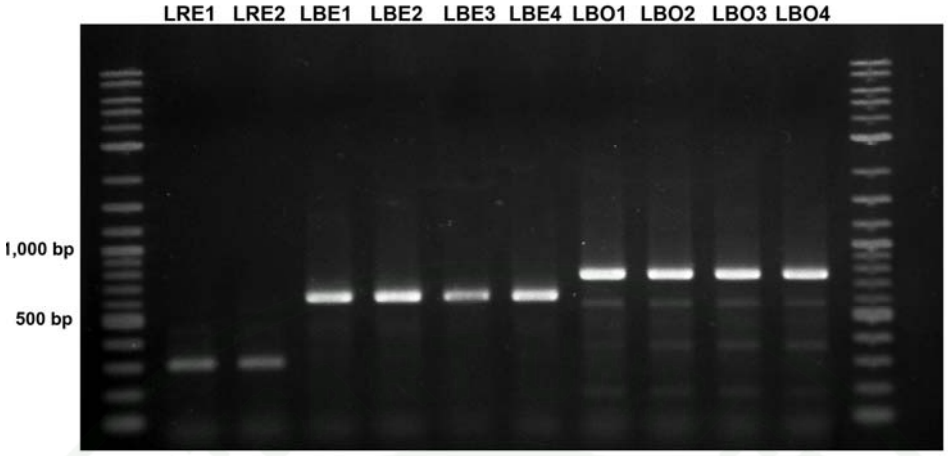

Figure 49 Agarose gel electrophoresis of semi-duplex PCR products using the primers BL16Sf, BLnd1r1 and BLnd1r2. *L. reevesii rubritaeniata* (LRE), *L. belliana belliana* (LBE) and *L. boehmei* (LBO).

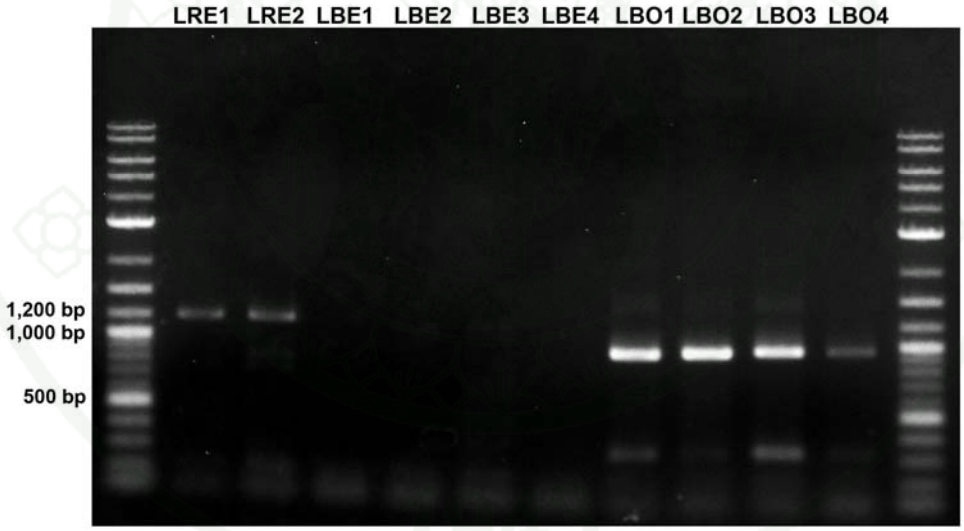

Figure 50 Agarose gel electrophoresis of semi-duplex PCR products using the primers LeiolepisND2f, BLND2COIr1 and BLND2COIr2. *L. reevesii rubritaeniata* (LRE), *L. belliana belliana* (LBE) and *L. boehmei* (LBO).

Figure 51 Agarose gel electrophoresis of semi-duplex PCR products using the primers BLCOIf, BLCOIr1 and BLCOIr2. *L. reevesii rubritaeniata* (LRE), *L. belliana belliana* (LBE) and *L. boehmei* (LBO).

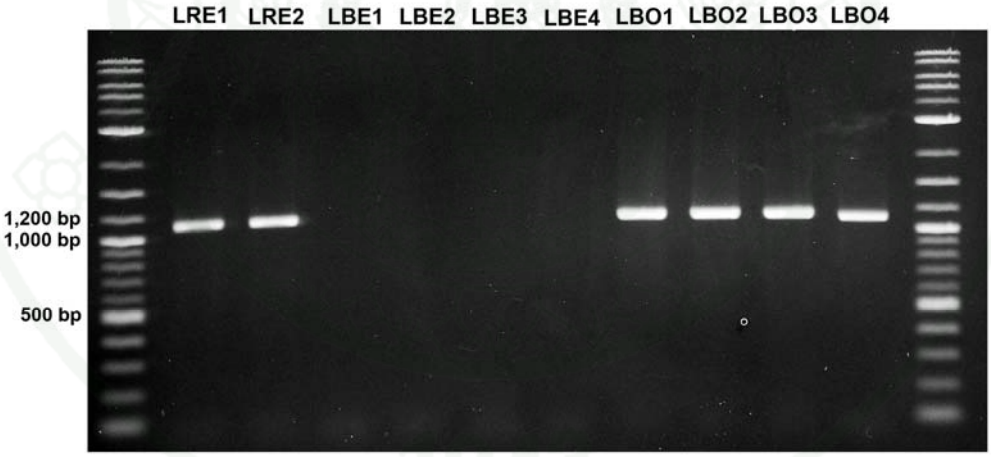

Figure 52 Agarose gel electrophoresis of PCR products using the primers LeiolepisND5f-LeiolepisND5r. *L. reevesii rubritaeniata* (LRE), *L. belliana belliana* (LBE) and *L. boehmei* (LBO).

CONCLUSION

1. Karyotypes in both male and female of *L. reevesii rubritaeniata* were composed of two distinct chromosomal components, macrochromosomes and microchromosomes: two pairs of large metacentric macrochromosomes (1st, 3rd), one pair of large submetacentric macrochromosomes (2nd), one pair of medium-sized metacentric macrochromosomes (4th), two pairs of small metacentric macrochromosomes (5th, 6th) and 12 pairs of microchromosomes (7th–18th) designated as 2n=2x=36 ($L_4^m + L_2^{sm} + M_2^m + S_4^m$ + 24 microchromosomes; NF=24 for macrochromosomes). Moreover, a large secondary constriction was also found in the subtelomeric region of the long arm of chromosome 1 in the karyotype of *L. reevesii rubritaeniata.*

2. CGH analysis failed to identify sex-specific region. No partial synapsis of differentiated chromosomal pair was observed at diakinesis-MI of primary spermatocytes, and there were no MII cells with condensed chromosomes either. Furthermore, heteromorphic sex chromosome could not be identified in *L. reevesii rubritaeniata*. These findings confirmed the deduction that this species might have a temperature sex determination (TSD) system or exhibit genetic sex determination (GSD) with morphologically undetectable cryptic sex chromosomes.

3. Homologues of six chicken Z-linked genes were all mapped to the short arm of *L. reevesii rubritaeniata* on chromosome 2 in the order of *ACO1*/*IREBP*-*RPS6*-*DMRT1*-*CHD1*-*GHR*-*ATP5A1* from the centromere to the distal end. This pattern was also identical with those of the *Pelodiscus sinensis* on chromosome 6, *Elaphe quadrivirgata* on chromosome 2p, *Gekko hokouensis* on Z chromosome, and also the ostrich on Z chromosome (*Struthio camelus*), suggesting that the conserved linkage homology of the genes has been highly maintained in reptiles and birds.

4. NORs and the 18S-28S rRNA genes were located at the secondary constriction of *L. reevesii rubritaeniata* on the long arm of chromosome 1. By contrast, the 18S-28S rRNA genes are generally located on a pair of

microchromosomes or chromosome 2 in other iguanian lizards. Therefore, cytogenetic studies should be performed on other lacertilian species to clarify the chromosomal locations of the 18S-28S rRNA genes in the ancestral karyotypes of Iguania and to find the process of their transposition to different chromosomes. Interestingly, the 5S rRNA genes were located in the pericentromeric region of the long arm of chromosome 6 in both male and female. This is the first report on the chromosomal location of the 5S rRNA genes in Squamata.

5. The location of 18S-28S rRNA and 5S rRNA genes was identified in the subtelomeric region of the long arm of chromosome 1 and the pericentromeric region of the long arm of chromosome 6 in *L. belliana belliana* and *L. boehmei*, indicating that the position of the major and minor ribosomal RNA genes might be the unique character of *Leiolepis* species in Iguania.

6. Fluorescence signals of $(TTAGGG)_{20}$ sequences were observed at telomeric ends of all chromosomes in the three species studied. The hybridization signals were weak on macrochromosomes; by contrast, high intensity of signals were observed on almost all microchromosomes, suggesting that the $(TTAGGG)_{20}$ sequences have been amplified site-specifically on microchromosomes. These features have not been reported in Squamata. In *L. reevesii rubritaeniata* and *L. boehmei*, interstitial telomeric sites (ITSs) were co-localized in the subtelomeric region of chromosome 1 with the 18S-28S rRNA genes, implying that a tandem fusion might have occurred between chromosome 1 and a microchromosome where the 18S-28S rRNA genes are located. However, ITSs was not found in that of *L. belliana belliana*, indicating that chromosomes might have very few copy number of (TTAGGG)*n* sequences, or there might be a gradual loss of the repeat sequences during chromosomal evolution.

7. The complete mitochondrial genome of the three butterfly lizards was determined. The sizes of the entire mitochondrial genome were 10,794 bp for *L. reevesii rubritaeniata* 10,812 bp for *L. belliana belliana* and 10,803 bp for *L. boehmei*. Twenty-two tRNA genes, two rRNA genes, thirteen protein-coding genes

and a control region were identified in three mitochondrial genomes of the three butterfly lizards. These relative position and orientation of all composition were similar to those of most vertebrate. The deletion of sequences approximately 47 bp in 12S rRNA gene has been revealed in *L. reevesii rubritaeniata* and *L. boehmei*. These results collectively suggested that the deletion in 12S rRNA gene might occur in the lineage of *Leiolepis* spp. before the divergence of *L. reevesii rubritaeniata* and *L. boehmei*.

8. Molecular sequence analyses comprising nuclear gene (*RAG1*, C-*mos*, *α-Enol* and *GAPD* genes) and concatenate 12 proteins coding mitochondrial gene revealed that *L. reevesii rubritaeniata* was more related to *L. belliana belliana* than *L. boehmei*. These results were also consistent with the morphological and chromosomal information of the butterfly lizard in Thailand. Comparing to the occurrence of the sequence deletion in 12S rRNA gene, *L. belliana belliana* might be the most primitive followed by *L. reevesii rubritaeniata* while *L. boehmei* is likely the most recent. However, the phylogenetic position among Leiolepidinae, Agaminae and Chamaelenidae remain uncertain, though there were additional taxa in Leiolepidinae in our analysis which was not in other previous study.

Further molecular cytogenetic characterization and comparative gene mapping are required for more butterfly lizard and lacertilian species to clarify the process of karyotypic evolution and the diversity of sex chromosomal origins in squamate reptiles. Additionally, sex determination system in butterfly are desired to speculate whether TSD system or GSD with morphologically undetectable cryptic sex chromosomes for deducing this properties in genus *Leiolepis*. Furthermore, complete mitochondrial genome analysis and nuclear gene molecular phylogeny for other species in Leiolepidinae is essential to identify the exclusive feature and reconstruct the phylogenetic relationship for all butterfly lizards and acrodont lizards.

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APPENDIX

Appendix Table 1 Degenerate oligonucleotide primers used for cloning cDNA fragments of the chicken Z-linked gene homologues.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ACO1/IREBP	GACAGYTTRCARAAGAATCARGAY	CCYTTRAATCCTTGCTTNGYTCC
	GTGCTCACYRTNACNAAGCACCT	AGGTCTCCCTGNGTDATNGCYTC
ATP5A1	GAARACTGGCACHGCWGARRTRTCCTC	GGCAATBGADGTTTTSCCMGTCTGYCTGTC
	CGYCTKCTGGARAGAGCAGCBAARATG	CTGKTCWGAGATYTTSCCMTCAGWCCTG
CHD1	TGTAACCATTGCTACCTCATTAARCC	AGATCATTYTGTGGATTCCARTCNGAATCR
	CTCCAGAAGATGTGGAATATTATAAYTGC	AGYTCYTTGTGNAGRCTTGCATAACC
DMRT1	GCAGCGGGTGATGGCNGCNCAGGT	GCCAGAATCTTGACTGCTGGGYGGYGA
GHR	TGAGTTTATTGAGYTGGAYATWGAYGA	GCTAHGGCAKGATTTTGTTCAGTTGG
RPS6	CACTGGCTGCCAGAAGCTCAT	GGCCTCCTTCATTCTCTTTG

Source: Kawai *et al*. (2009).

Appendix Table 2 Degenerate oligonucleotide primers used for cloning mitochondrial genome.

Primer name	Matching gene	Forward primer (5□-3□)	Primer name	Matching gene	Reverse primer (5□-3□)
Leiolepis12sf	12S rRNA	ACTACCTAGAGGAGCCTGTC	Leiolepis16sr	16S rRNA	TGACTCAGATCACGTAGGATT
Leiolepis16sf	16S rRNA	GATATCCTGATGGTGCAGAAGC	LeiolepisND1r	*ND1*	ATAATCGGTGTTTAAGTTGGTCGTA
LeiolepisND1f2	*ND1*	GGATTTAATGTAGAATACGCAGG	LeiolepisND2r2	*ND2*	ATTGTTGTTAGGTAGGCTATTCG
LeiolepisND2f	*ND2*	ATAATRGCCTAYTCATCAATCGC	LeiolepisCOIr2	*COI*	AATGCTGTATTTAGGTTTCGGTC
LeiolepisCOIf	*COI*	GACATAGCMTTYCCWCGMMTAAA	LeiolepisCOIr	*COI*	TCTGGGWARTCYGAGTATCG
LeiolepisCOIf2	*COI*	CAYTAGCAAARGCCCAATTCTG	LeiolepisCOIIr	*COII*	CAGATYTCTGARCAYTGTCCGTA
LeiolepisCOIIf2	*COII*	CAATGATACTGAAGYTACGAATAC	LeiolepisCOIIIr2	*COIII*	GAAGTGTCAGTATCAKGCWGCTGC
LeiolepisCOIIIf2	*COIII*	CATAGGAAAGCTTGCATTTAAGCGT	LeiolepisND4r2	*ND4*	GATTGTTGGTGGGAGTGCCATAT
LeiolepisgapCOIIIf3	*COIII*	TAACAGCCAACCTAACMGCAGGACA	LeiolepisND4r5	*ND4*	CCTATATGGCCAACTGAGGA
LeiolepisgapCOIIIf4	*COIII*	GGCCTACATGTWATYATTGGAACAAC	LeiolepisND4r6	*ND4*	GTGGTTTTGGCTGGCTATGATTATTA
LeiolepisND4f	*ND4*	GCCCACGGAYTMACCTCCTCAATA	LeiolepisND5r2	*ND5*	GGCCTTCTATTGCTGCRGGKAGTCA
LeiolepisND5f4	*ND5*	TCCGCAGCAATACAAGCCATC	LeioepisND5r3	*ND5*	GTTGAATGTTCTGGTTTTGAAGG
LeiolepisND5f3	*ND5*	ACMAACCCAATAYTACGACTAAC	LeiolepisCytbr2	*Cytb*	AGDGTTGGGTYRTCTACTGAG
LeiolepisCytbf	*Cytb*	CCCACATCAAACCGGAGTGAT	Leiolepis12Sr	12S rRNA	CTTAAAGGCAGACTGACAACG
Leiolepiscontrolf	control region	GCACATCTCATAAAACCACAGC	Leiolepis12sr3	12S rRNA	GTACGCTTACCATGTTACGAC

Appendix Table 3 Degenerate oligonucleotide primers used for cloning nuclear gene.

Primer name	Matching gene	Forward primer (5□-3□)	Primer name	Matching gene	Reverse primer (5□-3□)	Reference
	RAG1			*RAG1*		San Mauro *et al*. (2004)
MosF	C-*mos*	CTCTGGKGGCTTTGGKK CTGTSTACAAGG	MosR	C-*mos*	GGTGATGGCAAANGAGTAGATGTCTGC	Godinho *et al*. (2006)
EnolL 731	α-*Enolase*	TGGACTTCAAATCCCCCGA TGATCCCAGC	EnolH 912	α-*Enolase*	CCAGGCACCCCAGTCTACCTGGTCAAA	Friesen *et al*. (1997)
GapdL890	*GAPD*	ACCTTTAATGCGGGTGC TGGCATTGC	GapdH950	*GAPD*	CATCAAGTCCACAACACGGTTGCTGTA	Friesen *et al*. (1997)

Appendix Table 4 Degenerate oligonucleotide primers used for discriminating *Leiolepis* spp. as DNA marker.

Primer name	Matching gene	Forward primer (5'-3')	Primer name	Matching gene	Reverse primer (5'-3')
BL16Sf	16s rRNA	ATCTGAGTTCAGACCGGAGC	BLnd1r1	*ND1*	TGGAGATTGCTATTAGGAATAGG
			BLnd1r2	*ND1*	TGGCTAGACATATTAGGATGAGG
BLCOIf	*COI*	CATAAGCTTCTGACTCCTRCC	BLCOIr1	*COI*	GGCTGCTGCTAGAACAGGTAAA
			BLCOIr2	*COI*	TACAACGTAATAAGTGTCATGTAAA
LeiolepisND2f	*ND2*	ATAATRGCCTAYTCATCAATCGC	BLND2COIr1	*ND2-COI*	ATGCCAGGAGTAACAGAAAGGGC
			BLND2COIr2	*ND2-COI*	GTTGACCCAGCTAATCCGGTAGCAT
LeiolepisND5f	*ND5*	ATTTACAACCGHATCGGMGACAT	LeiolepisND5r	*ND5*	TTGGBCCDGATTTTTCTAGTCA

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

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Ala	GCG	8	0.21	3.00
Ala	GCA	115	3.07	42.00
Ala	GCT	48	1.28	17.00
Ala	GCC	106	2.83	38.00
Cys	TGT	11	0.29	34.00
Cys	TGC	21	0.56	66.00
Asp	GAT	15	0.40	25.00
Asp	GAC	45	1.20	75.00
Glu	GAG	13	0.35	15.00
Glu	GAA	72	1.92	85.00
Phe	TTT	94	2.51	48.00
Phe	TTC	100	2.67	52.00
Gly	GGG	34	0.91	17.00
Gly	GGA	99	2.64	48.00
Gly	GGT	25	0.67	12.00
Gly	GGC	47	1.25	23.00
His	CAT	38	1.01	37.00
His	CAC	64	1.71	63.00
Ile	ATT	137	3.66	45.00
Ile	ATC	167	4.46	55.00
Lys	AAG	10	0.27	9.00
Lys	AAA	97	2.59	91.00
Leu	TTG	23	0.61	4.00
Leu	TTA	111	2.96	19.00
Leu	CTG	46	1.23	8.00

Appendix Table 5 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Leu	CTA	264	7.04	44.00
Leu	CTT	71	1.89	12.00
Leu	CTC	80	2.13	13.00
Met	ATG	44	1.17	18.00
Met	ATA	203	5.42	82.00
Asn	AAT	47	1.25	30.00
Asn	AAC	110	2.94	70.00
Pro	CCG	12	0.32	5.00
Pro	CCA	124	3.31	57.00
Pro	CCT	26	0.69	12.00
Pro	CCC	57	1.52	26.00
Gln	CAG	8	0.21	8.00
Gln	CAA	98	2.62	92.00
Arg	CGG	5	0.13	7.00
Arg	CGA	40	1.07	60.00
Arg	CGT	11	0.29	16.00
Arg	CGC	11	0.29	16.00
Ser	AGT	19	0.51	7.00
Ser	AGC	33	0.88	13.00
Ser	TCG	3	0.08	1.00
Ser	TCA	108	2.88	42.00
Ser	TCT	40	1.07	16.00
Ser	TCC	55	1.47	21.00
Thr	ACG	6	0.16	2.00
Thr	ACA	170	4.54	45.00
Thr	ACT	65	1.73	17.00
Thr	ACC	135	3.60	36.00

Appendix Table 5 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Val	GTG	14	0.37	9.00
Val	GTA	64	1.71	43.00
Val	GTT	29	0.77	19.00
Val	GTC	42	1.12	28.00
Trp	TGG	10	0.27	10.00
Trp	TGA	88	2.35	90.00
Tyr	TAT	37	0.99	34.00
Tyr	TAC	73	1.95	66.00

Appendix Table 6 Codon pattern composition (% of total number) for each protein-coding genes found in the mitochondrial genome of *L. belliana belliana*.

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Ala	GCG	11	0.29	4.00
Ala	GCA	120	3.20	43.00
Ala	GCT	33	0.88	12.00
Ala	GCC	113	3.01	41.00
Cys	TGT	18	0.48	51.00
Cys	TGC	17	0.45	49.00
Asp	GAT	16	0.43	25.00
Asp	GAC	49	1.31	75.00
Glu	GAG	14	0.37	16.00
Glu	GAA	75	2.00	84.00

Appendix Table 6 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Phe	TTT	91	2.42	49.00
Phe	TTC	94	2.50	51.00
Gly	GGG	31	0.83	15.00
Gly	GGA	101	2.69	50.00
Gly	GGT	23	0.61	11.00
Gly	GGC	48	1.28	24.00
His	CAT	36	0.96	36.00
His	CAC	64	1.70	64.00
Ile	ATT	132	3.52	42.00
Ile	ATC	180	4.79	58.00
Lys	AAG	7	0.19	7.00
Lys	AAA	97	2.58	93.00
Leu	TTG	22	0.59	4.00
Leu	TTA	102	2.72	17.00
Leu	CTG	35	0.93	6.00
Leu	CTA	276	7.35	47.00
Leu	CTT	72	1.92	12.00
Leu	CTC	86	2.29	15.00
Met	ATG	28	0.75	12.00
Met	ATA	207	5.51	88.00
Asn	AAT	45	1.20	28.00
Asn	AAC	113	3.01	72.00
Pro	CCG	3	0.08	1.00
Pro	CCA	123	3.28	58.00
Pro	CCT	36	0.96	17.00
Pro	CCC	50	1.33	24.00
Gln	CAG	11	0.29	10.00

Appendix Table 6 (Continued)

Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Gln	CAA	98	2.61	90.00
Arg	CGG	4	0.11	5.00
Arg	CGA	45	1.20	60.00
Arg	CGT	12	0.32	16.00
Arg	CGC	14	0.37	19.00
Ser	AGT	13	0.35	5.00
Ser	AGC	36	0.96	14.00
Ser	TCG	8	0.21	3.00
Ser	TCA	105	2.80	41.00
Ser	TCT	44	1.17	17.00
Ser	TCC	52	1.39	20.00
Thr	ACG	6	0.16	2.00
Thr	ACA	162	4.31	43.00
Thr	ACT	60	1.60	16.00
Thr	ACC	151	4.02	40.00
Val	GTG	21	0.56	15.00
Val	GTA	59	1.57	42.00
Val	GTT	26	0.69	18.00
Val	GTC	36	0.96	25.00
Trp	TGG	13	0.35	12.00
Trp	TGA	94	2.50	88.00
Tyr	TAT	38	1.01	32.00
Tyr	TAC	79	2.10	68.00

Appendix Table 7 Codon pattern composition (% of total number) for each protein-coding genes found in the mitochondrial genome of *L. boehmei*.

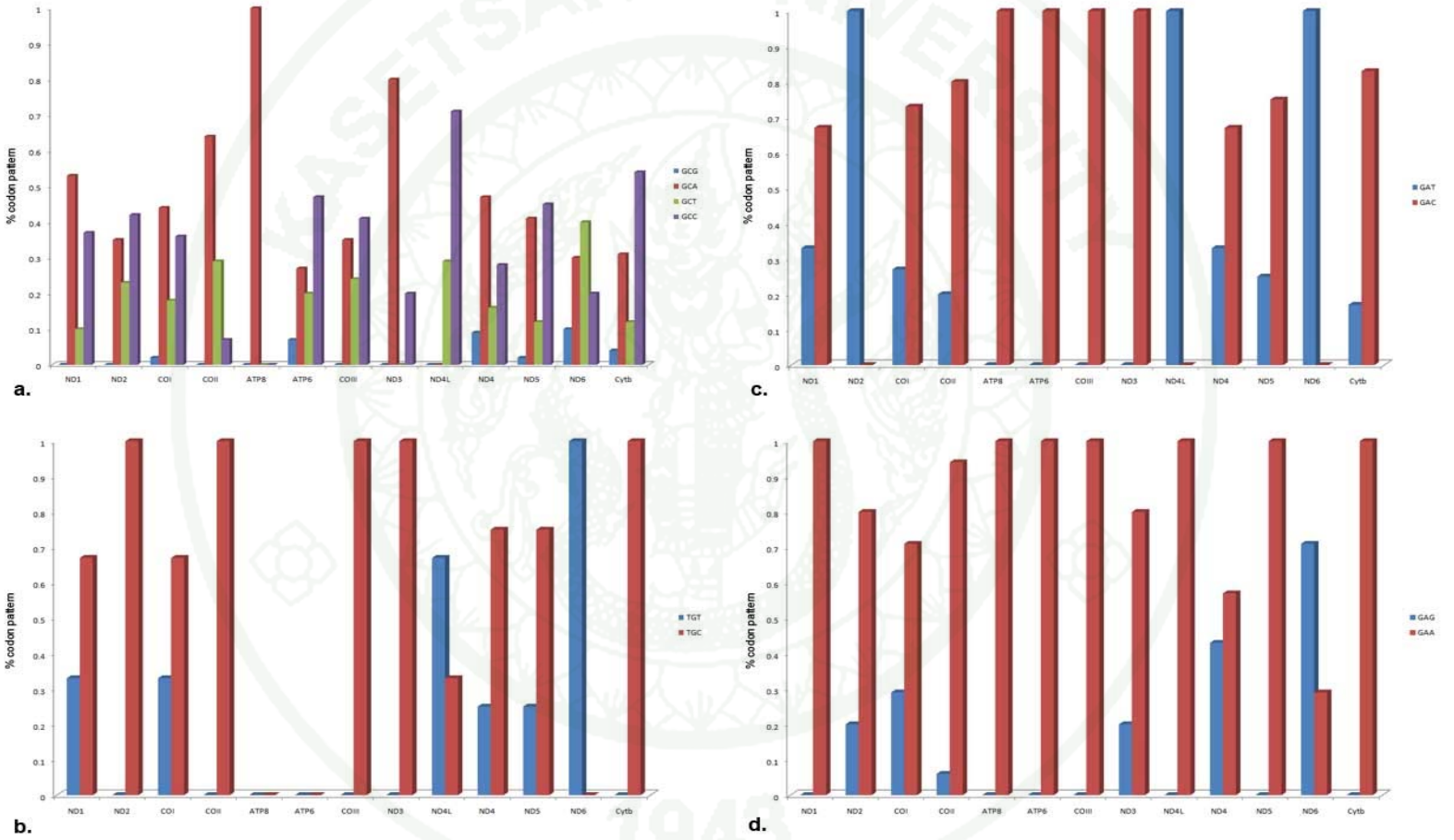
Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Ala	GCG	11	0.29	4.00
Ala	GCA	111	2.96	39.00
Ala	GCT	40	1.07	14.00
Ala	GCC	122	3.25	43.00
Cys	TGT	15	0.40	47.00
Cys	TGC	17	0.45	53.00
Asp	GAT	18	0.48	30.00
Asp	GAC	43	1.15	70.00
Glu	GAG	16	0.43	18.00
Glu	GAA	74	1.97	82.00
Phe	TTT	87	2.32	44.00
Phe	TTC	111	2.96	56.00
Gly	GGG	34	0.91	16.00
Gly	GGA	114	3.04	55.00
Gly	GGT	21	0.56	10.00
Gly	GGC	38	1.01	18.00
His	CAT	26	0.69	24.00
His	CAC	82	2.19	76.00
Ile	ATT	117	3.12	40.00
Ile	ATC	179	4.77	60.00
Lys	AAG	12	0.32	11.00
Lys	AAA	96	2.56	89.00
Leu	TTG	23	0.61	4.00
Leu	TTA	99	2.64	17.00
Leu	CTG	38	1.01	7.00

Appendix Table 7 (Continued)

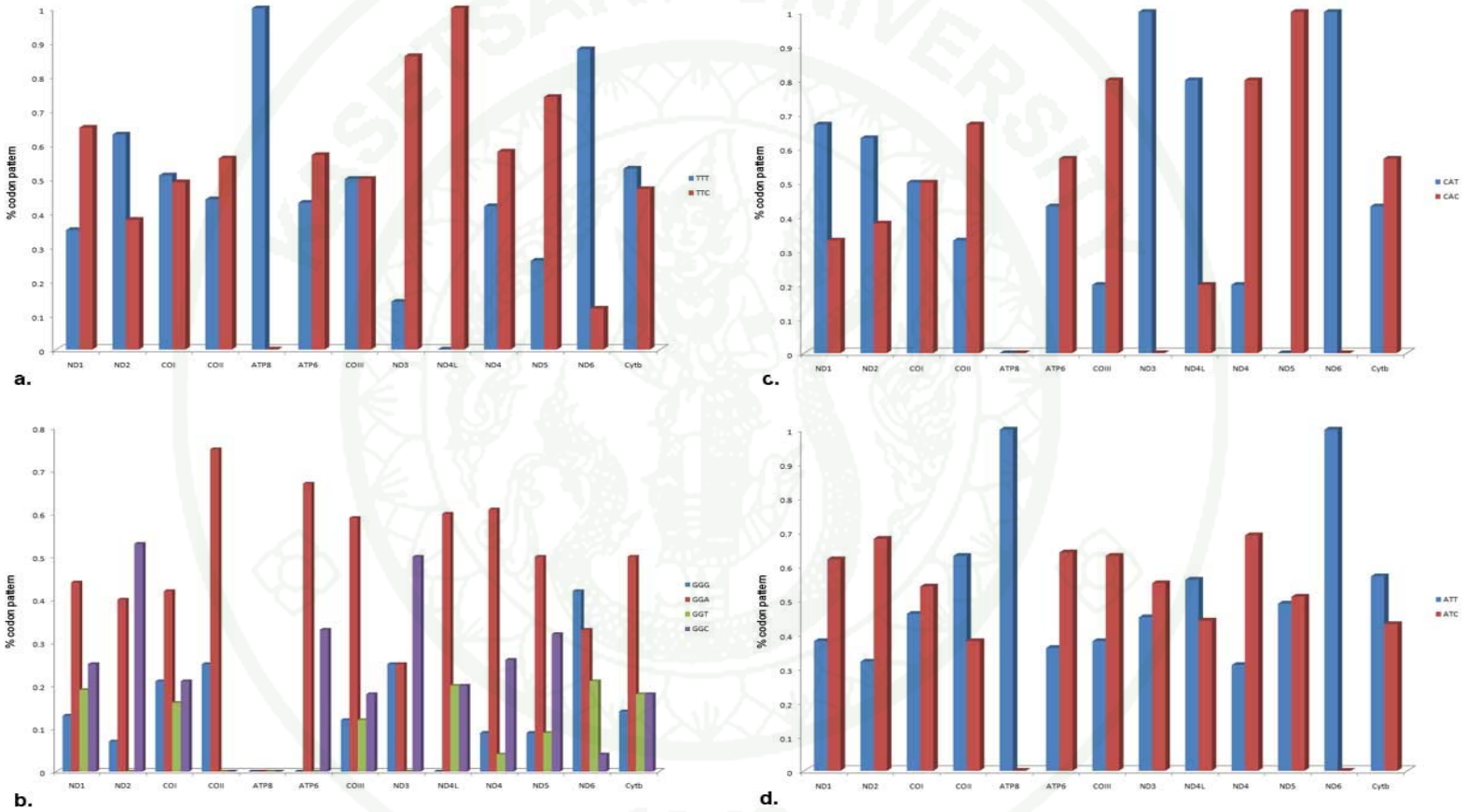
Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Leu	CTA	277	7.39	48.00
Leu	CTT	63	1.68	11.00
Leu	CTC	81	2.16	14.00
Met	ATG	44	1.17	17.00
Met	ATA	216	5.76	83.00
Asn	AAT	48	1.28	29.00
Asn	AAC	120	3.20	71.00
Pro	CCG	4	0.11	2.00
Pro	CCA	147	3.92	69.00
Pro	CCT	27	0.72	13.00
Pro	CCC	34	0.91	16.00
Gln	CAG	5	0.13	5.00
Gln	CAA	96	2.56	95.00
Arg	CGG	4	0.11	6.00
Arg	CGA	45	1.20	67.00
Arg	CGT	8	0.21	12.00
Arg	CGC	10	0.27	15.00
Ser	AGT	12	0.32	5.00
Ser	AGC	31	0.83	13.00
Ser	TCG	5	0.13	2.00
Ser	TCA	118	3.15	48.00
Ser	TCT	28	0.75	11.00
Ser	TCC	50	1.33	20.00
Thr	ACG	5	0.13	1.00
Thr	ACA	182	4.85	48.00
Thr	ACT	68	1.81	18.00
Thr	ACC	121	3.23	32.00

Appendix Table 7 (Continued)

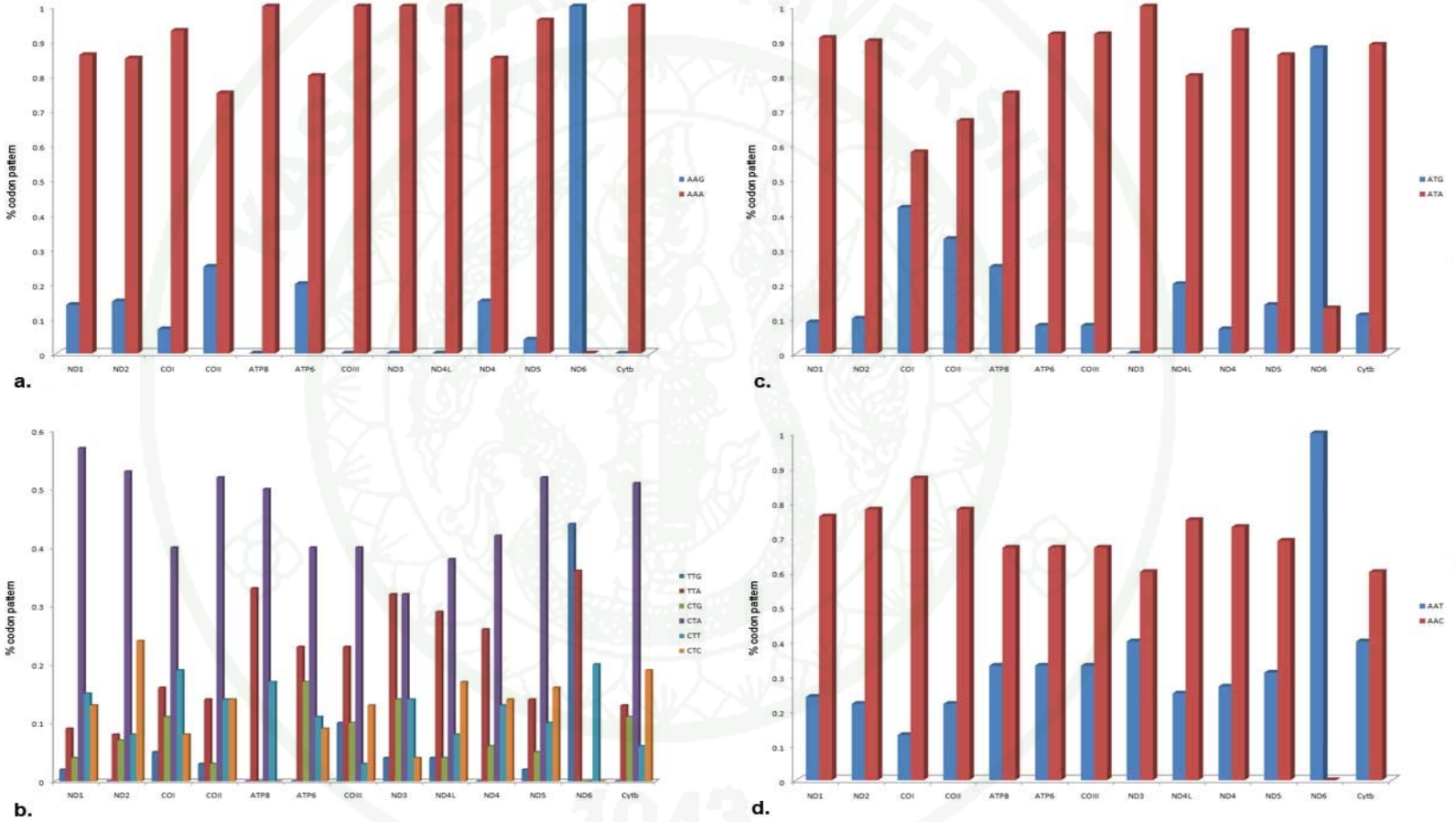
Amino acid	Codon	Number of codon	Codon composition in total (%)	Codon composition in each amino acid (%)
Val	GTG	10	0.27	7.00
Val	GTA	71	1.89	48.00
Val	GTT	37	0.99	25.00
Val	GTC	30	0.80	20.00
Trp	TGG	13	0.35	13.00
Trp	TGA	87	2.32	87.00
Tyr	TAT	42	1.12	39.00
Tyr	TAC	67	1.79	61.00



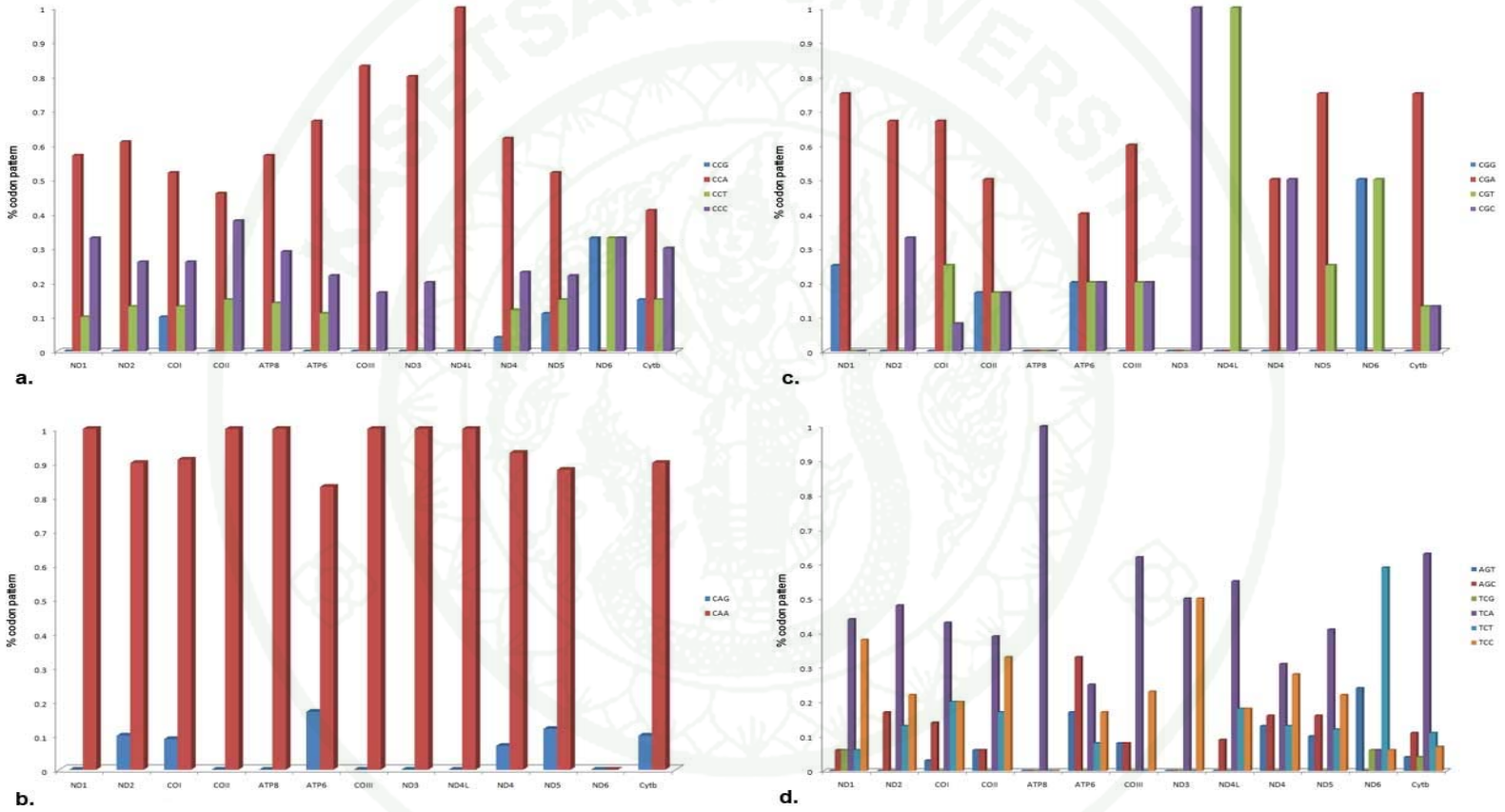
Appendix Figure 1 The relationship between the codon pattern (%) and each protein coding gene for Ala (a), Cys (b), Asp (c) and Glu (d) amino acid in *L. reevesii rubritaeniata*.



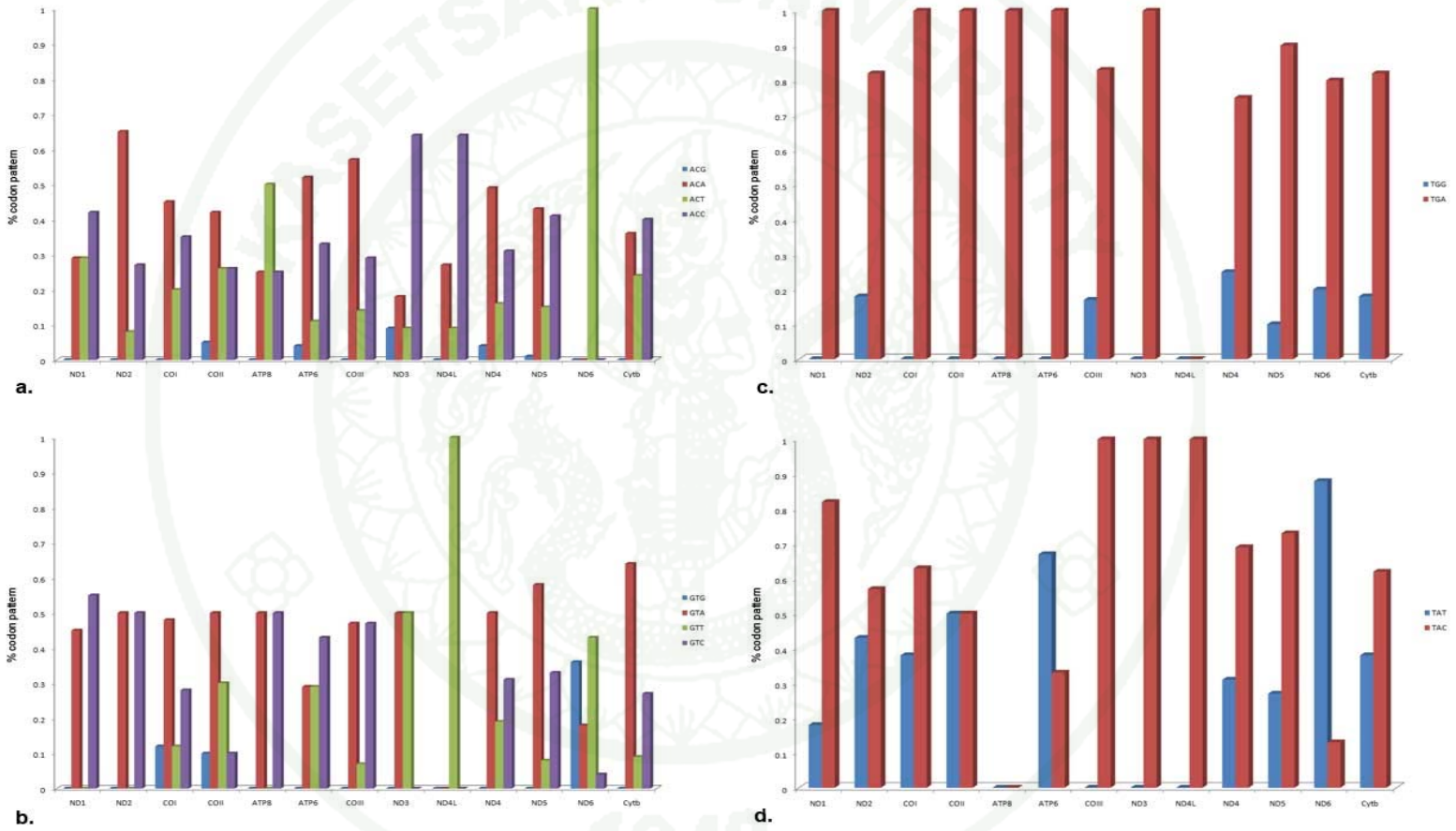
Appendix Figure 2 The relationship between the codon pattern (%) and each protein coding gene for Phe (a), Gly (b), His (c) and Ile (d) amino acid in *L. reevesii rubritaeniata*.



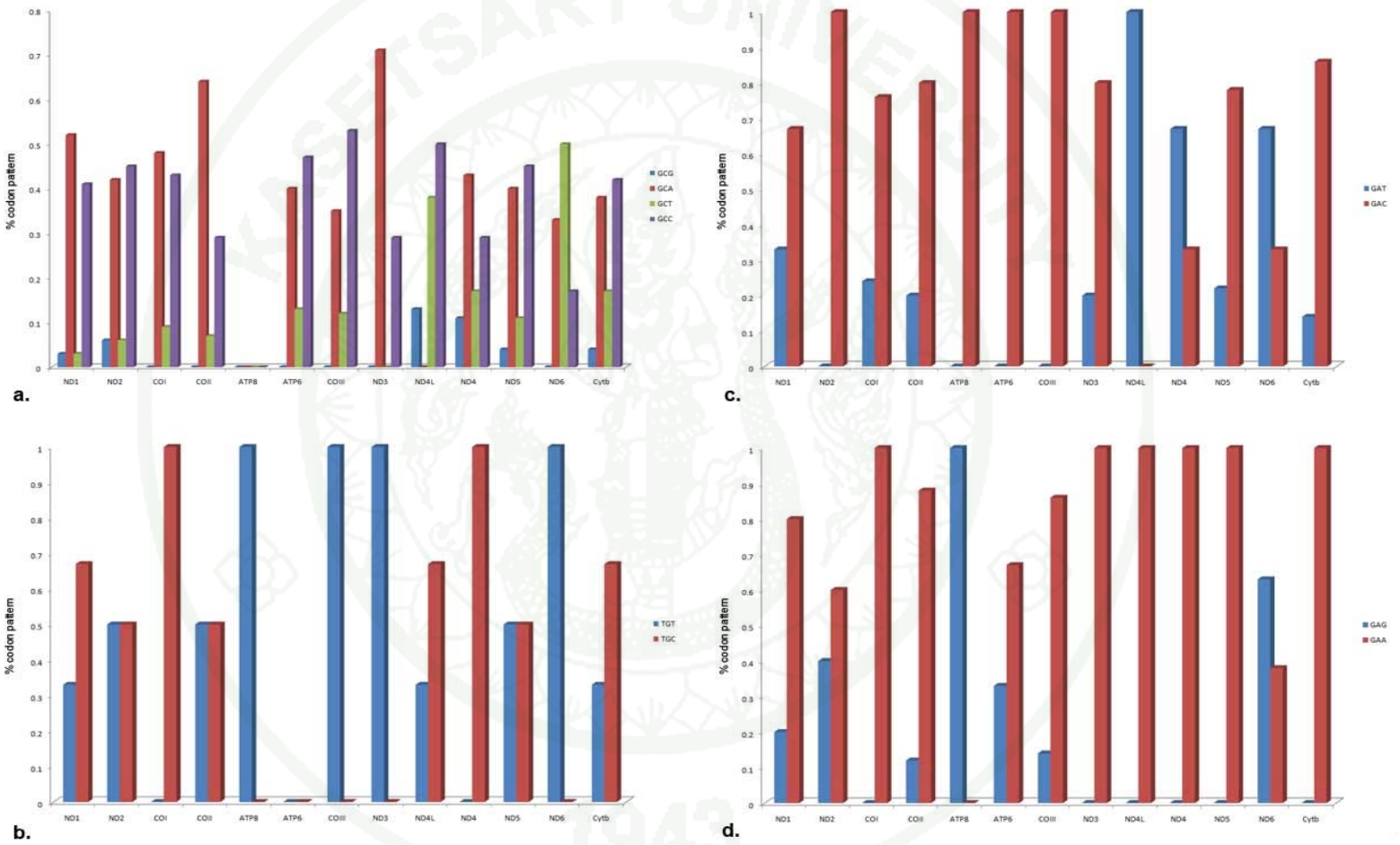
Appendix Figure 3 The relationship between the codon pattern (%) and each protein coding gene for Lys (a), Leu (b), Met (c) and Asn (d) amino acid in *L. reevesii rubritaeniata*.



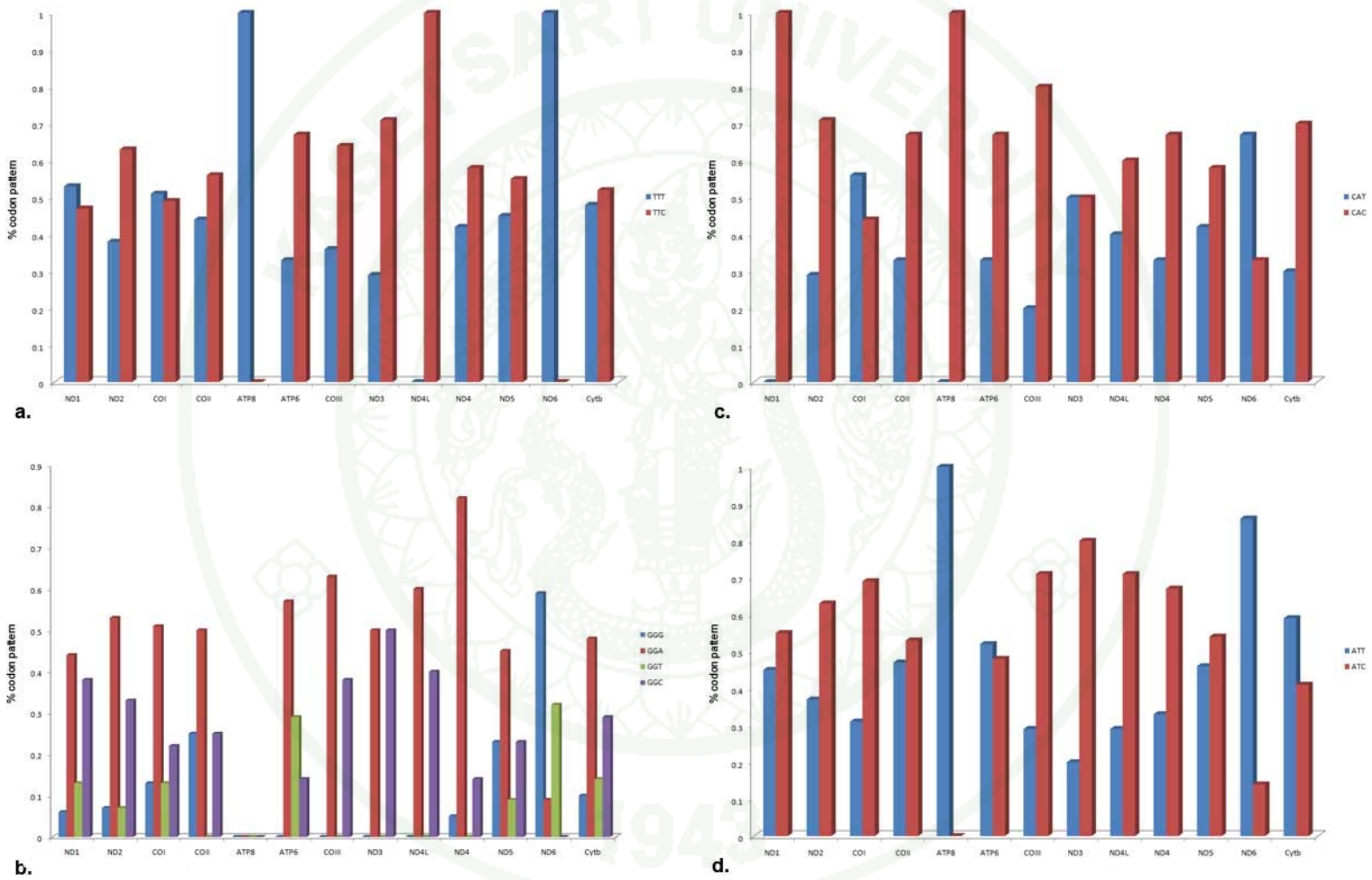
Appendix Figure 4 The relationship between the codon pattern (%) and each protein coding gene for Pro (a), Gln (b), Arg (c) and Ser (d) amino acid in *L. reevesii rubritaeniata*.



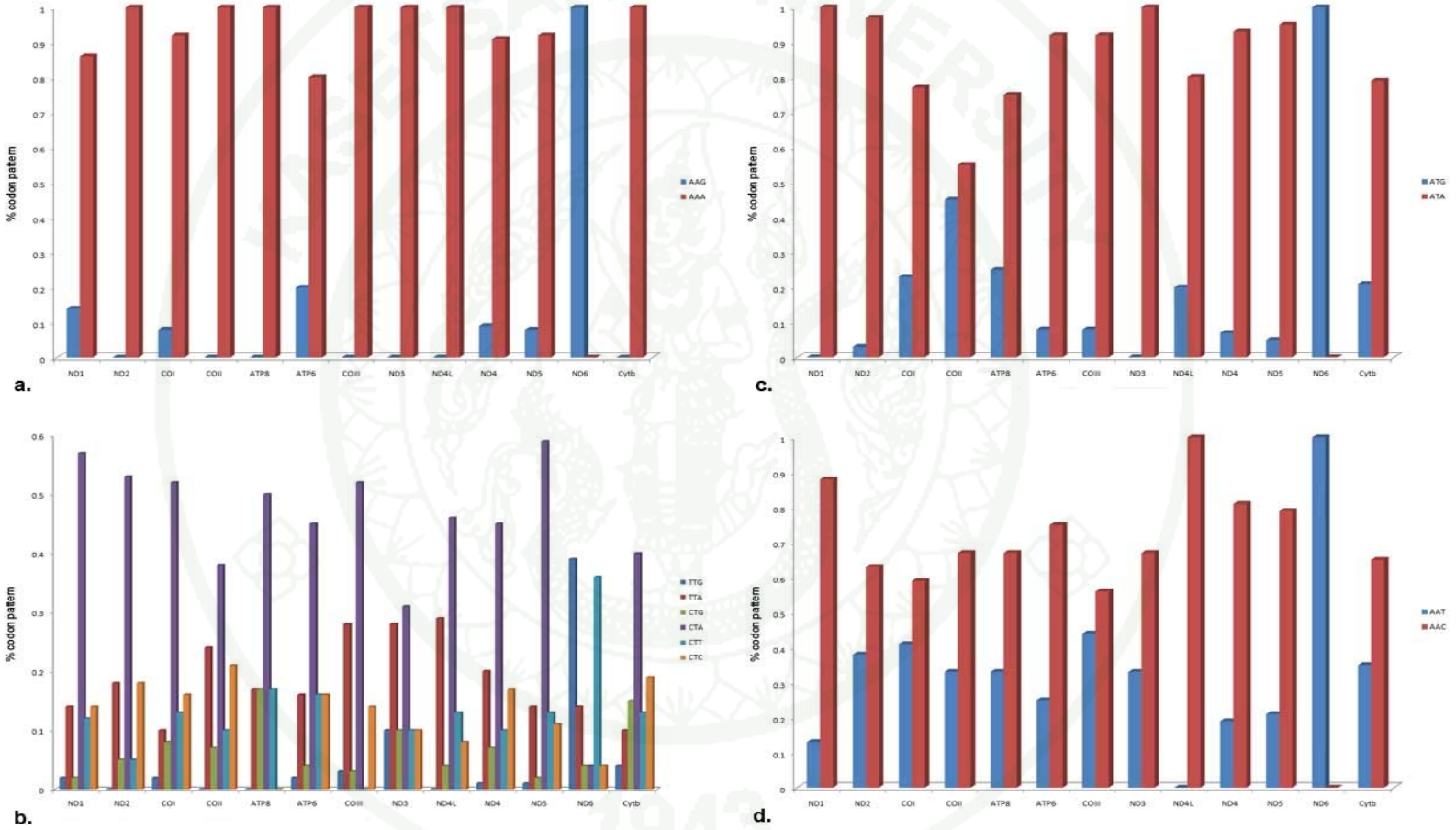
Appendix Figure 5 The relationship between the codon pattern (%) and each protein coding gene for Thr (a), Val (b), Trp (c) and Tyr (d) amino acid in *L. reevesii rubritaeniata*.



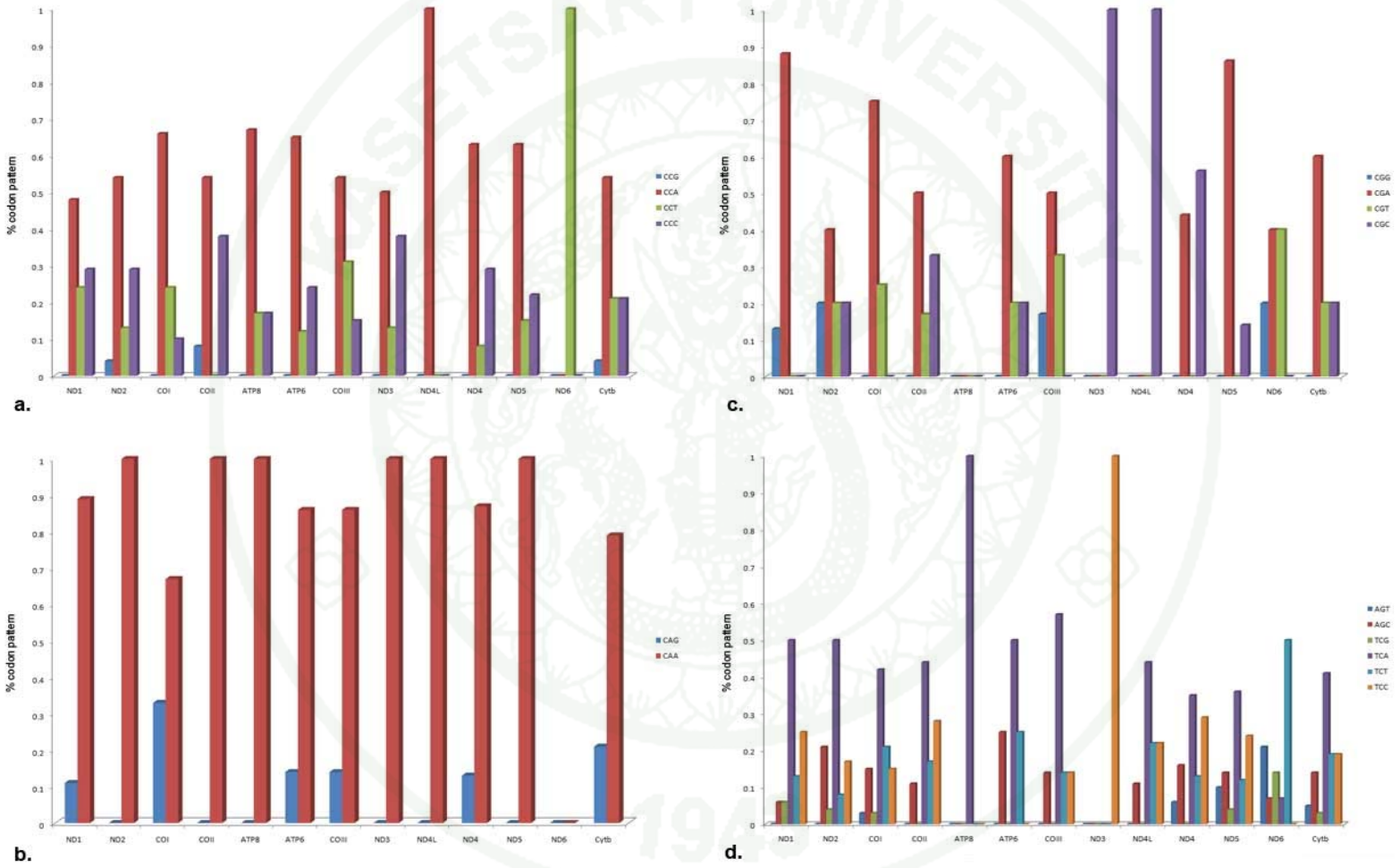
Appendix Figure 6 The relationship between the codon pattern (%) and each protein coding gene for Ala (a), Cys (b), Asp (c) and Glu (d) amino acid in *L. belliana belliana*.



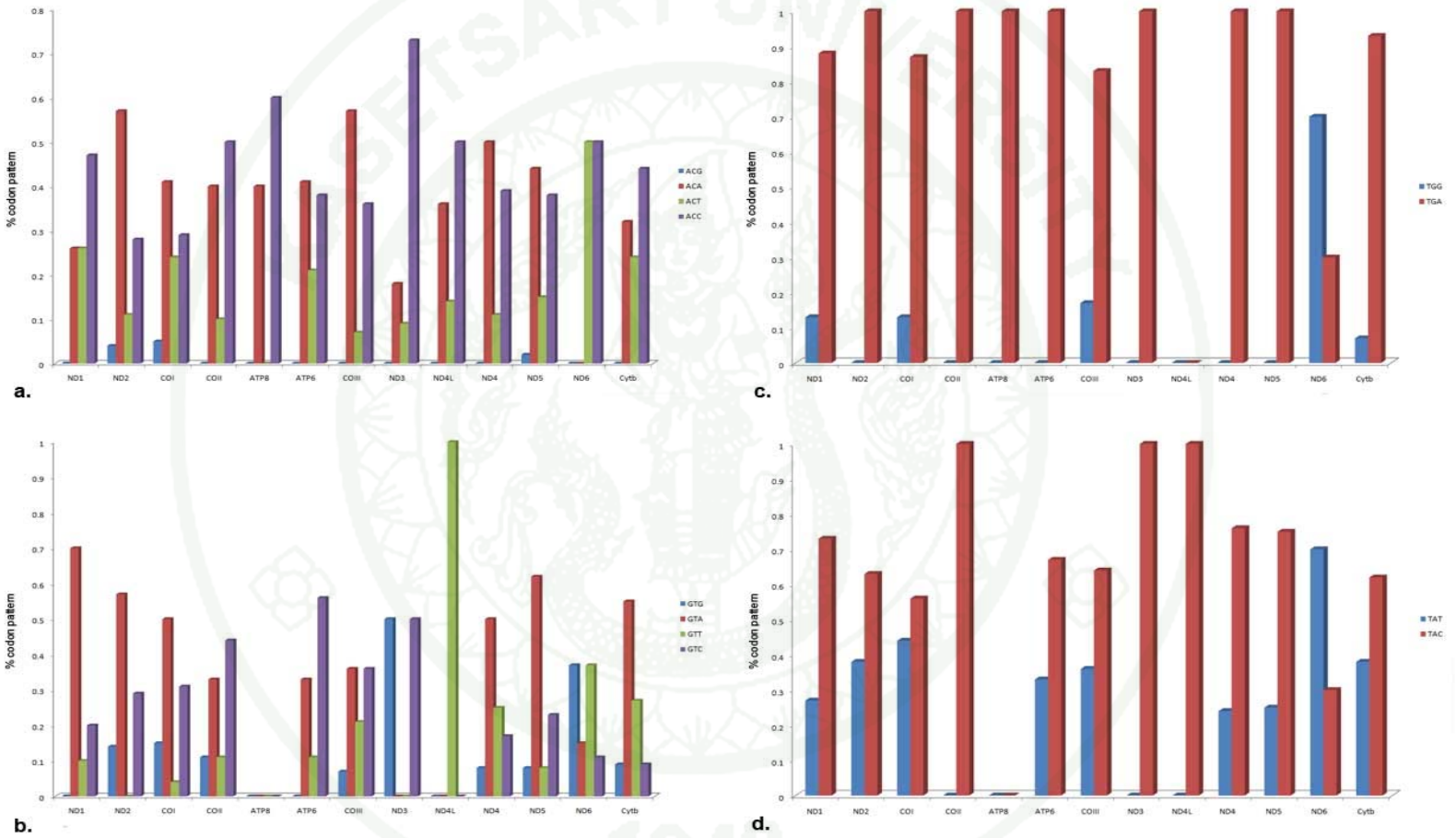
Appendix Figure 7 The relationship between the codon pattern (%) and each protein coding gene for Phe (a),



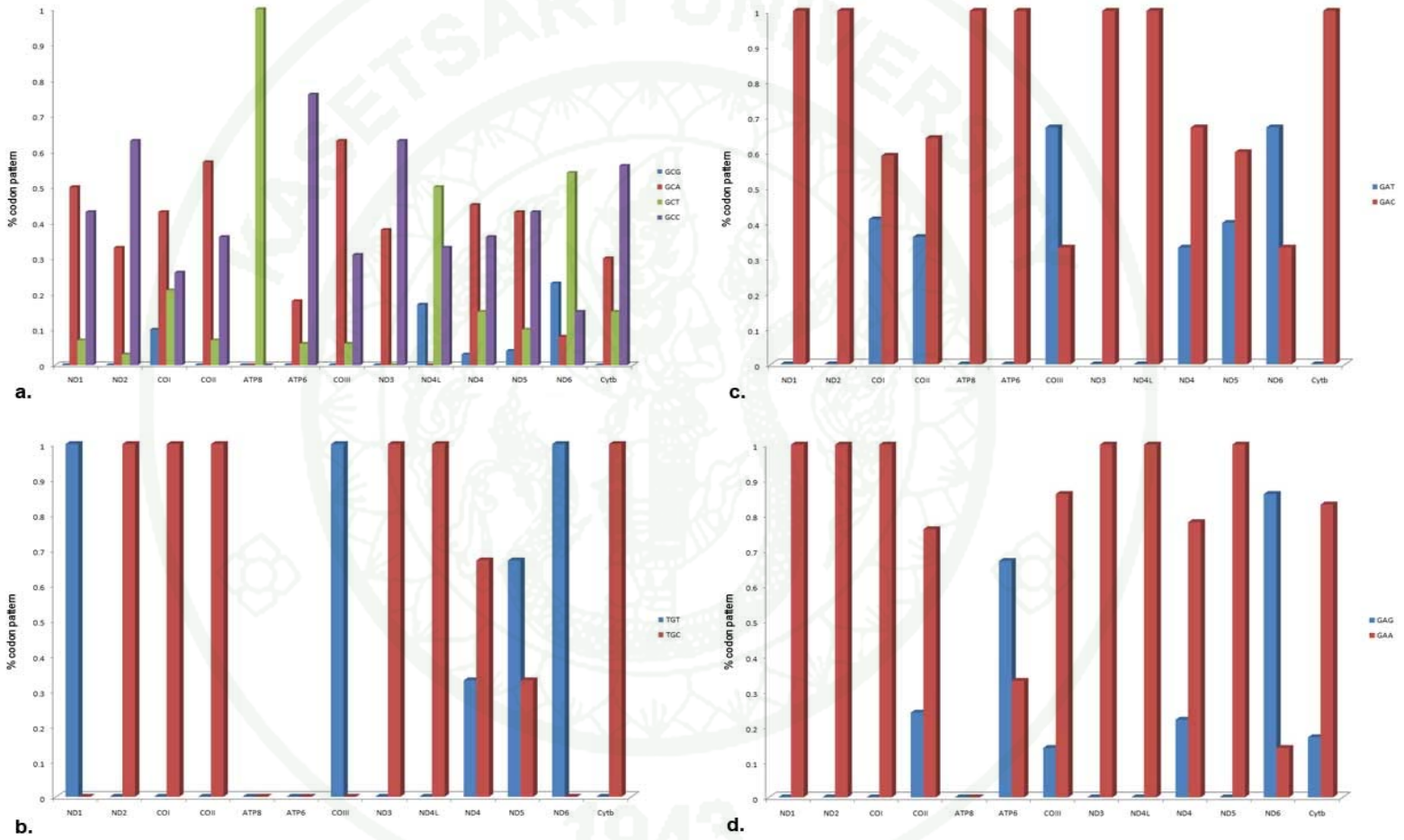
Appendix Figure 8 The relationship between the codon pattern (%) and each protein coding gene for Lys (a), Leu (b), Met (c) and Asn (d) amino acid in *L. belliana belliana*.



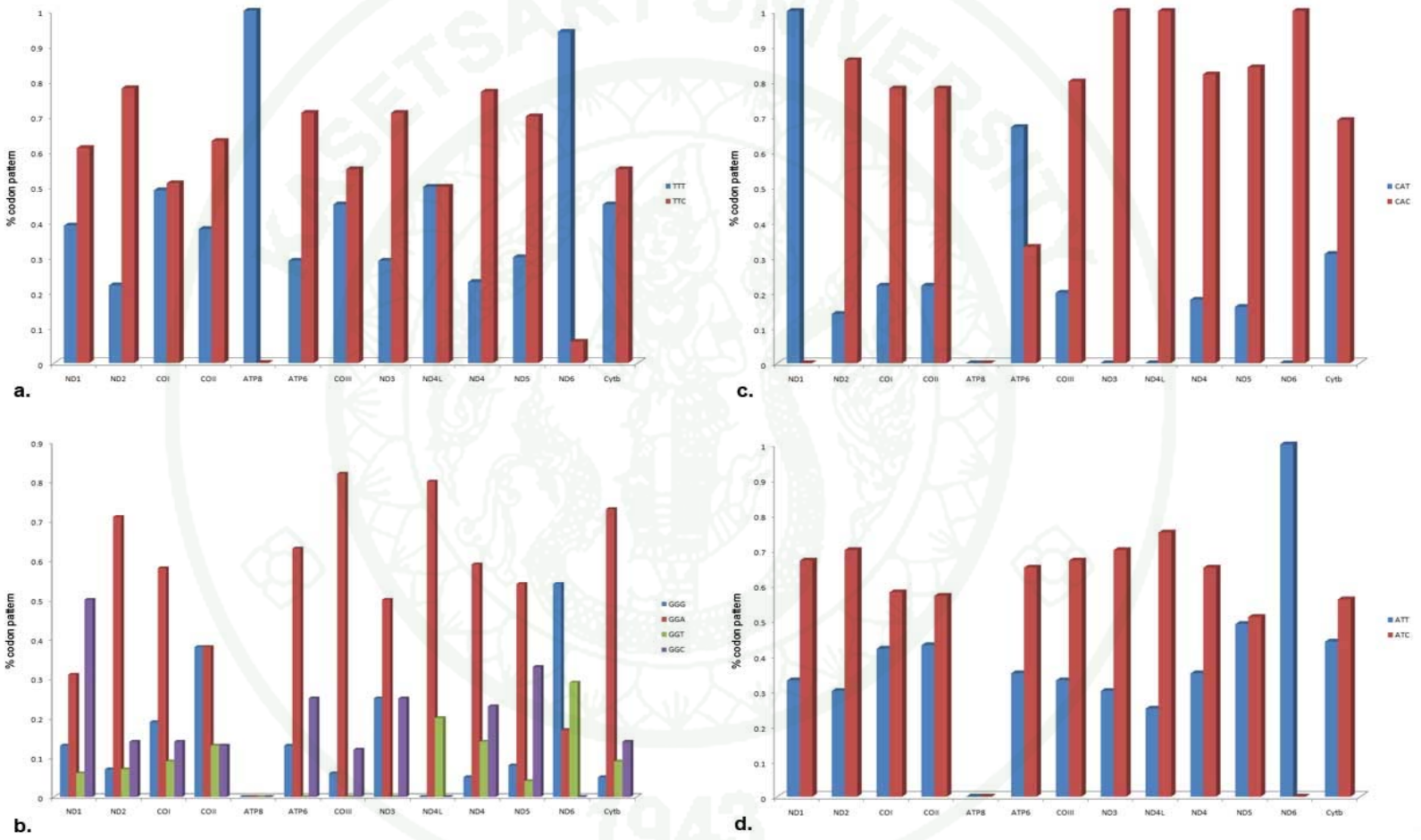
Appendix Figure 9 The relationship between the codon pattern (%) and each protein coding gene for Pro (a),



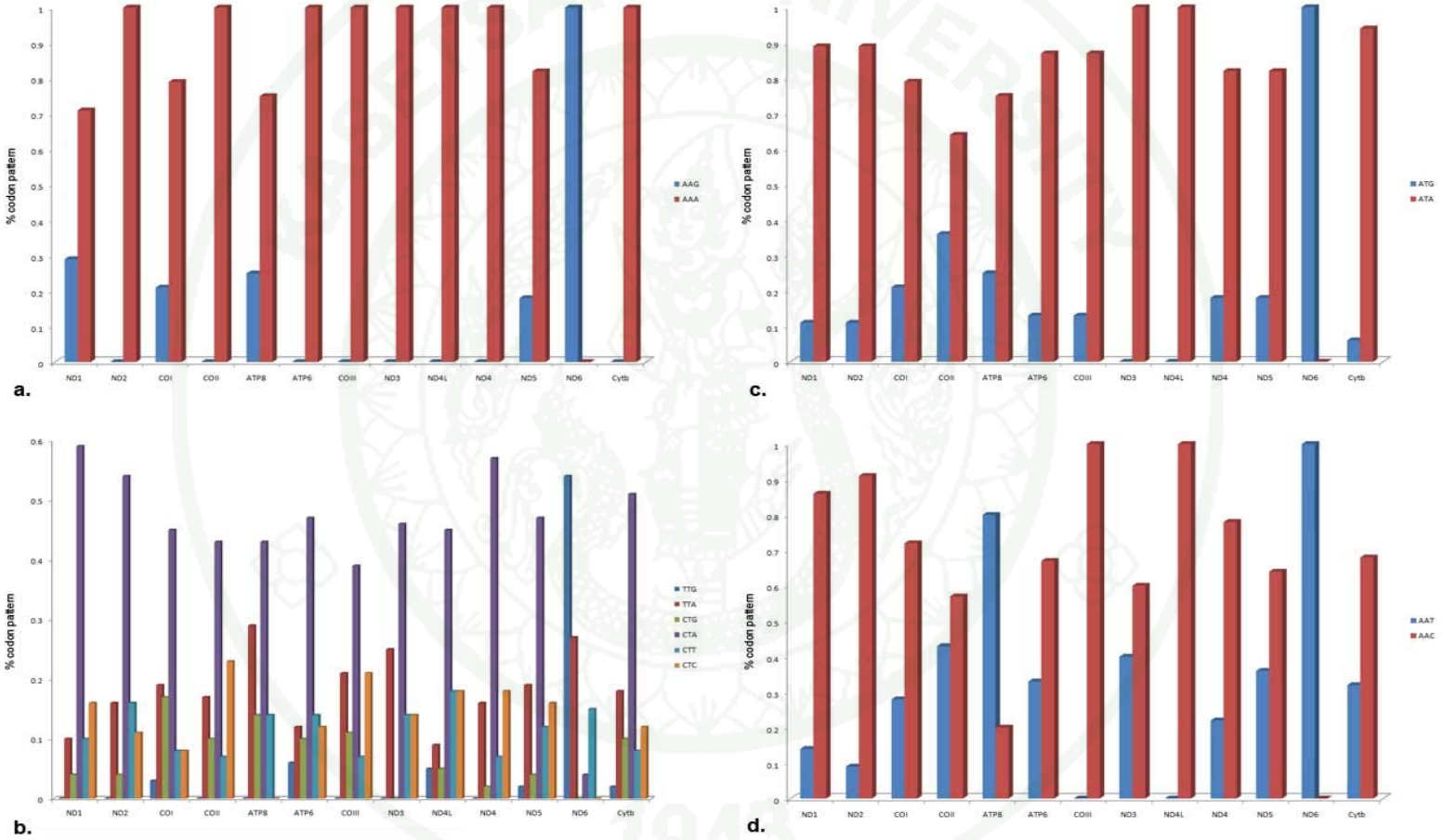
Appendix Figure 10 The relationship between the codon pattern (%) and each protein coding gene for Thr (a), Val (b), Trp (c) and Tyr (d) amino acid in *L. belliana belliana*.



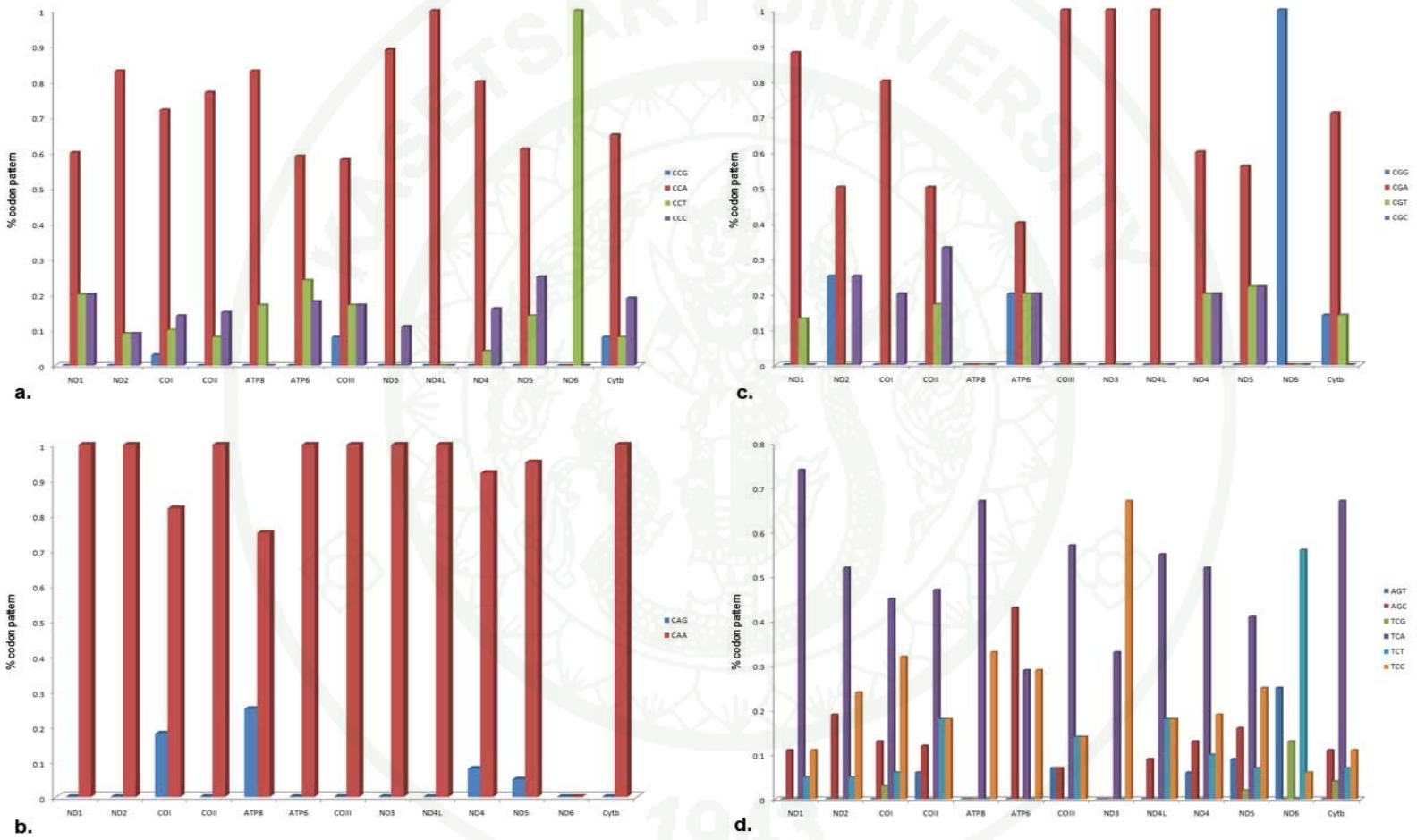
Appendix Figure 11 The relationship between the codon pattern (%) and each protein coding gene for Ala (a), Cys (b), Asp (c) and Glu (d) amino acid in *L. boehmei*.



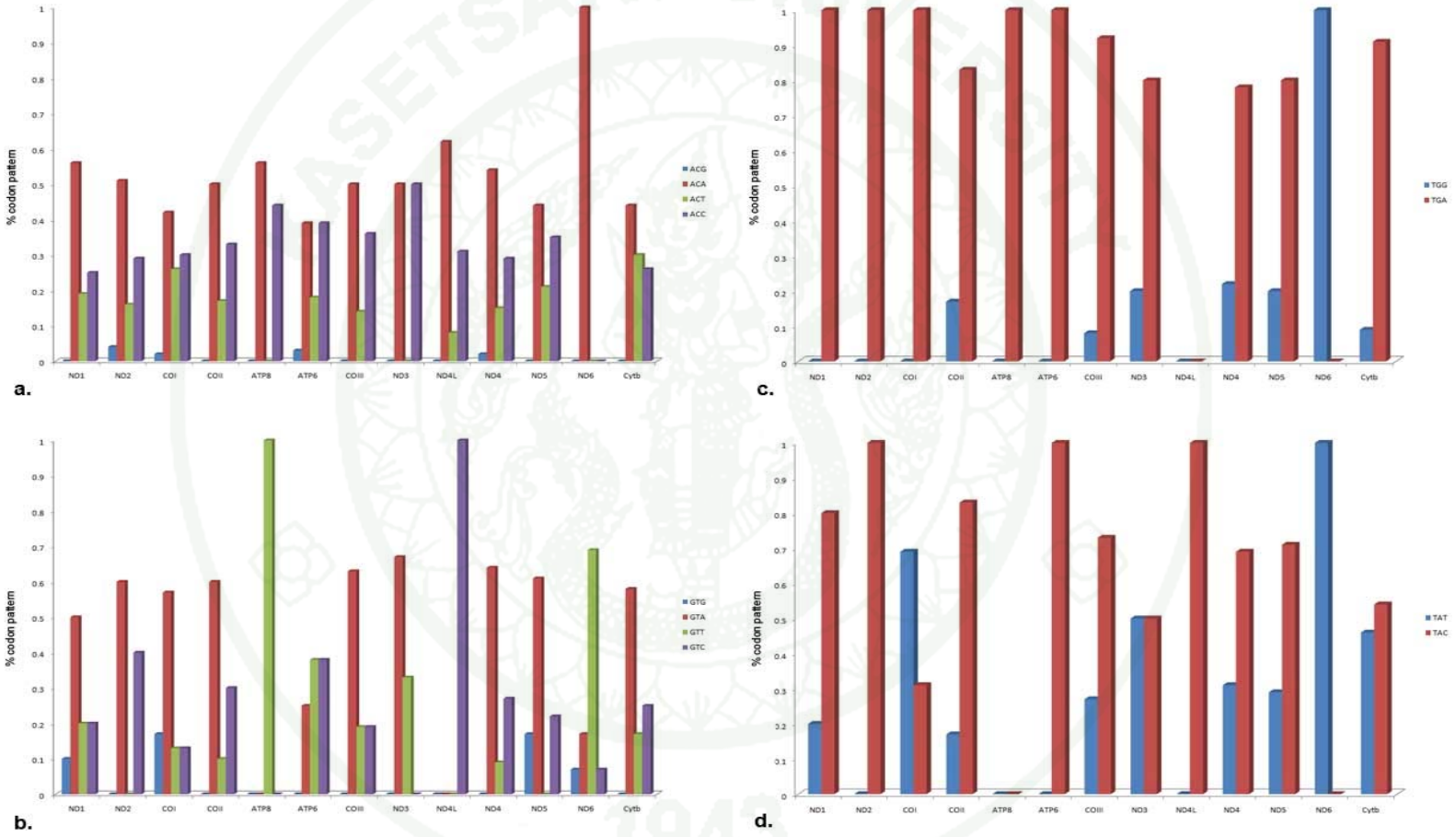
Appendix Figure 12 The relationship between the codon pattern (%) and each protein coding gene for Phe (a), Gly (b), His (c) and Ile (d) amino acid in *L. boehmei*.



Appendix Figure 13 The relationship between the codon pattern (%) and each protein coding gene for Lys (a), Leu (b), Met (c) and Asn (d) amino acid in *L. boehmei*.



Appendix Figure 14 The relationship between the codon pattern (%) and each protein coding gene for Pro (a), Gln (b), Arg (c) and Ser (d) amino acid in *L. boehmei*.



Appendix Figure 15 The relationship between the codon pattern (%) and each protein coding gene for Thr (a), Val (b), Trp (c) and Tyr (d) amino acid in *L. boehmei*.

Amino Acid Abbreviation

Ala (A)	=	Alanine
Arg (R)	=	Arginine
Asn (N)	=	Asparagine
Asp (D)	=	Aspartic acid (Aspartate)
Cys (C)	=	Cysteine
Gln (Q)	=	Glutamine
Glu (E)	=	Glutamic acid (Glutamate)
Gly (G)	=	Glycine
His (H)	=	Histidine
Ile (I)	=	Isoleucine
Leu (L)	=	Leucine
Lys (K)	=	Lysine
Met (M)	=	Methionine
Phe (F)	=	Phenylalanine
Pro (P)	=	Proline
Ser (S)	=	Serine
Thr (T)	=	Threonine
Trp (W)	=	Tryptophan
Tyr (Y)	=	Tyrosine
Val (V)	=	Valine
Asx (B)	=	Aspartic acid or Asparagine
Glx (Z)	=	Glutamine or Glutamic acid

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PROCEEDING : Srikulnath, K., A. Thongpan and S. Apisitwanich. 2007. Karyotypes of Siamese crocodile (*Crocodylus siamensis*) and saltwater crocodile (*Crocodylus porosus*) using Het- and G-banding. **Proceeding of the 15th National Genetic Conference.** Songkla. Thailand.

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