

Genetic Relationship of Three Butterfly Lizard Species (*Leiolepis reevesii rubritaeniata*, *Leiolepis belliana belliana*, *Leiolepis boehmei*, Agamidae, Squamata) Inferred from Nuclear Gene Sequence Analyses

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ABSTRACT

The genetic relationship was investigated of three butterfly lizard species (*Leiolepis reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*) selectively inhabiting Thailand. The findings were based on *RAG1* and *C-mos* gene analyses. The DNA sequences were also compared with the other squamate reptiles. The analysis strongly supported that *L. reevesii rubritaeniata* was related more closely to *L. belliana belliana* than to *L. boehmei*. The phylogenetic position of *Leiolepis* spp., however, was contentious with regard to its relationship among the Leiolepidinae, Agaminae and Chamaeleonidae, which suggested that their phylogeny remains uncertain.

Keywords: butterfly lizard, Leiolepidinae, phylogeny, *RAG1*, *C-mos*

INTRODUCTION

The Squamata is the most diverse reptilian order that has been classified traditionally into three suborders: Serpentes (snakes), Amphisbaenia (worm lizards) and Lacertilia (lizards). The extant lizards can be further categorized into five infraorders (the Iguania, Gekkota, Scincomorpha, Diploglossa, Dibamia, Platynota) (Uetz, 2009). Butterfly lizards (Agamidae, Iguania) are burrow diggers and

inhabit Southeast Asia. They show a great variety of karyotypes and sexual systems. In Thailand, there are three species, which barely can be discriminated from other congeneric species by their typical scale and skin coloration (Peters, 1971). Bisexualism has been described in *Leiolepis belliana belliana* ($2n=2x=36$), which is widely found throughout the country, *L. belliana ocellata* ($2n=2x=34$) found in upper northern, and *L. reevesii rubritaeniata* ($2n=2x=36$) which is distributed only in the northeast (Aranyavalai,

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2003; Srikulnath *et al.*, 2009). The putatively unisexual diploidy has been reported also in *L. boehmei* ($2n=2x=34$), in which all individuals are female in Songkhla and Nakhon Si Thammarat provinces, southern Thailand (Aranyavalai *et al.*, 2004). However, their genetic relationship has not been revealed. Even though their morphology (body color, pattern and shape) were previously explored as the key to species identification (Aranyavalai, 2003), this morphology might be misleading for constructing the correct phylogeny due to homoplasy. A consideration of molecular information and nuclear and mitochondrial gene sequences is appropriate for studies on both shallow and deep genetic relationships. However, the mitochondrial DNA sequences commonly demonstrate saturation at basal nodes and deeper nodes. Probably, only the nuclear data can be best assessed to rebuild nodes at the deepest level of the squamate tree (Townsend *et al.*, 2004).

C-mos (cellular moloney murine sarcoma), a candidate nuclear gene, is a proto-oncogene encoding a serine/threonine kinase expressed at high levels in germ cells, in which the protein regulates cell maturation and tubulin formation (Yew *et al.*, 1993). *RAG1* (recombination activating gene-1) is a nuclear gene encoding component of the recombinase enzyme, which is involved in the V(D)J recombination of the T-receptor and immunoglobulin genes (Schatz *et al.*, 1989). Both *C-mos* and *RAG1* genes are single-copy, without introns. Besides a few insertions and deletions, there are no repetitive sequences that can cause any 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 the Squamata (Saint *et al.*, 1998; Townsend *et al.*, 2004; Vidal and Hedges, 2004). In this study, phylogenetic trees were documented for *L. reevesii rubritaeniata*, *L. belliana belliana*, *L. boehmei*, and

46 other squamate reptiles, using *RAG1* and *C-mos* as individual and combined data sets to speculate on the relationships of the genus *Leiolepis* in Thailand and confirm its phylogenetic position in the Squamata.

MATERIALS AND METHODS

Specimen collection

One adult female each of *L. reevesii rubritaeniata* and *L. boehmei* was collected from Nakhon Ratchasima and Songkhla provinces, respectively. An adult male of *L. belliana belliana* was captured in Chon Buri province. All experimental procedures conducted on the animals conformed to the guidelines established by the Animal Care Committee, Hokkaido University. Although *L. belliana ocellata* used to be found in Thailand, it was not available for this study.

DNA extraction

Whole genomic DNA, used as a template for PCR, was extracted from the livers of all individuals, following a standard phenol-chloroform-isoamylalcohol protocol (Sambrook and Russell, 2001), with slight modification. Briefly, after homogenization, the tissue was digested at 37°C overnight using 25 µg/µL proteinase K in 0.5% (w/v) SDS in STE buffer (0.1 M NaCl, 50 mM Tris and 1 mM EDTA, pH 8.0). Then, the mixture was extracted with phenol-chloroform-isoamylalcohol (25:24:1) and the DNA was precipitated with 0.05 volume of 0.2 M NaCl and 2.5 volume of 100% ethanol. After washing in 70% ethanol, the genomic DNA was air-dried and resuspended in TE buffer.

PCR amplification

PCR primers and conditions for the *RAG1* gene and *C-mos* gene were taken from San Mauro *et al.* (2004) and Godinho *et al.* (2006), respectively. The standard PCR reaction was performed using 1× ThermalPoll reaction buffer

containing 1.5 mM MgCl₂, 0.2 mM dNTPs, 5 pM specific primers and 0.25 U of NEB *Taq* polymerase (New England Biolabs, Ipswich, England) and 25 ng genomic DNA in a final reaction volume of 20 µL.

Cloning and DNA sequencing

Amplified products were examined by electrophoresis on 1% agarose gel; the DNA fragments were subsequently extracted from the ethidium bromide-stained gel and were then ligated to pGEM-T Easy Vector System I (Promega, Madison, WI, USA). The ligated plasmids were transformed into *Escherichia coli* DH5⁺ competent cells. Nucleotide sequences of the DNA fragments were determined by 1stBase DNA sequencing service (Malaysia). Then, a nucleotide sequence comparison against the National Center for Biotechnology Information (NCBI) database was performed using the blastx and the blastn program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All nucleotide sequences were deposited in DDBJ (DNA data bank of Japan, <http://www.ddbj.nig.ac.jp/submission-e.html>) and accession numbers are shown in Table 1.

Sequence analysis and data set

The *RAG1* and *C-mos* gene nucleotide sequences of *L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei* were aligned, using the default parameters of clustalX (Thompson *et al.*, 1997), to 46 other squamate reptiles, and 6 other reptilian and avian species as an outgroup taken from the NCBI database (Table1). Phylogenetic analyses were conducted with three data sets (*RAG1*, *C-mos* and the combined data set of the two genes). All unalignable sites and gap-containing sites were carefully checked before they were removed from these data sets. The 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 all codon positions, as implemented in PAUP* v. 4.0b10. Nucleotide saturation was analyzed for individual and all codon positions in each nucleotide data set by plotting the total number of transitions (Ts) + transversions (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.*, 2004) and PAUP* v. 4.0b10. The level of incongruence between the two genes was examined using PAUP*. This approach used the incongruence length difference (ILD) test with parsimony criterion (Farris *et al.*, 1995); 100 randomizations were performed.

Phylogenetic analysis

The phylogenetic trees were reconstructed by four different methods: maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ) and Bayesian inference (BI). The ML trees were generated with PHYML v.2.4.4 (Guindon and Gascuel, 2003) using non-parametric bootstrapping with 1,000 pseudoreplicates. The model and parameters indicated by Modeltest 3.7 were used, based on the Akaike Information Criterion (AIC) (Posada and Crandall, 1998). 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 was carried out at every 100th 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 the Bayesian posterior nodal relationship in the sampled tree population was shown as a percentage of the Bayesian posterior probability (BPP) obtained from a majority-rule consensus tree. MP and NJ were carried out using PAUP* v.

Table 1 Classification and accession numbers of species used in this study^a.

Class	Order	Suborder	Infraorder	Family	Species	Accession for <i>RAG1</i> gene	Accession for <i>C-mos</i> gene
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Leirolepis reevesii rubritaeniata</i>	AB516967*	AB516970*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Leirolepis belliana belliana</i>	AB516968*	AB516971*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Leirolepis boehmei</i>	AB516969*	AB516972*
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Agama agama</i>	EU402825	AF137530
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Physignathus cocincinus</i>	FJ356737	AF039476
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Uromastix acanthinura</i>	AY988025	AY987992
Reptilia	Squamata	Lacertilia	Iguania	Agamidae	<i>Physignathus lesueurii</i>	AY662581	DQ340689
Reptilia	Squamata	Lacertilia	Iguania	Chamaeleonidae	<i>Chamaeleo jacksonii</i>	AY988023	AF137528
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Microlophus peruvianus</i>	EF616443	EF615791
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Oplurus cuvieri</i>	AY662601	EU099681
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Chalarodon madagascariensis</i>	AY988018	EU099654
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Diplolaemus darwini</i>	AY988019	AY987988
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Crotaphytus collaris</i>	FJ356749	AY987985
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Basiliscus plumifrons</i>	AY662599	AY987986
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Phrynosoma cornutum</i>	FJ356738	AY987989
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Liolaemus lineomaculatus</i>	FJ356740	AY367903
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Polychrus marmoratus</i>	FJ356748	AY987983
Reptilia	Squamata	Lacertilia	Iguania	Iguanidae	<i>Dipsosaurus dorsalis</i>	FJ356747	AF148705
Reptilia	Squamata	Lacertilia	Gekkota	Gekkonidae	<i>Gekko gekko</i>	AY444054	EU366455
Reptilia	Squamata	Lacertilia	Dibamia	Dibamidae	<i>Dibamus montanus</i>	AY444053	AY444027
Reptilia	Squamata	Lacertilia	Scincomorpha	Scincidae	<i>Scelotes angina</i>	AY662635	AY217878
Reptilia	Squamata	Lacertilia	Scincomorpha	Teiidae	<i>Aspidoscelis tigris</i>	EU402829	AF039481
Reptilia	Squamata	Lacertilia	Diploglossa	Anniellidae	<i>Anniella pulchra</i>	AY662605	AY487350

^a New sequences from our study are indicated by *

Table 1 (Cont.) Classification and accession numbers of species used in this study^a.

Class	Order	Suborder	Infraorder	Family	Species	Accession for <i>RAG1</i> gene	Accession for <i>C-mos</i> gene
Reptilia	Squamata	Lacertilia	Diploglossa	Anguidae	<i>Ophisaurus gracilis</i>	AY444056	AY444030
Reptilia	Squamata	Lacertilia	Diploglossa	Xenosauridae	<i>Xenosaurus grandis</i>	AY662607	AY662567
Reptilia	Squamata	Lacertilia	Platynota	Varanidae	<i>Varanus salvator</i>	EU402828	AF435017
Reptilia	Squamata	Lacertilia	Platynota	Helodermatidae	<i>Heloderma suspectum</i>	AY662606	AY487348
Reptilia	Squamata	Lacertilia	Platynota	Lanthanotidae	<i>Lanthanotus borneensis</i>	AY662609	AY662564
Reptilia	Squamata	Amphisbaenia		Blaniidae	<i>Blanus strauchi</i>	AY444050	AY444024
Reptilia	Squamata	Amphisbaenia		Bipedidae	<i>Bipes biporus</i>	AY662616	AF039482
Reptilia	Squamata	Amphisbaenia		Amphisbaenidae	<i>Geocalanus acutus</i>	AY444043	FI441784
Reptilia	Squamata	Amphisbaenia		Rhineuridae	<i>Rhineura floridana</i>	AY662618	AY444022
Reptilia	Squamata	Amphisbaenia		Trogonophidae	<i>Trogonophis wiegmanni</i>	AY662617	FI441727
Reptilia	Squamata	Serpentes		Pythionidae	<i>Python reticulatus</i>	EU624119	AF544675
Reptilia	Squamata	Serpentes		Viperidae	<i>Daboia russellii</i>	EU402843	AF471156
Reptilia	Squamata	Serpentes		Acrochordidae	<i>Acrochordus granulatus</i>	EU402831	AF544706
Reptilia	Squamata	Serpentes		Cylindrophidae	<i>Cylindrophis ruffus</i>	AY662613	AF471133
Reptilia	Squamata	Serpentes		Loxocemidae	<i>Loxocemus bicolor</i>	EU402854	AY099969
Reptilia	Squamata	Serpentes		Xenopeltidae	<i>Xenopeltis unicolor</i>	EU402870	DQ465561
Reptilia	Squamata	Serpentes		Boidae	<i>Charina trivirgata</i>	EU402852	AY099974
Reptilia	Squamata	Serpentes		Colubridae	<i>Xenodermus javanicus</i>	EU402869	AF544711
Reptilia	Squamata	Serpentes		Elapidae	<i>Naja kaouthia</i>	EU402857	AY058938
Reptilia	Squamata	Serpentes		Anomalepididae	<i>Liotyphlops albirostris</i>	EU402853	AF544727
Reptilia	Squamata	Serpentes		Typhlopidae	<i>Ramphotyphlops braminus</i>	AY444062	AF544717
Reptilia	Squamata	Serpentes		Leptotyphlopidae	<i>Leptotyphlops humilis</i>	EU402851	AY099979
Reptilia	Squamata	Serpentes		Aniliidae	<i>Anilius scytale</i>	EU402834	AF544722
Reptilia	Squamata	Serpentes		Bolyeriidae	<i>Casarea dussumieri</i>	EU402840	AF471114
Reptilia	Squamata	Serpentes		Tropidophiidae	<i>Ungaliophis continentalis</i>	EU402867	AY099970
Reptilia	Rhynchocephalia	Sphenodontida		Sphenodontidae	<i>Sphenodon punctatus</i>	AY662576	AF039483
Reptilia	Crocodylia	Eusuchia		Crocodylidae	<i>Crocodylus porosus</i>	EU375509	FI011695
Reptilia	Crocodylia	Eusuchia		Crocodylidae	<i>Alligator sinensis</i>	AY239171	AY447979
Reptilia	Crocodylia	Eusuchia		Crocodylidae	<i>Tomistoma schlegelii</i>	AY239176	EF414017
Reptilia	Testudines	Pleurodira		Pelomedusidae	<i>Pelomedusa subrufa</i>	AY988102	FI230876
Aves	Passeriformes			Callaeatidae	<i>Creadion carunculatus</i>	AY443317	DQ469305

^a New sequences from the current study are indicated by *.

4.0b10 by heuristic searches, with the tree bisection-reconnection branch swapping (TBR) and 10 random taxon additions, while the non-parametric bootstrap analyses with 1,000 pseudoreplicates 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

General properties of sequences

The individual *RAG1* and *C-mos* data sets, and the combined data set of the same species were used to determine the genetic relationship and phylogenetic position of *Leiolepis* spp. in the 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 positions (Table 2). Similarly, the third codon position of *C-mos* data set exposed 49.77% informative sites, whereas the respective details of the 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 contained mainly informative characters to find the phylogenetic relationship from the *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 the two gene data sets were subsequently analyzed as individual and as all codon positions (Tarrio *et al.*, 2000). The results showed that the nucleotide frequencies were generally similar between the two genes in the three butterfly lizard species, and there were also no statistically significant proportion differences between squamate and other reptile species (Table 2), indicating that the two data set analyses had no heterogeneity of base frequencies, and the

Table 2 Properties of character variation for all data sets.

Data set	All aligned sequence	Parsimony-informative sites	Variable sites	Nucleotide bias			χ^2	d.f.	p-value	Best model ^a	I ^b	G ^c
				%A	%C	%G						
<i>RAG1</i>	657	295	353	28.99	19.95	24.18	26.88	51.86	159	1.0000	TrN+I+G	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		
<i>C-mos</i>	348	215	197	27.40	20.65	23.70	28.25	124.37	159	0.9805	HKY+I+G	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	2.2296

^a Best models were selected with Modeltest version 3.7

^b I : Proportion of invariable site

^c G : Gamma shape parameter

codon bias might not have distorted phylogenetic inference. Surprisingly, the GC contents of the *C-mos* gene sequences were clearly different between squamate reptiles (average 43.72 and 19.92% for the all codon and third codon position, respectively) and other reptile species (average 53.71 and 29.53% for the all codon and third codon position, respectively; Table 3 and data not shown), although base frequencies at the third codon position were not significantly heterogeneous. The substantial base composition difference between ingroup taxa and outgroup taxa might also have been responsible for incorrect rooting (Tarrio *et al.*, 2000). Harris (2003) found that there was a large difference in the GC content and codon usage between teiid lizards and other squamates, indicating that codon bias could cause the misconstruction of phylogeny. However, all of the topologies of the MP and ML analyses of the full *C-mos* data set were similar to those of the MP and ML analyses of the *C-mos* data set using the first and second codon positions. This situation was comparable to the *C-mos* data set, according to Townsend *et al.* (2004), which had a different third codon position GC content between outgroup taxa (average 63.2%) and ingroup 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 1). The regression lines were not momentarily different from straight lines, implying that saturation of third codon positions did not cause a problem in the two nuclear gene sequences at the level of homoplasy, and that there was a phylogenetic cue for all codon positions.

The ILD test revealed that there was some incongruences 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 have been the cause of this incongruence. However, the combination of partial *RAG1* and *C-mos* sequences has been used commonly (Townsend *et al.*, 2004; Vidal and Hedges, 2004) to reconstruct reliable phylogenetic trees. The current study also found that all topologies from the *RAG1* data set were highly similar to those from the combined data set. Therefore, the two data sets were combined and the results considered.

Phylogenetic analyses

The cladistic analysis was reconstructed, based on the *RAG1* and *C-mos* genes as separated and combined data sets, using BI, ML, MP and NJ. The Squamata was distinctly presented as a monophyletic group (Figure 2), but the phylogenetic pattern (chiefly within the basal

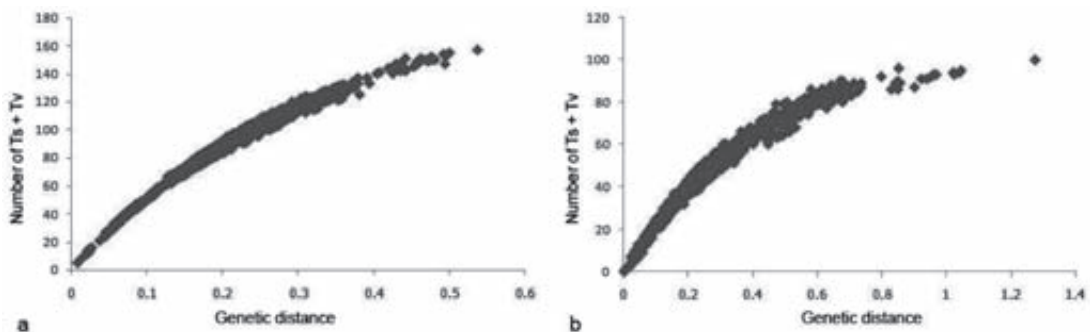


Figure 1 The relationship between the total number of transitions (Ts) + transversions (Tv) and corrected distance for all pairwise comparisons in: (a) *RAG1* sequence data set; and (b) *C-mos* sequence data set.

Table 3 Comparison of the base contents within *RAG1* and *C-mos* data sets.

Taxonomic organism	Percentage of bases with presented <i>RAG1</i> sequences					Percentage of bases with presented <i>C-mos</i> sequences				
	A	C	G	T	GC	A	C	G	T	GC
<i>L. reevesii rubritaeniata</i>	29.68	18.57	24.20	27.55	42.77	27.30	20.98	22.14	29.60	43.12
<i>L. belliana belliana</i>	29.68	19.03	24.05	27.25	43.08	27.01	20.69	22.13	30.17	42.82
<i>L. boehmei</i>	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
Amphibiaenia	29.28	20.40	24.20	26.12	44.60	28.84	21.44	22.86	26.86	44.30
<i>Sphenodon punctatus</i>	29.38	21.01	23.74	25.88	44.75	23.06	23.92	27.09	25.94	51.01
<i>Crocodylus porosus</i>	27.40	21.01	25.42	26.18	46.43	22.70	25.00	28.45	23.85	53.45
<i>Alligator sinensis</i>	28.46	20.55	24.81	26.18	45.36	23.56	25.00	27.59	23.85	52.59
<i>Tomistoma sinensis</i>	27.85	20.40	25.42	26.33	45.82	22.99	26.15	28.16	22.70	54.31
<i>Pelomedusa subrufa</i>	28.62	21.16	24.81	25.42	45.97	22.13	27.01	28.45	22.41	55.46
<i>Creadion carunculatus</i>	29.22	19.48	26.79	24.51	46.27	23.56	24.14	31.32	20.98	55.46

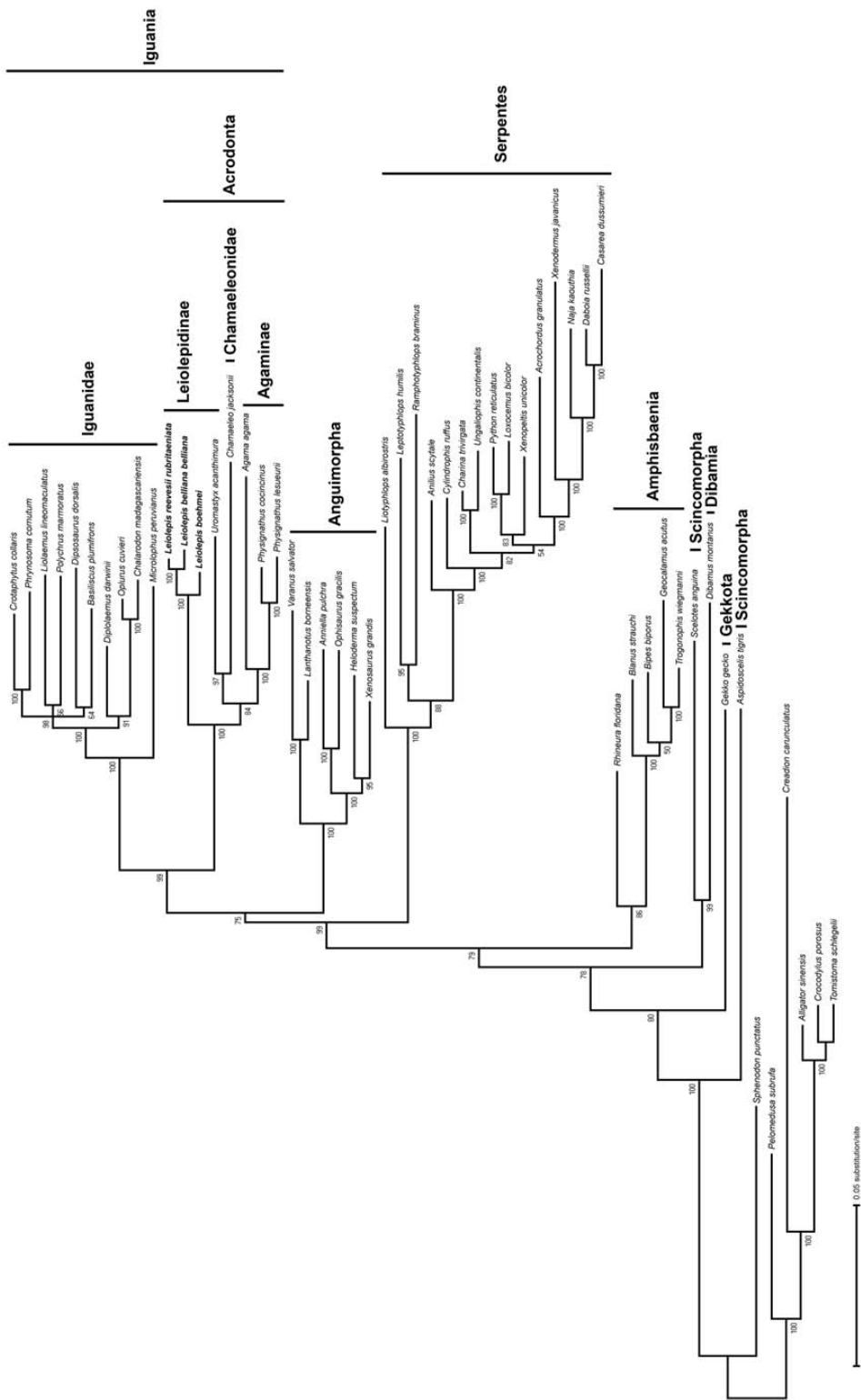


Figure 2 A Bayesian phylogram clarifying the phylogenetic relationship between *Leiolepis* spp. as a member of the Iguania and other squamate groups constructed using the combined *RAG1/C-mos* sequence data set. The 50% majority-rule consensus of post-burn-in sample trees from the Bayesian inference based on Tamura-Nei, AIC model is shown. Branch lengths are mean estimates.

splits) differed according to several of the methods of analysis. Specifically, the BI phylogram was quite similar to the ML phylogram, and closely resembled the previously reported molecular phylogenetic trees of the Squamata *RAG1* and *C-mos* gene tree (Vidal and Hedges, 2004), *RAG1*, *C-mos* and *ND2* gene tree (Townsend *et al.*, 2004), mitochondrial nucleotide sequence (Kumazawa, 2007) and *TSHZ1* and the *RAG1* gene tree (Schulte and Cartwright, 2009). However, all methods illustrated substantial agreement concerning the relationships within the infraorders and families of the Squamata. The grouping of the Gekkota, Dibamia and Scincomorpha strongly supported the basal position of the Squamata by all analyses. The large infraorder of the Iguania, comprising the two groups of Iguanidae and Acrodonta (Agamidae and Chamaeleonidae), formed a distinctive single clade with BI posterior probability (99%), which was also a sister relationship with the Anguimorpha (Diploglossa and Platyntota). The other significant clusters were the Serpentes and Amphisbaenia, which were strongly sustained with support values of 99 and 79%, respectively, in the BI analysis.

The Agamidae was categorized into two subfamilies, the Agaminae and Leiolepidinae, which were classified at the genus level as *Leiolepis* and *Uromastix* (Uets, 2009). However, the position of *Leiolepis*, *Uromastix* and the Chamaeleonidae could be diversely grouped in the phylogram from the current study. *Uromastix* and the Chamaeleonidae were sister taxon in *RAG1* and combined *RAG1/C-mos* BI analyses, whereas the latter taxon was monophyletic in the *C-mos* analysis. These inconclusive results were comparable to the individual and combined *RAG1/C-mos* gene trees and the *ND2* gene tree (Townsend *et al.*, 2004) and the combined *TSHZ1-RAG1* gene tree (Schulte and Cartwright, 2009), suggesting that the phylogenetic topology was influenced by many parameters. Therefore, outgroups, genes and taxon sampling might be explored as a relative effect (Albert *et al.*, 2009).

On the other hand, the morphological characters, lizard skull character (Stayton, 2005) and osteological and soft anatomical data (Lee, 2005) strongly supported the Agamidae as a monophyletic group. Schulte *et al.* (1998) suggested that the phylogenetic relationship of the Agamidae and Leiolepidinae was as metataxon, where monophyly was not found, but not statistically rejected. Thus, more molecular and morphological markers, and taxon sampling require further study to examine the relationship status of the Acrodonta.

In *Leiolepis* spp., all methods of statistical analyses strongly supported (100%) that *L. reevesii rubritaeniata* was more adjacent to *L. belliana belliana* than *L. boehmei* in the *RAG1* and combined *RAG1/C-mos* analyses (Figure 2). 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*. Fragments of the *C-mos* gene have been performed to assess the relationship across squamate reptiles (Saint *et al.*, 1998; Harris *et al.*, 2001); however, most relationships between families were not quite robust. This might have been caused by rapid cladogenesis or have been an artifact of limited sampling. Nevertheless, contrary to the chromosome number of 36 for *L. reevesii rubritaeniata* and *L. belliana belliana*, containing 12 bi-armed macrochromosomes (NF=24) and 24 microchromosomes, *L. boehmei* had a chromosome number of 34, containing 12 bi-armed macrochromosomes (NF=24) and 22 microchromosomes (Aranyavalai, 2003; Aranyavalai *et al.*, 2004; Srikuhnath *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 strongly suggested that *L. reevesii rubritaeniata* was closely related to *L. belliana belliana* rather than to *L.*

boehmei. Graybeal (1998), however, has shown that the addition of taxa improved the accuracy of a relationship rather than the addition of characters. Therefore, other molecular and morphological studies with additional taxa for the genus *Leiolepis* are also desirable to delineate precisely the phylogenetic relationship and hierarchy.

CONCLUSION

This is the first report on the genetic relationship of three butterfly lizard species (*L. reevesii rubritaeniata*, *L. belliana belliana* and *L. boehmei*) determined by molecular sequence analyses (*RAG1* and *C-mos* genes). Their molecular phylogeny revealed that *L. reevesii rubritaeniata* is related more closely to *L. belliana belliana* than to *L. boehmei*. This finding was also consistent with the morphological and chromosomal information of the butterfly lizard in Thailand. The phylogenetic position among the Leiolepidinae, Agaminae and Chamaeleonidae remained uncertain, though there were additional taxa in the Leiolepidinae in the current analysis that had not been used in other previous studies. Further study with additional taxa for the Leiolepidinae, Agaminae and Chamaeleonidae should be considered to elucidate their genetic relationship.

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