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Mitochondrial genomes from major lizard families suggest their phylogenetic relationships and ancient radiations

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Abstract

In placental mammals and birds, molecular data generally support a view that they diverged into their ordinal groups in good response to mid-Cretaceous continental fragmentations. However, such divergence patterns have rarely been studied for reptiles for which phylogenetic relationships among their major groups have not yet been established molecularly. Here, I determined complete or nearly complete mitochondrial DNA sequences from seven lizard families and reconstructed phylogenetic relationships between major lizard families. When snakes were included, maximum likelihood analysis did not support a morphological view of the snakes–varanoids affinity, although several other competing hypotheses on the position of snakes still cannot be discriminated presumably due to extremely long branches of the snake lineages. I also conducted clock-free Bayesian analyses to show that divergence times between major lizard families were centered in Triassic–Jurassic times. Thus, lizards include much deeper divergences than the mammals and birds and they appear to have already radiated into various families prior to the mid-Cretaceous major continental fragmentation.

Keywords: Squamata; Serpentes; Mitochondrial DNA; Molecular phylogeny; Divergence time; Biogeography

1. Introduction

Squamata is the most diversified reptilian order that has been traditionally classified into three suborders: Lacertilia (lizards) with 16–19 families, Serpentes (snakes) with 14–17 families and Amphisbaenia (worm lizards) with 3–4 families (e.g., Pough et al., 2004). Since squamates include groups with specialized or adapted morphological features (e.g., reduction of limbs and adaptation to fossorial life style on multiple lineages), phylogenetic relationships among these suborders and their families inferred based on morphological characters (e.g., Estes et al., 1988; Lee, 1998, 2000; Kearney, 2003) have been highly controversial.

Estes et al. (1988) suggested that extant lizard families can be divided into four infraorders (Iguania, Anguimorpha, Scincomorpha and Gekkota) and that Iguania has a sister relationship to Scleroglossa consisting of the other three infraorders. This sister relationship has been supported by a number of synapomorphies, such as tongue structure, feeding behavior, and brain morphology (e.g., Pough et al., 2004). Iguanians (agamids, chamaeleonids, and iguanids) mostly consist of visually oriented ambush-foraging species with lingual prehension for prey capture, whereas scleroglossans freed the tongue from involvement in prey acquisition and developed prey capture depending on use of jaws (e.g., Vitt et al., 2003; Pough et al., 2004). Gekkotans (geckos and relatives) then developed nocturnality and nasal olfaction, while active foraging and increased vomeronasal development gave autarchoglossans (Anguimorpha and Scincomorpha) access to hidden and sedentary prey (Vitt et al., 2003).

Although previous morphological studies (e.g., Estes et al., 1988; Lee, 1998) generally agreed to this relationship among

Abbreviations: cytb, cytochrome *b*; LA-PCR, long-and-accurate polymerase chain reaction; MCMC, Markov Chain Monte Carlo; ML, maximum likelihood; mtDNA, mitochondrial DNA; ND1–6, NADH dehydrogenase subunits 1–6.

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lacertilian infraorders, they provided different views on the placement of snakes, amphisbaenians, and dibamids (a minor fossorial family of lizards). For example, snakes have been placed at positions which are sister to varanoids (monitor lizards and relatives) (Lee, 1998, 2000), all lacertilians (Underwood, 1970), or amphisbaenians+dibamids (Estes et al., 1988; Kearney, 2003). The association of snakes with varanoids and mosasauroids (extinct marine lizards) led to a controversial hypothesis for marine snake origins (see, e.g., Lee and Caldwell, 2000).

Recent molecular work using various mitochondrial or nuclear gene regions (e.g., Forstner et al., 1995; Saint et al., 1998; Harris, 2003; Rest et al., 2003; Townsend et al., 2004; Vidal and Hedges, 2004, 2005) has tackled above-mentioned issues but more scrutinization using ample molecular characters is clearly needed. One of potentially useful targets for this is complete mitochondrial DNA (mtDNA) sequences that are still underrepresented in the database for reptiles as compared to mammals and fishes. In this study, I thus determined complete or nearly complete mtDNA sequences from seven lizard families and conducted phylogenetic analyses in order to gain insights into the tangled phylogenetic relationships and radiation processes in Squamata.

2. Materials and methods

2.1. Samples, DNA amplification, and sequencing

Table 1 lists eight squamate taxa sequenced for their mtDNAs in this study. Although a mtDNA sequence from an amphisbaenian taxa (the wedge-snouted worm lizard) has already been reported by Macey et al. (2004), phylogenetic analyses of this study were carried out with the sequence accidentally determined by me from the second individual of the same species. Samples were obtained from various sources. Samples for the Japanese grass lizard were caught inside the Nagoya University campus. Samples for the Western banded gecko were provided by Museum of Vertebrate Zoology, University of California at Berkeley. Samples for the Gila monster were provided by Higashiyama Zoo, Nagoya. Other samples originated from dead individuals in the pet shops.

Mitochondrial DNAs for the eight taxa were sequenced as described in Kumazawa and Endo (2004). Briefly, total DNA was extracted from a small quantity (20 mg) of tissues or blood, with which several short mtDNA fragments were amplified and sequenced in order to design taxon-specific primers for the long-and-accurate polymerase chain reaction (LA-PCR) amplifications (see Supplementary Table S1 for these LA-PCR primers). The typical condition for the LA-PCR using an LA-Taq (Takara Shuzo Co.) was 32 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 15 min. Using these LA-PCR products as a template, nested PCR amplifications for shorter regions were carried out with a set of reptile-oriented primers (Kumazawa and Endo, 2004). Sequences of these nested PCR products together with those obtained by the primer walking were assembled into a continuous mtDNA sequence.

In the mtDNA sequences thus obtained, individual 37 genes were identified in light of their counterparts from other vertebrates. Identification of tRNA genes was based on their secondary structures (Kumazawa and Nishida, 1993) whereas boundaries of rRNA genes and control regions were tentatively recognized by the range of adjacent coding genes. Sequences and annotations of these mtDNAs will appear in the DDBJ/EMBL/GenBank databases with accession numbers shown in Table 1.

2.2. Data sets

Individual gene sequences identified in the eight mtDNAs were aligned with counterparts of other 31 vertebrates, which were taken from the database: bearded dragon (AB166795), green iguana (AJ278511), fence lizard (AB079242), crocodile lizard (AB080274), alligator lizard (AB080273), Komodo

Table 1
Squamate taxa sequenced for their mtDNAs in this study

Scientific name (common name)	Family	mtDNA size (bp)	Accession no.	Voucher no.
Lacertilia				
Gekkota				
Coleonyx variegatus (Western banded gecko)	Eublepharidae	17,110	AB114446	MVZ12988
Gekko vittatus (lined gecko)	Gekkonidae	16,946*	AB178897	NUM-Az373
Iguania				
Furcifer oustaleti (Oustalet's chameleon)	Chamaeleonidae	18,021	AB185326	NUM-Az380
Scincomorpha				
Takydromus tachydromoides (Japanese grass lizard)	Lacertidae	18,245*	AB080237	NUM-Az364
Lepidophyma flavimaculatum (yellow-spotted night lizard)	Xantusiidae	16,158	AB162908	NUM-Az372
Anguimorpha				
Heloderma suspectum (Gila monster)	Helodermatidae	16,846	AB167711	–
Varanus niloticus (Nile monitor)	Varanidae	18,511	AB185327	NUM-Az381
Amphisbaenia				
Geocalamus acutus (wedge-snouted worm lizard)	Amphisbaenidae	16,682	AB162909	NUM-Az379

Asterisks mean that the corresponding mtDNAs could not be completely sequenced because of long tandem duplications or heteroplasmy in the major noncoding region. MVZ and NUM in voucher numbers mean Museum of Vertebrate Zoology, University of California at Berkeley and Nagoya University Museum, respectively. There is no voucher number for the Gila monster because we used blood samples taken from a live individual of the Higashiyama Zoo. Note that the complete mtDNA sequence of the wedge-snouted worm lizard was first determined by Macey et al. (2004).

monitor (AB080275 and AB080276), mole skink (AB016606), spinytail lizard (AB079613), Texas blind snake (AB079597), akamata (AB008539), himehabu (AB175670), little file snake (AB177879), boa constrictor (AB177354), red pipe snake (AB179619), sunbeam snake (AB179620), ball python (AB177878), tuatara (AF534390), chicken (X52392), indigo bird (AF090341), alligator (Y13113), caiman (AJ404872), green turtle (AB012104), helmeted turtle (AF039066), cow (J01394), platypus (X83427), toad (M10217), caecilian (AF154051), lungfish (L42813), coelacanth (U82228), loach (M91245), and trout (L29771). The gekkonid *Teratoscincus keyserlingii* (Macey et al., 2005) was not included because its mtDNA showed an approximately 300 bp of truncation inside cytochrome *b* (cytb) gene. Gekkota was represented by other two taxa in my data set (Western banded gecko and lined gecko; see Table 1).

Phylogenetic analyses were conducted with both nucleotide and amino acid sequences. A nucleotide data set consisted of concatenated light-strand sequences of first and second codon positions of 12 heavy-strand-encoded protein genes, 22 tRNA genes, and 2 rRNA genes, whereas an amino acid data set consisted of concatenated amino acid sequences of 12 heavy-strand-encoded protein genes. All unalignable sites, as well as gap-containing sites were carefully removed from these data sets.

Because Rest et al. (2003) found the tuatara mtDNA not to encode genes for NADH dehydrogenase subunit 5 (ND5) (the longest of all mitochondrial protein genes), $tRNA^{His}$, and $tRNA^{Thr}$, inclusion of this taxon into the analyses resulted in a considerably reduced number of aligned sites, which may have a profound effect on phylogenetic analyses. I therefore examined two different conditions (i.e., with and without tuatara) for some statistical analyses in which phylogenetic hypotheses were compared by the maximum likelihood (ML) criterion.

2.3. Phylogenetic analyses

Phylogenetic trees were made by ML and Bayesian methods primarily using the nucleotide data set. The ML trees were made with PAUP* 4.0b10 (Swofford, 2003) by heuristic searches with the TBR branch swapping and 10 random taxon additions. The general reversible model (GTR+I+G) and parameters optimized by modeltest 3.0 (Posada and Crandall, 1998) were used. For the Bayesian analyses, MrBayes v3.0 (Huelsenbeck and Ronquist, 2001) was used with the GTR+I+G model by separating the data into three partitions (codon first positions, codon second positions, and rRNA and tRNA positions). The Markov Chain Monte Carlo (MCMC) process was set to run four chains simultaneously. After the log-likelihood value reached stationarity, 10,000 trees were sampled in every 100 generation to provide a 50% majority-rule consensus tree with averaged branch lengths and Bayesian posterior probability values in which the frequency of a specific nodal relationship in the sampled tree population is shown as a percentage.

Tree-support values were assessed with the above-mentioned Bayesian posterior probabilities, ML bootstrap values obtained with Treefinder (Jobb, 2004) using the GTR+G model, and neighbor-joining bootstrap values obtained with PAUP* 4.0b10 (Swofford, 2003) using the ML distances under the model and parameters optimized by modeltest 3.0 (Posada and Crandall, 1998).

Statistical evaluation of alternative phylogenetic hypotheses was done with TREE-PUZZLE 5.2 (Schmidt et al., 2002) using the two-sided Kishino and Hasegawa (1989) test and the Shimodaira and Hasegawa (1999) test. As stated above, both nucleotide and amino acid data sets were examined with and without the tuatara. The GTR+I+G model and its parameters optimized by modeltest 3.0 were used for the nucleotide data set, whereas the mtREV24+I+G model was used for the amino acid data set using parameters optimized with TREE-PUZZLE 5.2.

2.4. Divergence time estimation

For the divergence time estimation, both the nucleotide and amino acid data sets were used with the multidistribute program (Thorne et al., 1998) by assuming a topological relationship thus obtained (see Sections 3.2 and 3.3 for details) but without assuming the molecular clock (i.e., by allowing the rate evolution along branches). Upper and/or lower time constraints at selected nodes were set for the Bayesian MCMC processes to estimate divergence times and relative rates at ingroup nodes. I first used PAML (Yang, 1997) to optimize parameters for the model (F84 for the nucleotide sequence data and mtREV24-F for the amino acid sequence data) and the gamma distribution for 8 categories to account for the site-heterogeneity. The estbranches/multidivtime programs were then used to estimate divergence times using four reliable time constraints shown in Table S2.

3. Results and discussion

3.1. Mitochondrial genomes from major lizard families

In this study, I determined eight complete or nearly complete mtDNA sequences from seven lizard families and one amphisbaenian (Table 1). Five of the seven lizard families (Eublepharidae, Chamaeleonidae, Lacertidae, Xantusiidae, and Helodermatidae) have been previously unrepresented for the complete mitochondrial genomes. All 37 genes encoding 2 rRNAs, 22 tRNAs and 13 proteins were identified in these eight mtDNAs basically in the same order and orientation as found for most other vertebrates, albeit with some exceptions: 1) switch of the $tRNA^{Ile}$ and $tRNA^{Gln}$ genes (Macey et al., 1997) for the Oustalet's chameleon, 2) juxtaposition of the $tRNA^{Pro}$ gene not to the $tRNA^{Thr}$ gene but to the $tRNA^{Phe}$ gene for the Oustalet's chameleon, and 3) extensive shuffling of five adjacent genes (i.e., ND6, $tRNA^{Glu}$, cytb, $tRNA^{Thr}$, and $tRNA^{Pro}$), duplication of the control region, and insertion of a repetitive noncoding region for the Nile monitor as found for the Komodo monitor (Kumazawa and Endo, 2004). Since a molecular phylogeny on varanids (Ast, 2001) showed basal divergence of lineages leading to the Komodo and Nile monitors, sharing of the rearranged gene organization by these species but not by any other anguimorphans suggests occurrence of the gene rearrangements in an ancestral varanid lineage. Base compositions of

these new mtDNAs are similarly skewed as those of other vertebrates (e.g., Asakawa et al., 1991) (data not shown).

3.2. Phylogenetic relationships among major lizard families

Fig. 1 shows a Bayesian tree in which all available lacertilian taxa are included without snakes that have highly accelerated evolutionary rates of mtDNA sequences (Kumazawa et al., 1998). Four major groups corresponding to morphology-based lacertilian infraorders (Iguania, Anguimorpha, Scincomorpha and Gekkota) were recognized. Although Scincomorpha appeared to be paraphyletic relative to Iguania and Anguimorpha (Fig. 1), the Kishino–Hasegawa and Shimodaira–Hasegawa tests (Table 2) showed that monophyly of scincomorphan families was not statistically rejectable. Paraphyly of Scincomorpha has been suggested by some morphological studies (e.g., Lee, 1998), albeit with different scincomorphan relationships.

Contrary to previous morphological studies (Estes et al., 1988; Lee, 1998) but in agreement with recent molecular ones mostly using nuclear genes (Harris, 2003; Townsend et al., 2004; Vidal and Hedges, 2004, 2005), Iguania did not represent the earliest shoot-off among the four major groups and Amphisbaenia was nested within Lacertilia at a sister position to lacertids. Inclusion of more amphisbaenian taxa (Macey et al., 2004) did not change the sister position of Amphisbaenia to the lacertids (data not shown). Statistical tests (Table 2) showed that hypotheses for the earliest shoot-off of Iguania (including two morphology-based topologies) and the origin of Amphisbaenia outside Lacertilia are very unlikely. Thus, the independent suborder status for Amphisbaenia is now very questionable from molecular points of view using both nuclear and mitochondrial sequences.

To the best of my knowledge, molecular phylogeny based on the longest nuclear gene sequences is by Vidal and Hedges (2005). It is noteworthy that the nuclearly constructed tree (Vidal and Hedges, 2005) is very similar to my mitochondrial tree (Fig. 1) with respect to relationships between commonly represented families. In both trees, Gokkota diverged first among the four infraorders, and Scincomorpha is a paraphyletic assemblage in which a scincid+cordylid+xantusiid clade has a

node	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Bayes-PP	100	100	100	100	100	100	100	100	100	100	99	83	91	100	100
ML-BP	100	100	68	57	80	51	98	56	98	100	78	—	—	100	100
NJ-BP	100	100	—	69	80	—	99	81	89	100	97	—	—	100	100

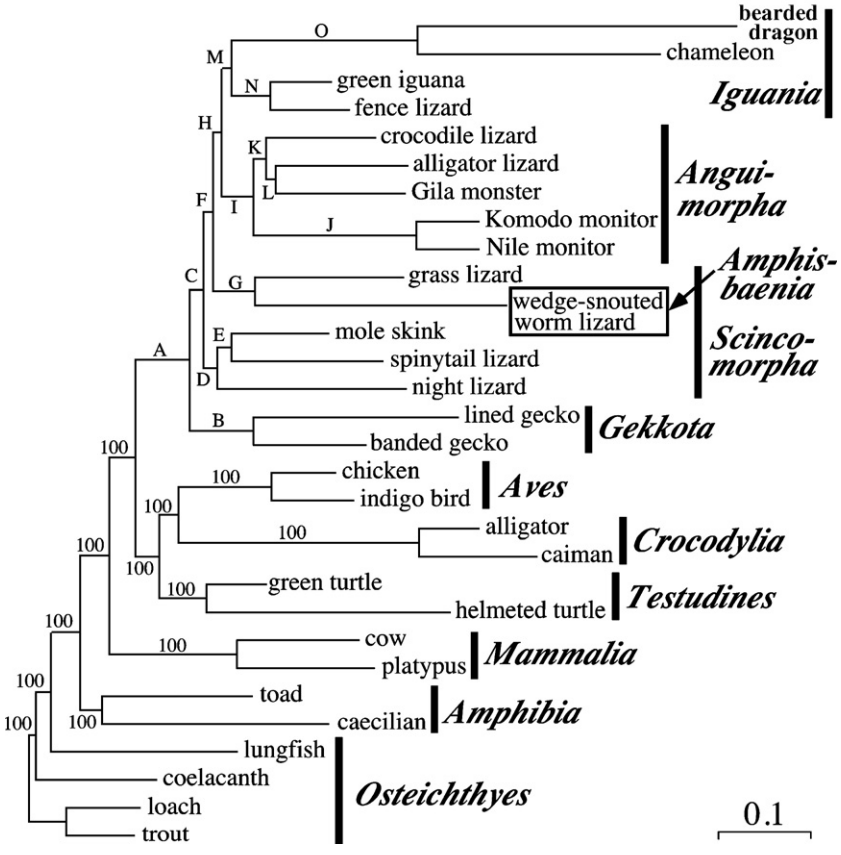

Fig. 1. A Bayesian tree constructed using the mitochondrial nucleotide sequences (9542 alignable sites for 30 taxa). Shown here is a 50% majority-rule consensus tree with averaged branch lengths and Bayesian posterior probability (Bayes-PP) values on branches. The same topological relationships were obtained with the ML method (data not shown). See Section 2.3 for details in analytical procedures. *Inset*, tree-support values for branches A–O obtained using different methods, i.e., the Bayes-PP, bootstrap probabilities from heuristic ML analyses (ML-BP) based on the GTR+G model with Treefinder (Jobb, 2004), and bootstrap probabilities from neighbor-joining analyses (NJ-BP) using the ML distances based on the GTR+I+G model with PAUP*4.0b10 (Swofford, 2003). Both BP values were obtained from 500 replications. Tree-support values are shown only when they are more than 50%.

Table 2
Evaluation of alternative hypotheses on lizard relationships

Tree		Nucleotide sequences (1st+2nd positions of L-strand proteins, rRNAs, and tRNAs)				Amino acid sequences (L-strand proteins)			
		−Tuatara (9542 sites)		+Tuatara (8407 sites)		−Tuatara (3361 sites)		+Tuatara (2848 sites)	
		pKH	pSH	pKH	pSH	pKH	pSH	pKH	pSH
1	Lizards' topology as in Fig. 1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
2	Monophyly of Scincomorpha	0.140	0.574	0.285	0.703	0.130	0.439	0.492	0.737
3	Earliest shoot-off of Iguania	0.001*	0.004*	0.001*	0.007*	0.026*	0.102	0.104	0.222
4	Origin of Amphisbaenia outside Lacertilia	0.000*	0.012*	0.001*	0.043*	0.000*	0.017*	0.001*	0.022*
5	Morphological tree (Estes et al., 1988)	0.000*	0.003*	0.001*	0.000*	0.006*	0.011*	0.112	0.128
6	Morphological tree (Lee, 1998)	0.000*	0.000*	0.000*	0.000*	0.001*	0.001*	0.004*	0.004*

Probabilities for alternative phylogenetic relationships were assessed using the two-sided Kishino–Hasegawa test (pKH) and the Shimodaira–Hasegawa test (pSH). Four concatenated data sets using either the nucleotide sequences (GTR+I+G model) or amino acid sequences (mtREV24+I+G model) with or without the tuatara were examined with TREE-PUZZLE 5.2 (Schmidt et al., 2002). Probabilities with an asterisk mean significant ($p<0.05$) rejection of the corresponding hypotheses. See Supplementary Table S3 for Newick representations of topological relationships.

sister relationship with a lacertid+amphisbaenian clade and an iguanian + anguimorphan clade. A major difference between the trees lies only in the placement of snakes (see below).

3.3. Phylogenetic position of snakes

Phylogenetic affiliation of snakes relative to lizards and amphisbaenians has been another controversial matter. Addition of eight snakes to the 30 taxa of Fig. 1 resulted in clustering of snakes with acrodonts (agamids and chamaeleonids) (data not shown). However, as suggested by Townsend et al. (2004), this seems to be misled by the long branch attraction between rapidly evolving acrodont and snake sequences because exclusion of two acrodont taxa did not result in connection of the snakes to the iguanids (remaining members of Iguania) but to a more basal lineage, either to the lacertid–amphisbaenian clade or to the sister position of all lacertilian taxa depending on the exclusion or inclusion of the tuatara (Supplementary Figs. S1 and S2).

Statistical tests on alternative positions of snakes (Table 3) indicated that these two Bayesian trees, which correspond, respectively, to Tree 7 and Tree 1 in Table 3, and other hypotheses that connect snakes to several alternative basal positions within Lacertilia (e.g., Tree 2 connecting snakes to ancestral lineages of Gekkota) are not significantly discriminable, although probabilities for the snakes–varanids affinity (Tree 11) (Lee, 1998) were close to a rejectable level using the nucleotide data set ($0.04<p<0.11$). The snakes' affinity to an iguanian +anguimorphan clade (Tree 9) (Vidal and Hedges, 2005) was not rejected in most analytical conditions. Only using the amino acid data set without the tuatara, the Kishino–Hasegawa test regarded this hypothesis rejectable.

Table 3
Evaluation of alternative hypotheses on phylogenetic affiliation of snakes

Tree		Nucleotide sequences (1st+2nd positions of L-strand proteins, rRNAs, and tRNAs)				Amino acid sequences (L-strand proteins)			
		−Tuatara (9462 sites)		+Tuatara (8336 sites)		−Tuatara (3353 sites)		+Tuatara (2843 sites)	
No	Position of snakes	pKH	pSH	pKH	pSH	pKH	pSH	pKH	pSH
1	Branch A	1.000	1.000	1.000	1.000	0.257	0.368	0.757	0.740
2	Branch B	0.095	0.578	0.154	0.612	0.122	0.237	0.591	0.649
3	Branch C	0.767	0.931	0.833	0.940	0.035*	0.148	0.418	0.575
4	Branch D	0.337	0.598	0.320	0.516	0.002*	0.056	0.024*	0.210
5	Branch E	0.058	0.174	0.134	0.274	0.001*	0.018*	0.027*	0.182
6	Branch F	0.655	0.864	0.316	0.543	0.021*	0.408	0.064	0.559
7	Branch G	0.980	0.951	0.411	0.518	1.000	1.000	1.000	1.000
8	The worm lizard	0.440	0.459	0.163	0.197	0.892	0.776	0.882	0.768
9	Branch H	0.416	0.596	0.125	0.208	0.025*	0.427	0.058	0.553
10	Branch I	0.337	0.434	0.070	0.070	0.186	0.618	0.496	0.808
11	Branch J	0.108	0.113	0.041*	0.043*	0.084	0.136	0.376	0.420

Phylogenetic relationships shown in Fig. 1 (but without two acrodont taxa) were assumed and eight snake taxa were joined to each of A–M internal branches and terminal branches of Fig. 1. Among these alternative hypotheses, only those with competitive log-likelihood values and those corresponding to previous hypotheses are listed in this table. Tree 1 represents a hypothesis for the mutual monophyly of lizards and snakes (Underwood, 1970; see also Fig. S2). Trees 7, 9 and 11 represent hypotheses for the snakes' affinity to the lacertid–amphisbaenian clade (Fig. S1), the iguanian+anguimorphan clade (Vidal and Hedges, 2005) and varanids (Lee, 1998), respectively. Probabilities for alternative phylogenetic relationships were assessed using the two-sided Kishino–Hasegawa test (pKH) and the Shimodaira–Hasegawa test (pSH). Four concatenated data sets using either the nucleotide sequences (GTR+I+G model) or amino acid sequences (mtREV24+I+G model) with or without the tuatara were examined with TREE-PUZZLE 5.2 (Schmidt et al., 2002). Probabilities with an asterisk mean significant ($p<0.05$) rejection of the corresponding hypotheses.

Kumazawa (2004) and Dong and Kumazawa (2005) previously supported a hypothesis for the mutual monophyly of snakes and lizards based on complete mtDNA sequences. However, this study was based on analyses using only seven lizards (no gekkotans). The present study considerably increased taxon numbers for lizards (14 taxa including an amphisbaenian) with a hope to provide this issue with an increased resolution, which does not seem to have been realized well. The mitogenomic data indeed discriminated a morphology-based hypothesis (Tree 11 in Table 3) but could not point to a single tree among several alternative hypotheses. This may be primarily due to the accelerated molecular evolutionary rates for snakes. The long branch attraction may erroneously urge the snakes to cluster with other long branches in lacertilian or non-lacertilian groups. If this is the case, overcoming the long branch attraction problem by increasing taxon samplings (Page and Holmes, 1998) and/or development of novel models to describe distinct molecular evolutionary rates and modes for different branches may be future ways.

Recently, Piskurek et al. (2006) characterized short interspersed elements from saurian nuclear genomes. Based on the structural similarities of the elements, they referred to a possibility that snakes and anguimorphans are closely related. Thus, current molecular approaches have not converged to a clear conclusion on the phylogenetic affiliation of snakes. Further investigation of this issue using different disciplines should be encouraged in the future.

3.4. Radiation of major lizard families

Gamma-corrected distances of mtDNA sequences were shown to be applicable to dating deep divergences (Kumazawa et al., 2004). Using the Bayesian method that allows evolution of molecular evolutionary rates over time (Thorne et al., 1998), divergence times were estimated between major squamate lineages (Fig. 2). The early squamate fossil record is extremely patchy and there seems to be a major gap between time of the first fossil occurrence of a particular family and real divergence time from its sister family (Evans, 2003). Thus, my time estimates between squamate families basically rely on several reliable time constraints between outgroup taxa (Supplementary Table S2).

Fig. 2 suggests a Permian–early Triassic origin of Squamata, though there is no certain fossil record of squamates before the early Jurassic (Evans, 2003). It was also suggested that lizard families diverged from each other during Triassic–Jurassic times, with an exception of the mid-Cretaceous divergence between Agamidae and Chamaeleonidae, whereas families of alethinophidian snakes ('typical snakes' other than blind snakes) did so much more recently in the Tertiary (Fig. 2). These results were significantly affected neither by the equivocal phylogenetic position of snakes (e.g., whether sister to the lacertid–amphisbaenian clade or to all the lizard families; data not shown) nor by use of the amino acid data set (data not shown).

There is no contradiction between the time estimates in Fig. 2 and fossil evidence. The occurrence of some crown-group lizards

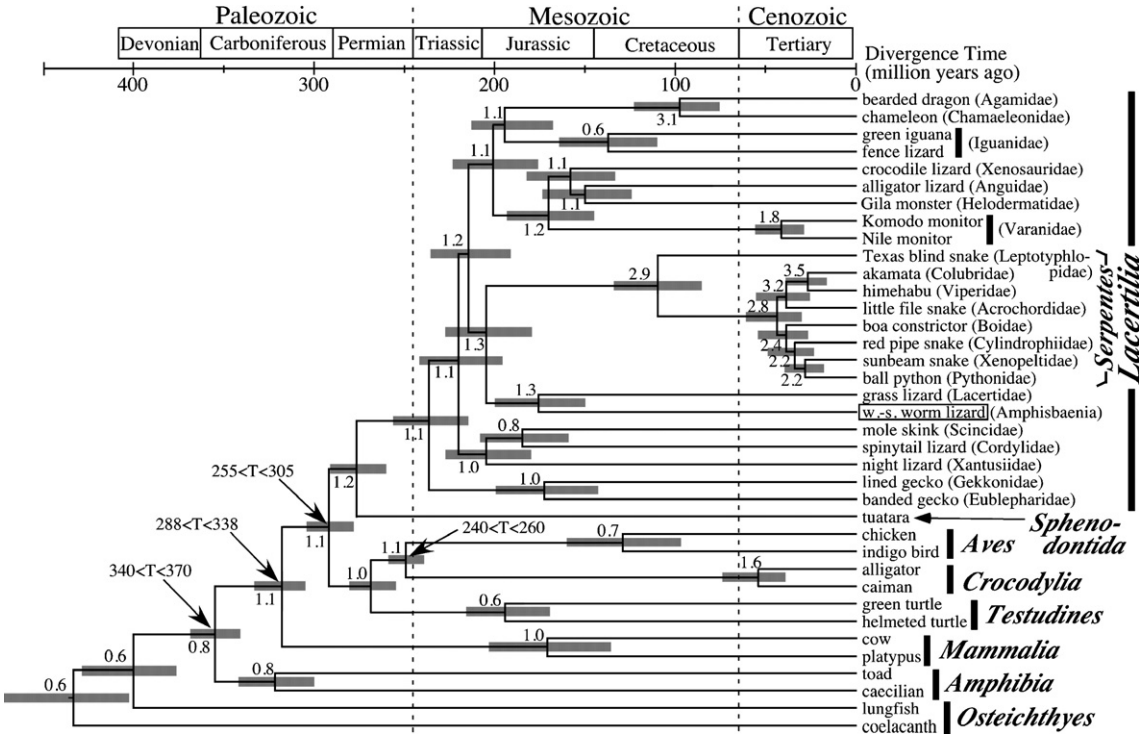

Fig. 2. Estimated divergence times and their 95% credibility intervals (hatched rectangles) inferred using the nucleotide data set (8310 sites for 39 taxa). These 39 taxa include 8 snakes and the tuatara in addition to the 30 taxa used for Fig. 1. Estimated rates at internal nodes ($\times 10^{-3}$/million years) are also shown. Phylogenetic relationships were assumed based on results of Supplementary Fig. S1, Table 3, and an argument on the phylogenetic position of snakes and acrodont lizards (see text). The multidistribute program (Thorne et al., 1998) was used with four reliable time constraints shown in this figure and Table S2. Inclusion of some more time constraints within Lepidosauria (lower constraints based on fossil evidence; see Table S2) hardly changed estimated divergence times between squamate groups (data not shown) presumably because the lower limitation values for these constraints still underestimate their true divergence times.

from the middle Jurassic does imply the substantially older origin of Squamata (Evans, 2003). The mid-Cretaceous divergence between Agamidae and Chamaeleonidae is consistent with some recent molecular work within Agamidae (Hugall and Lee, 2004; Amer and Kumazawa, 2005).

These results have taxonomic and biogeographic implications. The substantially older divergence times and much more genetic divergences between lizard families than those between snake families (Fig. 2) and between mammalian and avian orders (reviewed in Hasegawa et al., 2003) may request taxonomists to reevaluate morphological diversities between lizard families to evaluate the adequacy of their taxonomic rank.

Estes (1983) hypothesized that Iguania diverged from the other lizard groups vicariantly by the break-up of Pangea into Laurasia and Gondwanaland by the middle Jurassic. However, my results are not in agreement with this hypothesis with respect to both phylogenetic relationships (Fig. 1) and divergence times (Fig. 2). Rather, I suggest that this vicariant event may correspond to divergences between crown lizard groups, such as between Eublepharidae and Gekkonidae (Kluge, 1987). Finally, I conclude that a large scale of continental break-ups during Cretaceous time, which have been postulated to be responsible for the divergence and preservation of major mammalian lineages (Hedges et al., 1996), may have correlated with divergences of lizards, if any, not at the family level but at the subfamily or genus level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2006.09.026.

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