Mitochondrial DNA Sequences of Five Squamates: Phylogenetic Affiliation of Snakes

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Abstract

Complete or nearly complete mitochondrial DNA sequences were determined from four lizards (Western fence lizard, Warren’s spinytail lizard, Terrestrial arboreal alligator lizard, and Chinese crocodile lizard) and a snake (Texas blind snake). These genomes had a typical gene organization found in those of most mammals and fishes, except for a translocation of the glutamine tRNA gene in the blind snake and a tandem duplication of the threonine and proline tRNA genes in the spinytail lizard. Although previous work showed the existence of duplicate control regions in mitochondrial DNAs of several snakes, the blind snake did not have this characteristic. Phylogenetic analyses based on different tree-building methods consistently supported that the blind snake and a colubrid snake (akamata) make a sister clade relative to all the lizard taxa from six different families. An alternative hypothesis that snakes evolved from a lineage of varanoids was not favored and nearly statistically rejected by the Kishino-Hasegawa test. It is therefore likely that the apparent similarity of the tongue structure between snakes and varanoids independently evolved and that the duplication of the control region occurred on a snake lineage after divergence of the blind snake.

Key words: reptile; mitochondrial genome; molecular phylogeny; gene rearrangement

Mitochondrial DNAs (mtDNAs) of vertebrates are 16–18 kbp double-stranded circular DNAs that encode genes for 2 rRNAs, 22 tRNAs, and 13 respiratory proteins (reviewed in Boore1). The mtDNA sequences have been frequently used for phylogenetic studies because of the conservative gene organization, the lack of introns, their relative abundance in cells, and the presumed orthology of genes collected from different species. However, it is often difficult to resolve phylogenetic relationships confidently among distantly related animals with single or a few mitochondrial gene sequences (see, e.g., Cao et al.3). Multiple substitutions at the same site may give rise to homoplasious changes that become noise in phylogenetic analyses.4 The use of a large quantity of data (e.g., complete encoded genes of mtDNA) could overcome this problem by allowing phylogenetic signals to be amplified over random noise.5

Reptiles are a paraphyletic group of vertebrates, from which mammals and birds evolved.6 By acquiring the amnion (a membrane in an egg) and being free from the need to lay eggs in an aquatic environment, they adapted themselves to various terrestrial (and aquatic) environments and developed remarkable morphological variations.6 Because of the adaptational or specialized features of many morphological characteristics in reptiles, phylogenetic relationships among major reptilian groups have often been debated, leaving a number of open questions and testable hypotheses that may be addressed at the molecular level.6 Extant members of the class Reptilia have been traditionally classified into four orders: Testudines (turtles), Squamata (lizards and snakes), Sphenodontida (tuataras), and Crocodylia (crocodilians).6 Among them, squamates are the most diversified group, containing approximately 7800 species distributed throughout the world.7 Complete mtDNA sequences have been reported from relatively few species of squamates8–10 In an accompanying paper,11 we described a method to sequence squamate mtDNAs efficiently by designing a set of primers conserved among known squamate mtDNAs and reported sequences and some relevant features of the Komodo dragon mtDNA. In order to prove the usefulness of this methodology, it seems important to apply it to various taxa of squamates and other nonguamate reptiles.

In the present study, I report mtDNA sequences from four lizards and a snake. The data were used to address a
Table 1. Five squamate taxa sequenced in this study.

| scientific name (common name) | family              | mtDNA size | accession No. | voucher No. |
|-------------------------------|---------------------|------------|---------------|-------------|
| Sceloporus occidentalis       | Iguanidae           | 17072      | AB079242      |             |
| (Western fence lizard)        |                     |            |               |             |
| Cordylus warreni              | Cordylidae          | 17184      | AB079613      | NUM-Az 0371* |
| (Warren’s spinytail lizard)   |                     |            |               |             |
| Abronia graminea              | Anguidae            | 16016*     | AB080273      | NUM-Az 0369* |
| (Terrestrial arboreal alligator lizard) |         |            |               |             |
| Shinisaurus crocodilurus      | Xenosauridae        | 16583*     | AB080274      | NUM-Az 0370* |
| (Chinese crocodile lizard)    |                     |            |               |             |
| Leptotyphlops dulcis          | Leptotyphlopidae    | 16218      | AB079597      | MVZ 14037   |
| (Texas blind snake)           |                     |            |               |             |

Sample for the blind snake was provided by the Museum of Vertebrate Zoology of the University of California at Berkeley (MVZ), and other samples were gifted by Mr. Kosho Yagi. Asterisks mean that the corresponding mtDNAs could not be completely sequenced because of the presence of long tandem duplications in the major noncoding region. *Specimens deposited to Nagoya University Museum (NUM).

long-standing question with respect to the phylogenetic position of snakes relative to lizards. I also discuss the origin of the duplicate states of the control regions in squamate mitochondrial genomes.

1. Sequencing the Mitochondrial Genomes

Table 1 lists names of five squamate taxa sequenced in this study. Four lizard taxa were chosen from separate families. Using the procedure described in the footnote of Table 2, complete or nearly complete mtDNA sequences were determined from the five squamates (see Table 1 for the total size of each mtDNA). These sequences will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers shown in Table 1.

2. Characteristics of the Mitochondrial Genomes

Encoded genes were identified in light of sequence similarity to orthologous genes for other species, as well as secondary structures of tRNA and rRNA genes. All 37 genes encoding 2 rRNAs, 22 tRNAs and 13 proteins were identified in these mtDNAs basically in the same order and orientation as found for most other vertebrates. However, there were some exceptions. First, the glutamine tRNA gene was translocated from the IQM tRNA gene cluster to the WANCY tRNA gene cluster, giving rise to IM plus WQANCY organizations in mtDNA of the blind snake. This is a reconfirmation of our previous finding. Second, the threonine and proline tRNA genes were tandemly duplicated in mtDNA of the spinytail lizard. Since two copies of the proline tRNA gene had exactly the same nucleotide sequence, both copies were judged to be functional. Two copies of the threonine tRNA gene were different in two positions and the second copy seemed to allow two mismatched base pairs in the acceptor and anticodon stem regions (data not shown). I therefore tentatively assumed that the first copy represents a primary threonine tRNA gene.

A major noncoding region or the control region was typically present between the proline and phenylalanine tRNA genes for all the five squamates. Conserved sequence blocks (CSB) I, II and III have been known to be conserved sequence elements among mammalian control regions (reviewed in Clayton13). CSB I and III were identified for three squamate taxa whose major noncoding region was completely sequenced (i.e., the fence lizard, the spinytail lizard, and the blind snake). CSB II was also found for the fence lizard, the spinytail lizard, and the crocodile lizard. It is therefore suggested that the major noncoding regions for these species include controlling signals for replication, as demonstrated for mammals.13

Another feature related to DNA replication is the light-strand replication origin recognizable as a stable stem-and-loop structure between the asparagine and cysteine tRNA genes in the WANCY tRNA gene cluster.13 This structure was found for all the lizard taxa, but not for the blind snake. This finding suggests that the replication mechanism of at least lizards is similar to the asymmetric mechanism of mammals. On the other hand, transcriptional promoter sequences, which were biochem-
Table 2. Primers used for initial long PCR amplifications of mtDNA segments for each taxon.

| primer name [length] | matching gene | sequence (5' to 3') |
|----------------------|---------------|---------------------|
| **Western fence lizard** |
| Socc-L2 [22mer]   | Leu(CUN)  | CTCCAGTAAAAGTACATGCAC |
| Socc-L3 [25mer]  | 12S      | TTAAGATAAAAACACAACCGCATGGT |
| Socc-L4 [20mer]  | cytb    | CAGACAAATGCCCTTTCCAC |
| Socc-H2 [23mer]  | His     | TTAATTTAGCAGGATTAGGCC |
| Socc-H3 [9mer]   | 12S     | TCGTATAACCGGCCGTTGCT |
| ucytb-1H [26mer] | cytb    | GCCCTCAGAATATTTGCTCTCA |
| **Warren’s spinytail lizard** |
| Cwar-L1 [22mer]  | Gln      | AGGAATTGACACTATATTTGAG |
| Cwar-L3 [23mer]  | COIII   | GCCTACGCTATTATTTGCTCA |
| Cwar-H1 [23mer]  | Ile     | GTAGTCCTTTAATTAAGGCACAG |
| Cwar-H3 [22mer]  | COIII   | CGCCTGATGCCAGAAGACTGC |
| **Terrestrial arboreal alligator lizard** |
| Agra-L1 [27mer]  | Gln      | CTAGAAAGACAGGAATGATAGTTAC |
| Agra-L2 [24mer]  | cytb    | GGCGAGGGCTCTACTACGGCTCA |
| rPhe-1L [21mer]  | Phe     | AAAGACGGCACTGAAARATGC |
| Agra-H1 [27mer]  | Ile     | AATAGTCCTTTTGTTCAGGCACGCTTC |
| Agra-H2 [28mer]  | cytb    | GTAATAGTCATCCGTAATTAACGTCTCG |
| Agra-H3 [23mer]  | 12S     | CATCTCAGGAAATTTTGATGC |
| **Chinese crocodile lizard** |
| Scro-L1 [22mer]  | 12S     | CGCTATCGGCGAAAAGCGTGAC |
| Scro-L2 [26mer]  | Gln     | CAGATTGACCTGACCTTCTGAG |
| Scro-H1 [26mer]  | 12S     | CGGTTCGTCGAGAAATTTACGACG |
| Scro-H2 [21mer]  | Ile     | AAGACCTGACATCGAGATTTGG |
| **Texas blind snake** |
| Ldul-L1 [20mer]  | Met     | CAAGCTATTGGGCCATACC |
| Ldul-L2 [21mer]  | Leu(CUN) | CAACAACTTTGCTGAAAATCC |
| Ldul-L3 [17mer]  | 16S    | ATGTTGGATCAGGAC |
| Ldul-H1 [21mer]  | 16S    | TGGAAAGTGATATTGCTACC |
| Ldul-H2 [21mer]  | His    | TACACCTGCTCTAGTCTAGG |
| H4433 [20mer]    | Met    | AACCAACATTTGCAGGTAT |

Mitochondrial DNAs for the five squamates were sequenced as described in Kumazawa and Endo.11 Briefly, total DNA was extracted from a small quantity (20 mg) of tissues, 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.11 Initial attempts to amplify an almost entire region of mtDNA (e.g., by primers Cwar-L1 and Cwar-H3) did not work efficiently. I therefore divided the mtDNA into two or three segments that can be amplified with four or six primers, respectively. Combinations for the LA-PCR primers were: Socc-L3 and Socc-H2 (10.7 kbp), Socc-L2 and ucytb-1H (2.8 kbp), and Socc-L4 and Socc-H3 (2.7 kbp) for the fence lizard; Cwar-L1 and Cwar-H3 (5.2 kbp), and Cwar-L3 and Cwar-H1 (11.7 kbp) for the spinytail lizard; rPhe-1L and Agra-H1 (3.7 kbp), Agra-L1 and Agra-H2 (10.6 kbp), and Agra-L2 and Agra-H3 (4.2 kbp) for the alligator lizard; Scro-L1 and Scro-H2 (3.3 kbp), and Scro-L2 and Scro-H1 (15.0 kbp) for the crocodile lizard; and Ldul-L1 and Ldul-H2 (7.7 kbp), Ldul-L2 and Ldul-H1 (6.7 kbp), and Ldul-L3 and H4433 (1.4 kbp) for the blind snake. Using these LA-PCR products as a template, nested PCR amplifications for shorter regions were carried out with a set of conserved primers among squamates.12 Sequences of these nested PCR products together with those obtained by the primer walking were assembled to produce a continuous mtDNA sequence. Abbreviations for the matching gene are: 12S, 12S rRNA; 16S, 16S rRNA; cytb, cytochrome b; COIII, cytochrome oxidase subunit III; and three-letter amino acids, tRNA genes specifying them. H4433 is a primer reported in Kumazawa and Nishida.12 See Kumazawa and Endo11 for primers rPhe-1L and ucytb-1H.
ically identified inside the control region for only a few mammals, are known not to be conserved across mammalian species.\textsuperscript{13} It thus seems difficult to infer transcriptional mechanisms of the squamate mtDNAs based only on the mtDNA sequences.

Inside the major noncoding region of mtDNAs for the alligator lizard and the crocodile lizard, 54-bp and 141-bp repeat units, respectively, were tandemly duplicated in many copies. There were at least 11 repeats for the former and at least 5 repeats for the latter in the unambiguously determined sequences. However, the copy number appeared to be much greater, judging from the electropherogram profile for longer electrophoresis by the DNA sequencer (data not shown). The unusually long tandem repeats were also found in mtDNA of the Komodo dragon, another representative taxon from the infraorder Anguimorpha.\textsuperscript{11} Tandem repeats often occur within the control region of animal mtDNAs,\textsuperscript{14} but the repeats for the three anguimorphs seem noteworthy for their scales.

Heteroplasmy (sequence polymorphism within an individual) was also detected in four out of five squamates: (AT)\textsubscript{12} and (AT)\textsubscript{13} in the major noncoding region of the fence lizard, C\textsubscript{8} and C\textsubscript{9} in the 16S rRNA gene of the spinytailed lizard, C\textsubscript{9} and C\textsubscript{10} in the 12S rRNA gene of the crocodile lizard, and C\textsubscript{8}–C\textsubscript{11} in the 12S rRNA gene and T\textsubscript{9}–T\textsubscript{12} in the major noncoding region of the blind snake. These length variations were only seen in regions of simple repetitions of a nucleotide or dinucleotide, suggesting that replicational slippage\textsuperscript{15} plays a role in creating length mutations. Strong secondary structures in rRNA genes or the control region might provide sites where the mitochondrial DNA polymerase may pause, thus increasing the chance for slipped-strand mispairing.

Kumazawa et al.\textsuperscript{16} found that the control regions with nearly identical nucleotide sequences are present in two locations of mtDNA (one in the typical position and the other after the isoleucine tRNA gene within the IQM tRNA gene cluster) in several snakes from the families Boidae, Colubridae and Viperidae. The present study showed that the blind snake does not have the duplicate control region in its mtDNA, raising the possibilities that snakes originally did not have this characteristic and that the duplication occurred on a snake lineage after divergence of the blind snake (scolecodophidian) lineage. In this regard, it is noteworthy that duplicate control regions for the Komodo dragon were found in different gene arrangements and may have arisen independently.\textsuperscript{11} It thus does not support a close phylogenetic relationship between snakes and varanids.

3. Phylogenetic Analyses

All 37 genes of the squamate mtDNAs were aligned with homologous genes for 21 other vertebrates with known mtDNA sequences (see Fig. 1 legend for details). Two data sets were prepared for concatenated amino acid sequences of 12 protein genes encoded by the heavy strand (3392 sites) and for concatenated nucleotide sequences of the 12 protein genes, as well as 22 tRNA and 2 rRNA genes (9641 sites). NADH dehydrogenase subunit 6 (ND6) gene, encoded by the light strand, was not used due to the presumed difference in the base and amino acid compositions. The codon third positions were removed from the nucleotide data set due to the high substitution rates and subsequent multiple substitutions as a source of noise in phylogenetic analyses.\textsuperscript{4} The aligned data files used for phylogenetic analyses can be obtained by the author upon request.

Figure 1 depicts a maximum likelihood (ML) tree obtained using the nucleotide data set. Previous studies\textsuperscript{9,17,18} showed the importance of taking into consideration the rate heterogeneity across sites by modeling it with the gamma function for analyzing phylogenetic relationships among distantly related taxa. This point was incorporated in the GTR+I+G model used to make the ML tree of Fig. 1. Phylogenetic relationships for the outgroup taxa have been either well-established or repeatedly addressed with similar data sets.\textsuperscript{3,9,10,18–20} I therefore focus on the ingroup relationships of squamates in this report.

Heuristic tree-building methods using the amino acid or nucleotide data set consistently supported topological relationships at nodes A–D, F and H with relatively high bootstrap values (Fig. 1), suggesting independent evolutionary origins for lizards and snakes. Although these methods did not reconstruct the topological relationship at node E and supported that iguanids make a sister group with scincids and cordylids, it turned out that a tree with this alternative relationship had a slightly worse likelihood value than that of the ML tree shown in Fig. 1 (data not shown). One shortcoming in the heuristic ML analyses was that the gamma parameter was not used due to a technical reason. It was therefore necessary to evaluate the squamate relationships more fully by the Kishino-Hasegawa test\textsuperscript{21} under the gamma model.

To conduct this, I first excluded the two snake taxa having accelerated mtDNA evolutionary rates\textsuperscript{8} and compared likelihood values among all 945 rooted trees within lizards by constraining the outgroup relationships and the sister group relationship of the green iguana and the fence lizard. This test resulted in the same topological relationship of lizards shown in Fig. 1 (data not shown). I then fixed this topological relationship and compared likelihood values (Table 3) among 14 alternative hypotheses with respect to the phylogenetic position of snakes (Fig. 2). Both the amino acid and nucleotide data set were used with or without the gamma correction. In all cases, tree 1 (the same topology as in Fig. 1) became an ML tree topology. When the nucleotide data set was used with the gamma model, all the other trees, including tree 7 that represents the sister-group relationship...
**Figure 1.** Phylogenetic relationship of 26 vertebrates inferred from the whole mtDNA sequences. Concatenated light-strand nucleotide sequences of the first and second codon positions of 12 protein-coding genes encoded by the heavy strand, as well as 22 tRNA and 2 rRNA genes were used after unalignable sites and gap-containing sites were removed (9641 sites in total). A total of 56 DNA substitution models were compared using Modeltest version 3.06 to show that the GTR+I+G model best explains the DNA substitution process for the data set. With this model and optimized parameter values, an ML tree was constructed using PAUP* version 4.0b10 by an exhaustive search among 10,395 possible tree topologies among the iguanids, the mole skink, the spinytail lizard, the crocodile lizard, the Komodo dragon, and the snakes by fixing the outgroup relationship shown in this figure. Note that initial heuristic analyses using ML and neighbor-joining (NJ) methods all supported the outgroup relationships and provided two nodes B and H with 100% bootstrap probabilities (see the inset). It is therefore reasonable to cluster two iguanids, as well as two snakes in the constrained exhaustive search. Bootstrap probabilities shown on branches were estimated using MOLPHY version 2.3 with Tamura-Nei substitution model and parameters estimated from the data set ($\alpha/\beta = 2.92$, $\alpha_Y/\alpha_R = 1.06$). As recommended by the authors of MOLPHY, we first obtained a tree by the star decomposition search, from which the local rearrangement search was conducted to provide the ML tree and the local bootstrap values from 1000 replications. Inset, bootstrap probabilities for internal nodes A–H estimated by multiple methods. The first row shows bootstrap values from ML analyses using amino acid sequences of 12 protein genes (3392 sites). MOLPHY version 2.3 was used with the mtREV24 substitution matrix and amino acid frequency estimated from the data set. The second row shows bootstrap values from NJ analyses of the nucleotide sequence data. NJBOOT in LINTRE package was used with Tamura-Nei gamma option ($\alpha = 0.36$). All bootstrap values were from 1000 replications. At nodes where bootstrap values are underlined or no bootstrap value is given, the corresponding nodal relationship was not reconstructed in the best tree topology. Nucleotide sequences for the taxa which were not sequenced in this study were taken from a public database with the following accession numbers: green iguana (AJ278511), mole skink (AB016606), Komodo dragon (AB080275 and AB080276), akamata (AB008539), chicken (X52392), ostrich (Y1205), indigo bird (AF090341), alligator (Y13113), caiman (AJ404872), green turtle (AB012104), painted turtle (AF069423), helmed turtle (AF032966), cow (J01994), opossum (Z29573), platypus (X83427), toad (M10217), caecilian (AF154051), lungfish (L42813), coelacanth (U82228), loach (M91245), and trout (L29771).
Table 3. Kishino-Hasegawa test on the phylogenetic affiliation of snakes.

| Tree | 12 proteins (amino acid seq.) | all genes (nucleotide seq.) |
|------|-------------------------------|-----------------------------|
|      | -γ   | +γ   | -γ   | +γ   |
| 1    | ML   | ML   | ML   | ML   |
| 2    | 2.73** | 2.40* | 3.79** | 3.28** |
| 3    | 1.60 | 1.68 | 2.76** | 3.19** |
| 4    | 4.29** | 3.24** | 5.21** | 4.55** |
| 5    | 2.02* | 1.38 | 3.79** | 2.70** |
| 6    | 1.69 | 1.06 | 2.64* | 2.16* |
| 7    | 0.83 | 1.13 | 1.77 | 2.03* |
| 8    | 2.70** | 2.42* | 3.94** | 3.17** |
| 9    | 3.36** | 3.29** | 5.48** | 4.49** |
| 10   | 3.62** | 3.22** | 5.66** | 4.49** |
| 11   | 1.65 | 1.11 | 3.51** | 2.69** |
| 12   | 4.22** | 3.25** | 7.32** | 6.04** |
| 13   | 4.52** | 3.31** | 7.59** | 6.13** |
| 14   | 4.90** | 3.78** | 7.44** | 5.85** |

Kishino-Hasegawa test\textsuperscript{21} was conducted with PUZZLE 5.0\textsuperscript{14} with either amino acid sequences of 12 protein genes (3392 sites) or nucleotide sequences of all mitochondrial genes except ND6 (9641 sites). For each data set, two conditions (i.e., with or without gamma correction for the site heterogeneity of rates) were tested. ML stands for the ML tree topology (see Fig. 1). By fixing the phylogenetic relationship for 24 taxa other than snakes, 14 alternative hypotheses in which only the phylogenetic position of snakes differed were compared. Numbers in Fig. 2 for the position of snakes correspond to tree numbers in this table. Values shown in this table are the difference in log-likelihood from the ML tree topology divided by the standard error (Δ lnL/SE). An asterisk means that the corresponding phylogenetic hypothesis can be statistically rejected in 5% significance level by the standard criterion of Δ lnL/SE > 1.96, while double asterisk means stronger rejection in 1% significance level by Δ lnL/SE > 2.58.

Figure 2. Phylogenetic hypotheses on the affiliation of snakes used for the Kishino-Hasegawa test in Table 3. This figure illustrates 14 different branches to which two snake taxa (the blind snake and akamata) may join. Tree 1 corresponds to the ML tree topology shown in Fig. 1 and Table 3.

between snakes and varanoids, were rejected at the 5% significance level. Similar results were obtained even if the Kishino-Hasegawa test was conducted based on the alternative lizard relationship clustering iguanids, scincids and cordylids (data not shown).

There are two major hypotheses on the evolutionary origin of snakes relative to lizards.\textsuperscript{6} The first one\textsuperscript{22,23} postulates that snakes arose within the lizards, likely from a varanoid stock. As a supportive morphological characteristic, they have the forked tongue in common in which
the distal portion retracts into the proximal portion.\textsuperscript{6} The second hypothesis\textsuperscript{24} supports the view that snakes and lizards are separate monophyletic groups.

Previous molecular studies using parts of mtDNA sequences (see, e.g., Forstner et al.,\textsuperscript{25} Macey and Verma,\textsuperscript{26} and Rest et al.\textsuperscript{20}) and nuclear gene sequences (see, e.g., Saint et al.,\textsuperscript{27} Harris\textsuperscript{28}) generally favored a view that snakes arose within lizards. To the best of my knowledge, there has been little molecular support for separate origins of snakes and lizards (see, e.g., the viper hemoglobin sequence in Fushitani et al.\textsuperscript{29}) However, the whole mtDNA data set of the present study did not support this general view but strongly suggested the second hypothesis. I suspect that possible reasons for this discrepancy may lie in insufficient sampling of sites or taxa in the previous studies. In fact, I found that preliminary phylogenetic analyses using the whole mtDNA data set with only the snakes, the iguanids, the mole skink, and the Komodo dragon as squamate taxa erroneously led them to support the snake-varanid relationship, presumably due to the long branch attraction\textsuperscript{3} (data not shown). It is well known that the long branch attraction error may be tackled by sufficient taxon sampling.\textsuperscript{5} Thus, I think that my conclusion should also be evaluated for its robustness by incorporating more squamate taxa into the analyses in the future.

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