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Molecular systematics of primary reptilian lineages and the tuatara mitochondrial genome

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Abstract

We provide phylogenetic analyses for primary Reptilia lineages including, for the first time, *Sphenodon punctatus* (tuatara) using data from whole mitochondrial genomes. Our analyses firmly support a sister relationship between *Sphenodon* and Squamata, which includes lizards and snakes. Using *Sphenodon* as an outgroup for select squamates, we found evidence indicating a sister relationship, among our study taxa, between Serpentes (represented by *Dinodon*) and Varanidae. Our analyses support monophyly of Archosauria, and a sister relationship between turtles and archosaurs. This latter relationship is congruent with a growing set of morphological and molecular analyses placing turtles within crown Diapsida and recognizing them as secondarily anapsid (lacking a skull fenestration). Inclusion of *Sphenodon*, as the only surviving member of Sphenodontia (with fossils from the mid-Triassic), helps to fill a sampling gap within previous analyses of reptilian phylogeny. We also report a unique configuration for the mitochondrial genome of *Sphenodon*, including two $tRNA^{Lys}$ copies and an absence of ND5, $tRNA^{His}$, and $tRNA^{Thr}$ genes.

Keywords: *Sphenodon punctatus*; Reptilia phylogeny; Gene order; Archosauria; Turtles

1. Introduction

Phylogenetic relationships among the primary lineages of Reptilia remain controversial. Based on recent analyses of morphological characters, turtles have been placed as an early diverging lineage, representing ancient anapsids (lacking a temporal opening in the skull) (Lee, 2001) or as a younger lineage, descended from diapsid ancestors (with two temporal skull openings) and most closely related to the lepidosaurs (lizards, snakes, and tuatara) (Rieppel and deBraga, 1996). Analyses of molecular data also indicate turtles to be diapsids, but sister to either archosaurs (crocodilians and birds), crocodilians or lepidosaurs (Cao et al., 2000; Hedges and Poling, 1999; Zardoya and Meyer, 2000). Similarly, the relationship of snakes (Serpentes) relative to other lepidosaurs is uncertain. Some studies place snakes among lizards within Anguimorpha (Lee, 2000), which includes monitor lizards, whereas others place them as sister to a clade including all the lizards (Underwood, 1970). Two closely related tuatara species (*Sphenodon punctatus* and *Sphenodon guntheri*) are the only surviving taxa in the order Sphenodontia and are usually placed as sister to the combined group of lizards and snakes (squamates) based on morphology (Rieppel and Reisz, 1999), although analyses of limited molecular data suggest they are closer to archosaurs and turtles (Hedges and Poling, 1999; Zardoya and Meyer, 2000). Resolution of Reptilia phylogeny has important consequences for understanding the evolution of vertebrate morphology, genomes, and development, as well as informing the appropriate

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choice of outgroup taxa for phylogenetic analyses within Reptilia lineages, including birds.

Sphenodon is a medium-sized, predatory, nocturnal reptile, characterized by prominent spines on the neck and back, lack of an external ear, hook-like extensions on the ribs, fused premaxillary teeth, and a simple pineal "eye" on top of the head which is covered by scales in adults. *Sphenodon* remains quite similar in skeletal morphology to fossil forms over 225 million years old (Carroll, 1988). This distinctive lineage has had only a few gene sequences available for analyses, and its inclusion, with a larger set of characters, is necessary to provide a more comprehensive sampling of taxa and characters for enhanced resolution of Reptilia phylogeny. Toward this end, we have sequenced the entire mitochondrial (mt) genome for a tuatara (*S. punctatus*) and used it in combined analyses of homologous mitochondrial genes from representative mammals, lizards, snake, turtles, crocodilians, and birds.

2. Materials and methods

2.1. Sequencing Sphenodon mitochondria

Total genomic DNA was extracted from liver tissue from two *S. punctatus* individuals using a QiaAmp Tissue Kit (Qiagen). The entire mt genome was sequenced from one (CD540, C. Daugherty, Victoria University of Wellington), and relevant gene junctions were amplified and sequenced from the other (UMFS 10956, U. Michigan Museum of Zoology) as necessary to confirm gene order novelties relative to other vertebrates. Large pieces (2–5 kb) of mtDNA were amplified initially, and smaller fragments (500–1000 base pairs [bp]) amplified from them for sequencing. Large fragment PCR profiles consisted of an initial denaturing step of 2 min at 94 °C, followed by 10 cycles of a 94 °C 10 s denaturing step, a 45–55 °C 20 s annealing step, and a 68 °C 2–5 min extension step; these 10 cycles were followed by 21 more with the same profile with 10 s added to each successive extension step. A typical short PCR profile consisted of initial denaturing at 94 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 10 s, annealing at 45–55 °C for 20 s, and extension at 68 or 72 °C for 30–60 s. PCR products were purified and sequenced using standard protocols (Mindell et al., 1999). We used published (Sorenson et al., 1999) and newly designed primers (Table 1).

2.2. Taxa, alignment, and phylogenetic analyses

To assess phylogeny for primary lineages of Reptilia we assembled comparative sequence data from 14 taxa with complete mitochondrial genomes available. Study taxa names and GenBank accession numbers are as follows. *Didelphis virginiana* (North American opossum) NC_001610; *Mus musculus* (house mouse) NC_001569; *Eumeces egregius* (mole skink) NC_000888; *Chelonia mydas* (green sea-turtle) NC_000886; *Chrysemys picta* (painted turtle) NC_002073; *Pelomedusa subrufa* (African helmeted turtle) NC_001947; *Caiman crocodylus* (spectacled caiman) NC_002744; *Alligator mississippiensis* (American alligator) AF069428; *Vidua chalybeata* (village indigobird) NC_000880; *Buteo buteo* (common buzzard) NC_003128; *Rhea americana* (greater rhea) AF090339; *Iguana iguana* (green iguana) NC_002793; *Dinodon semicarinatus* (Ryukyu odd-tooth snake) NC_001945; *S. punctatus* (tuatara) AF534388, AF534389, AF534390 (new sequences). We assembled a second dataset, allow-

Table 1
Primer names and sequences

Primer name	5′–3′ sequence	Gene location
H02313	TTG CGG TAC TGT CTC TAT AGC TCC	16S rRNA
L04595	CCA CGC AAG AAC CAA TAT GAC TC	ND-1
H05761	CCG AGG CTG GTC AAT AAG TGA TG	ND-2
L08124	TAC CAA CCA CAA GAG AGG AAG G	CO-1/tRNA^{Ser}
L11634	AAG TTC TCT CTA GCC CTT ACA CAG G	ND-4
L12013	AAT CAC AAT CCT TCA CTT CCT GC	ND-4
L12074	AAC AAG TTT CTT TCT GTG AGG TGC	ND-4
H12094	GCT ATT ATA CAT GCA CCT CAC AG	ND-4
L13272	AAT CCA CCA CCA ACA GCA GAA GC	ND-6
H13363	CGT CTT TGG TTT GTT ATT AGT GGC	ND-6
CRrev	TAT AAT ATA AGG CGG CGT GCC GCC TGC	d-loop
CRfor	TAC CTC TGG TTA CTC TTT CCA GTG C	d-loop
H15036	CAT AAT CCT AGG AGA GAG CCA AAG	cyt b
H15150	AGT CAA CCG TAG TTT ACA TTC CGG C	cyt b
H16030	GGT GCC AGT GAT AGG AAT TAG GAC	cyt b
CRfor2	CGT ACC TAC AAC CAC ACC ATG CAC	d-loop

Primer names reflect approximate position relative to existing primers (Sorenson et al., 1999), with the prefix L and H referring to light and heavy strand primers, respectively. The control region primers CRfor and CRrev are heavy and light strand primers respectively, and match both copies of the d-loop (see Fig. 1). The primer CRfor2 matches the end of the 926 bp d-loop only.

ing us to use *Sphenodon* as an outgroup in an assessment of the phylogenetic position for snakes (*Dinodon*) relative to *Eumeces*, *Iguana* and three representatives of Anguimorpha, a group thought to be closely related to snakes: *Anniella pulchra* (California legless lizard) AF407537, *Varanus acanthurus* (spiny-tailed monitor) AF407488, and *Varanus griseus* (grey monitor) AF407503.

Correspondingly, two mitochondrial alignments were made. For protein coding genes, inferred amino acid sequences were aligned via ClustalX (Jeanmougin et al., 1998) and by eye, and this alignment was then imposed on the DNA sequences. tRNAs were aligned by eye and adjusted to fit secondary structure profiles, to maintain alignment among double-stranded stems. tRNA loops could not be reliably aligned and were excluded from analyses. Alignments for the two rRNAs were more problematic than the other genes and were also excluded. ND5, $tRNA^{Thr}$, and $tRNA^{His}$ were absent from *Sphenodon* and were similarly excluded. We focus our analyses on nucleotides from all genes combined (protein coding genes and tRNAs) in order to use the largest set of combined sequence characters. The primary alignment was 9851 nucleotides in length and included 11 mt protein coding genes and 20 tRNAs from 14 taxa. The secondary alignment was 2335 nucleotides long and included two protein coding genes (ND1, ND2) and eight tRNAs (I,Q,M,W,A,N,C,Y) for seven taxa. Analyses were performed on the 14 taxa alignment both with and without *D. semicarinatus* which has a relatively fast rate of mtDNA evolution (Kumazawa et al., 1998).

To estimate phylogeny we used a hierarchical Bayesian inference (BI) approach (Mau et al., 1999; Yang and Rannala, 1997) with Metropolis-coupled Markov chain Monte Carlo, or $(MC)^3$, to approximate the posterior probabilities of the trees with a GTR (reversible, 6 substitution types) model for DNA sequence evolution, as implemented in MrBayes 2.1 (Huelsenbeck and Ronquist, 2001). Each search was run twice, starting from random trees, for one million generations, each with four simultaneous Markov chains, sampling every 50 generations. Base frequencies and a gamma distribution with eight rate categories were estimated for each run, and the starting prior probabilities were uniform. Generations sampled before the chain reached stationarity (''burn-in'') were discarded. The proportion of searches in which any given node (set of relationships) is found during the chain constitutes the Bayesian posterior probability (PP) for that node.

BI has advantages over some methods of phylogenetic inference in computational speed (Larget and Simon, 1999) and consistency (Wilcox et al., 2002); however, PP values should be interpreted with caution, as simulations have demonstrated the potential for artifactually high values (Suzuki et al., 2002). Several analyses also indicate that the reliability of PP estimates depends on appropriateness of the model (Buckley, 2002; Waddell et al., 2001; Wilcox et al., 2002). Alfaro et al. (2003) have found PP values to be strongly correlated with conventional ML bootstrap values except when internodes are short and that PP estimates do support correct monophyletic groups more often than ML or MP bootstrap values, even with fewer characters. Douady et al. (2003) describe a more conservative approach to estimating nodal support in BI, by bootstrapping data matrices and performing BI on each replicate. They found these bootstrap posterior probabilities (BPP) to be strongly correlated with ML bootstrap percentages. We provide both PP and BPP values in this study. To calculate BPP values, 100 bootstrap replicates were run in MrBayes under the same parameters described above, except each replicate was run for 200,000 generations. To be consistent, the first 100,000 generations were discarded as ''burn-in'' for each replicate.

For comparative purposes, we also assessed phylogeny with maximum parsimony (MP) and bootstrap estimates for nodal support using PAUP* (Swofford, 2001). For each of 1000 bootstrap replicates, a heuristic search was run with 10 random sequence additions using TBR branch swapping. All character-state changes were equally weighted. The 1000 bootstrap replicates were summarized as a 50% majority rule consensus tree.

We evaluated a set of alternative phylogenetic trees, including various sub-optimal and published hypotheses of particular interest, to see if they could be statistically rejected in favor of the optimal tree using the approximately unbiased (AU; Shimodaira, 2002) tree selection test in the software package CONSEL (Shimodaira and Hasegawa, 2001). For comparison, we also performed the Shimodaira–Hasegawa test (SH; Shimodaira and Hasegawa, 1999). Both of these tests are statistically appropriate in comparing both a priori and a posteriori phylogenetic hypothesis, unlike the Kishino and Hasegawa (1989) and Templeton (1983) tests. The SH test compensates for a posteriori hypotheses by adjusting the expected difference in log-likelihood values and sampling multiple alternative topologies. However, the SH test is conservative, as the number of trees included in the confidence set increases as the number of trees being considered increases (Strimmer and Rambaut, 2002). The AU test uses a multiscale bootstrap technique to remove this bias, and thus it is recommended for general tree selection problems (Shimodaira, 2002). Log-likelihood values for sites were determined in PAML 3.11 (Yang, 1997) with GTR model parameters including eight rate categories in the gamma distribution and estimated base frequencies using the 14 taxa, 9851 character dataset for all of the alternative trees.

We estimated dates for lineage divergences using multiple fossil-based calibration points and the penalized likelihood method with truncated Newton optimization in the program r8s 1.50, which accommodates

heterogeneity in absolute evolutionary rates among lineages (Sanderson, 1997). The four fossil-based divergences for stem lineages used as calibration points are: archosaurs, 245 million years ago (mya); turtles, 220 mya; lepidosaurs, 230 mya; turtles + archosaurs, 272 mya (see Benton, 1993 and references therein).

3. Results and discussion

3.1. Sphenodon mitochondrial gene order

Gene order, with several unique features, was found as shown in Fig. 1. Unlike other vertebrates, *Sphenodon* has two $tRNA^{Lys}$ copies. Both appear to have functional anticodon loops and the potential to be folded into a cloverleaf structure, although the 5′-most $tRNA^{Lys}$ (adjacent to CO2) has more weakly pairing TΨCG and acceptor stems, which may affect its ability to function. *Sphenodon* lacks the origin of light strand replication sequence usually found within the WANCY tRNA cluster. This is a trait *Sphenodon* shares with all birds and crocodilians examined to date. In *Sphenodon*, ND4 is followed, in the 3′ direction, by ND6, $tRNA^{Glu}$, an 823 bp d-loop-like sequence, $tRNA^{Leu}$, cytochrome *b*, $tRNA^{Pro}$, $tRNA^{Ser}$, and a 926 bp d-loop-like sequence. The two, control region sequences are identical for the initial (5′) 750 bp, but differ thereafter. Apparent duplication of the control region explains recovery of two different PCR products in an earlier attempt to map the gene junctions in the *Sphenodon* mitochondria (Quinn and Mindell, 1996). Genes for ND5, $tRNA^{His}$, and $tRNA^{Thr}$, are found in this region in other vertebrates sequenced to date, but were absent from this region in *Sphenodon*. No sequences were found elsewhere in the mt genome showing similarity to these genes. All sequences represented in Fig. 1 had clear and extensive (>50 bp) overlap with neighboring sequences on both strands, and numerous PCR experiments were run with different primers pairs including sets designed using *Sphenodon* mtDNA sequence, to locate the missing genes, however no amplification products for those genes were recovered. Further, the gene order and gene absences (Fig. 1) were verified in two different *S. punctatus* individuals. Gene absences may reflect heteroplasmy or migration of mt genes to the nucleus (reviewed in Bensasson et al., 2001). It is also possible that the missing genes are present within an undetected duplicated region. We did find two PCR products (ca. 905 and 3000 bp) when the primers CRfor and H15150 were used together with long PCR protocols. However, sequence for both fragments showed only the 823 bp d-loop, $tRNA^{Leu}$, and cytochrome *b* gene region, and repeated PCR experiments using a light strand primer in cytochrome *b* and heavy strand primers in ND5, $tRNA^{His}$, and $tRNA^{Ser}$ yielded no products.

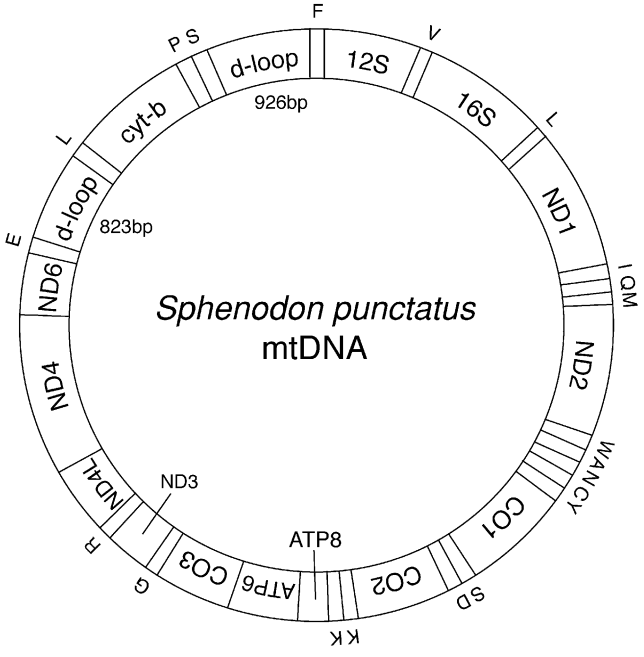

Fig. 1. Mitochondrial genome map for tuatara (*Sphenodon punctatus*). tRNAs are indicated by their single-letter amino acid code.

Variable mitochondrial gene order and gene constituency traits are useful phylogenetic markers, though several convergences have been found indicating the value of detailed sampling before assuming a single mitochondrial gene order for some clades (Boore et al., 1998; Dowton and Austin, 1999; Mindell et al., 1998; Roehrdanz et al., 2002). The novelties noted above (two $tRNA^{Lys}$ genes, absent ND5, $tRNA^{His}$, and $tRNA^{Thr}$; Fig. 1) in *Sphenodon* relative to other vertebrates examined to date are unique (autapomorphic), however, and do not currently provide any shared, derived traits (synapomorphies) indicating sister relationships with other taxa. Absence of the three genes in at least some *Sphenodon* mitochondria is similar to absences of ATP8 from some nematodes (Okimoto et al., 1992), platyhelminthes (Le et al., 2000), a bivalve (Hoffmann et al., 1992) and a tunicate (Yokobori et al., 1999), and absence of ATP6 from mitochondria for an alga, encoding it in the nucleus (Funes et al., 2002). Evidence for import of tRNAs into mitochondria has been reported for various invertebrates, plants, and marsupials (see Dorner et al., 2001; Tan et al., 2002).

3.2. Phylogeny of Reptilia

BI and BPP analyses for the 14 taxa dataset identified the topology shown in Fig. 2 tree A and in Fig. 3 as optimal, and we consider this to be our best estimate of phylogeny. For each BI analysis, two independent $(MC)^3$ runs starting from random trees, with four simultaneous chains yielded nearly identical posterior

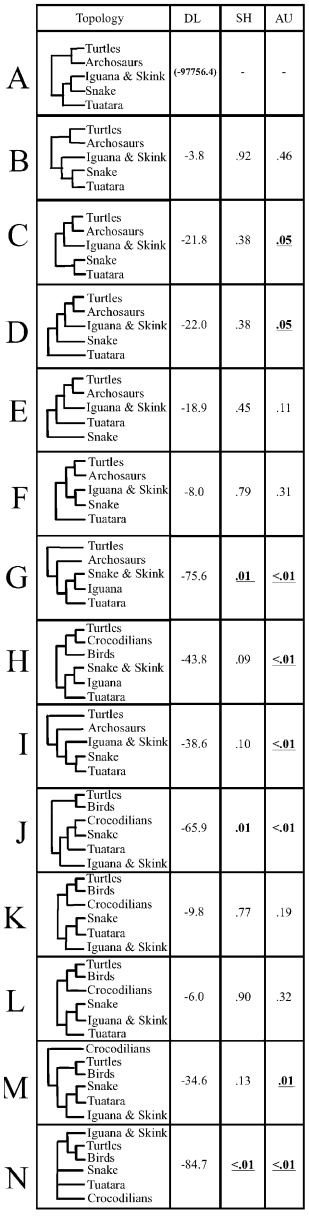

Fig. 2. Alternative tree topologies (B–N) in comparison to our optimal topology (A). The log-likelihood score for tree A is provided in parentheses, and the difference in log-likelihood is provided between A and all other topologies in the ΔLL column. The SH and AU tests indicate which alternative topologies can be rejected ($p \leqslant 0.05$, underlined) in favor of topology A. All topologies summarize relationships for the 14 study taxa, but are presented with either five or six higher-level terminal taxa for concision. In alternatives with six terminal taxa, Archosaurs are separated into crocodilians and birds.

probabilities and estimated model parameters for the same topologies, indicating the chains were run for a sufficient number of generations (1×10^6) and sampled the same posterior probability landscape. A 50,000 generation burn-in period was required for the chain to reach stationarity. Our overall optimal tree places a monophyletic Testudines (turtles) as sister to monophyletic Archosauria (birds and crocodilians). That clade is sister to monophyletic Lepidosauria, which includes a basal split between *Sphenodon* and Squamata (*Dinodon*, *Eumeces*, and *Iguana*). Within Squamata, *Dinodon* (snake) is placed as sister to the two lizards (*Eumeces*/*Iguana*) (Fig. 3). All Bayesian posterior probabilities (PP) for nodes in this tree were 98% or greater. All bootstrapped posterior probabilities (BPP) were 72% or greater, with the exception of a value of 66% for the node uniting birds and crocodilians. BI returned the same set of relationships (Figs. 2A and B and 3) for the study taxa regardless of *Dinodon* inclusion or exclusion.

All nodes in Fig. 3 are supported in MP 50% majority rule analyses of 1000 bootstrapped datasets for 14 taxa, with two exceptions. *Sphenodon* and *Dinodon* are placed as sister taxa and there is an unresolved trichotomy between the *Sphenodon*/*Dinodon* clade, the *Eumeces Iguana* clade, and the Archosauria/Testudines clade. We suspect the *Sphenodon*/*Dinodon* relationship is an artifact given the relatively fast rate of sequence change in *Dinodon* and the lack of explicit rate heterogeneity consideration in the MP analyses. Exclusion of *Dinodon* in MP analyses with 13 taxa left *Sphenodon* as a single lineage in the same unresolved trichotomy.

Comparison of log-likelihood scores and the AU and SH likelihood ratio tests for the set of alternative trees, suggested in the literature or in the course of our analyses, relative to our overall optimal tree are summarized in Fig. 2. The AU and SH test are employed to determine the confidence set of trees; that is, trees that cannot be statistically rejected in favor of the optimal tree. As noted previously, the AU test is a powerful test for general tree selection problems involving a priori and a posteriori hypotheses. The SH test is biased against rejecting trees when comparing many trees, but is provided here for comparison. Eight of the 13 suboptimal trees (trees C, D, G, H, I, J, M, and N) can be rejected in favor of tree A using the AU test, while three can be rejected using the more conservative SH test. This includes rejection by the AU test of all alternatives having a basal split between turtles and other reptilian lineages, as in traditional and some recent morphological analyses. We consider these tests to be conservative and failure to reject a particular topology does not mean that its constituent clades have compelling support.

Placement of *Chrysemys* and *Chelonia* as sisters relative to *Pelomedusa* is consistent with the primary division of turtles into pleurodires (side-necked species) and cryptodires (hidden-necked species) based on both morphological and molecular data (see Shaffer et al., 1997). Resolution of early divergences among birds has been difficult and we present a polytomy in Fig. 3 to indicate the variable support found for relationships among these three lineages whose collective monophyly is well supported.

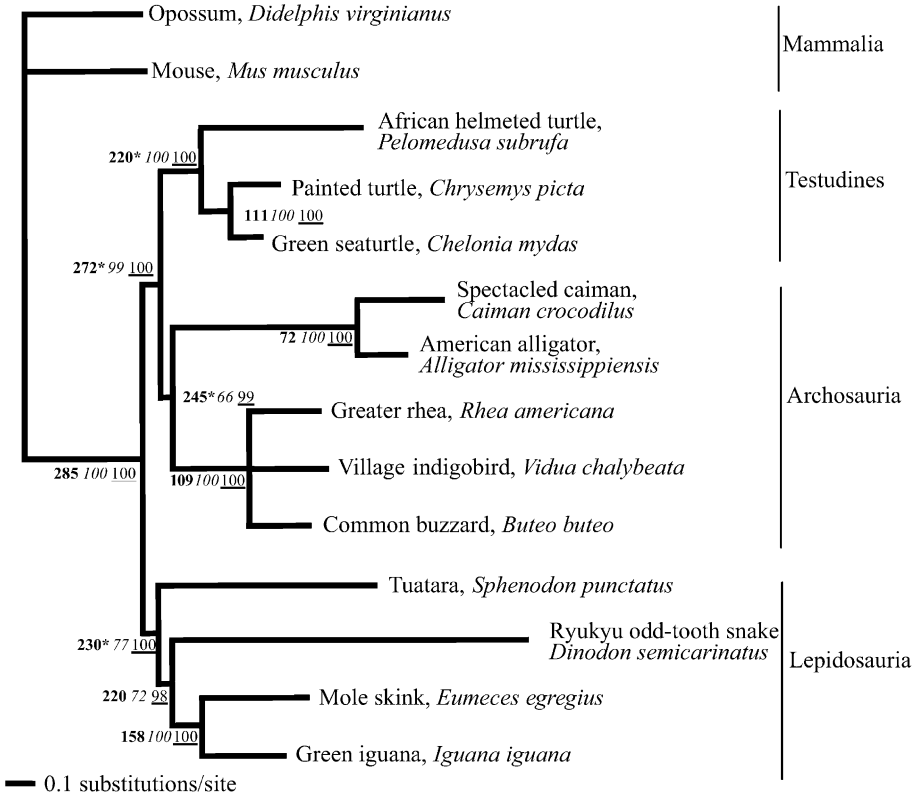

Fig. 3. Optimal phylogenetic hypothesis for representative lineages of Reptilia using a Bayesian inference approach. For this figure, branch lengths are calculated using a GTR + gamma model, with a dataset including 11 protein coding genes and 19 tRNAs from mitochondria. Numbers in bold at internodes are divergence time estimates in millions of years before the present. Asterisks designate calibration points from the fossil record (see Benton, 1993; Carroll, 1988). Numbers in italics are nonparametric bootstrapped Bayesian posterior probabilities (BPP) based on 100 replicates and 200,000 generations (see text). Bayesian posterior probabilities (PP) for nodes are underlined.

Our optimal tree places *Sphenodon* as sister to Squamata (Fig. 3). This is the first such diagnosis of *Sphenodon* based on a large molecular dataset and is congruent with morphological analyses (Rieppel and Reisz, 1999) rather than with analyses of smaller molecular datasets. *Sphenodon* was placed in an unresolved polytomy with birds, crocodilians, and turtles based on 785 amino acids from four nuclear genes (Hedges and Poling, 1999) or as sister to a turtle based on nucleotides from 12S and 16S mt rDNAs (Zardoya and Meyer, 2000). This also suggests that absence (loss) of the site for initiation of mitochondrial light-strand replication (O_L) from within the WANCY tRNA cluster for *Sphenodon* is independently derived, as the O_L is present in mammals, turtles, and most lepidosaurs, though absent from birds and crocodilians (see Seutin et al., 1994).

Phylogenetic placement of snakes has been controversial, with no consensus at present. Some studies place snakes among lizards within Anguimorpha and close to varanids (monitor lizards) (Lee, 2000; Macey and Verma, 1997), whereas others place them as sister to a clade including all the lizards (Underwood, 1970). Sequencing of the mt genome for *Sphenodon* and its supported position as sister to other Lepidosaurs (Figs. 2 and 3) allows us to focus further on the position of snakes using *Sphenodon* as an outgroup and three additional anguimorph taxa for mt ND1, ND2, and eight tRNA genes (see above). Addition of these three taxa may help reduce potential bias due to the relatively long branch for the representative snake (Fig. 3). BI and BPP analyses for seven taxa with *Sphenodon* as an outgroup are congruent with analyses of the larger set of taxa in Fig. 3. *Dinodon* is placed within Anguimorpha and well supported as sister to the two varanids within our set of study taxa, and the sister relationship between *Eumeces* and *Iguana* remains (Fig. 4). A sister relationship between Scincomorpha (skinks and relatives) and Anguimorpha is not supported. A tree placing *Dinodon* and *Eumeces* as sisters but with all other relationships as in our optimal tree (Fig. 3) was rejected using the AU test with the 14 taxa dataset (Fig. 2, tree H).

3.3. Estimated age of divergences

Estimating divergence times remains problematic, largely due to heterogeneity in rates of sequence change among taxa and over time, and because fossils provide only minimum (most recent possible) dates. Though we

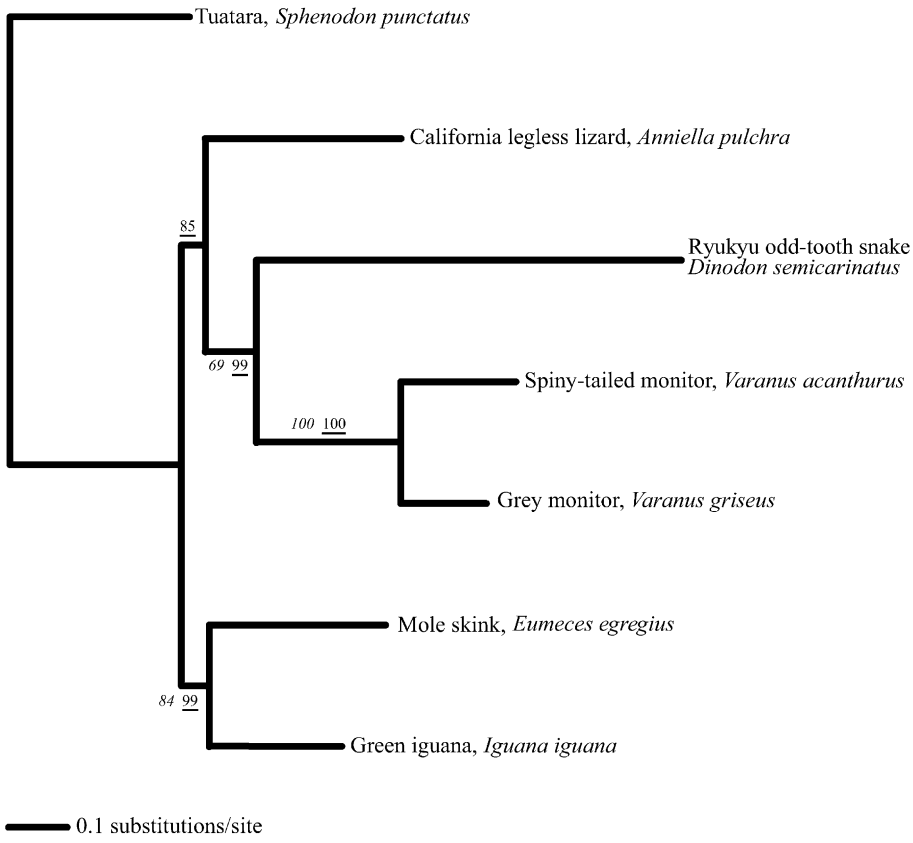

Fig. 4. Optimal phylogenetic hypothesis for representatives of seven lineages focusing on relative placement of snakes (e.g., *Dinodon*) using tuatara (*Sphenodon*) as an outgroup and a Bayesian inference approach. For this figure, branch lengths are calculated using a GTR + gamma model, with a dataset including two protein coding genes and eight tRNAs from mitochondria. Numbers in italics are nonparametric bootstrapped Bayesian posterior probabilities (BPP) based on 100 replicates and 200,000 generations. Bayesian posterior probabilities (PP) for nodes are underlined.

do not claim to have circumvented these difficulties, we have taken several steps to minimize their influence. We used the r8s program which estimates the variability of absolute rates of molecular evolution across lineages by relaxing the molecular clock assumption using likelihood and nonparametric smoothing methods, and enables use of multiple fossil-based estimates for divergences, and multiple, local calibration points within the tree.

Our estimates derived from these calibrations are 111 mya for the divergence between *Chrysemys* and *Chelonia*, 72 mya for divergence of *Caiman* and *Alligator*, 109 mya for the polytomy among three bird lineages, 285 mya for divergence between Lepidosauria and other diapsids (including turtles), 220 mya for the divergence of snakes from the representative other lizards, and 158 mya for the *Eumeces* and *Iguana* divergence (Fig. 3). The estimate of 109 mya for divergence among some extent avian lineages is roughly consistent with other mid-late Cretaceous estimates based on molecular data (e.g., Waddell et al., 1999) and with biogeographical analyses (Cracraft, 2001), but not with studies based on fossils alone (e.g., Feduccia, 1999). The estimate for the divergence between the stem snake lineage and lizards at about 220 mya is significantly older than the oldest fossils clearly attributable to crown-clade snakes (Serpentes) which have been dated to about 100 mya (Benton, 1993; Tchernov et al., 2000). Our estimate of 111 mya for the divergence between *Chrysemys* and *Chelonia* is in close agreement with the estimated range of 90–120 mya for these primary cryptodire turtle lineages based on fossils alone (Shaffer et al., 1997).

To summarize, our analyses firmly support a sister relationship between *Sphenodon* and Squamata, represented by *Eumeces*, *Iguana*, and *Dinodon*. Using *Sphenodon* as an outgroup for select squamates we found evidence indicating a sister relationship among *Dinodon* (representing Serpentes) and *Varanus* (Varanidae). Our analyses support monophyly of Archosauria, and a sister relationship between turtles and archosaurs. Morphological analyses by Rieppel and Reisz (1999), however, place turtles as sister to lepidosaurs rather than to archosaurs.

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