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# Amniotes (Amniota)

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## Abstract

**Amniota is a remarkably diverse clade of tetrapod vertebrates comprising more than 23,000 living species of mammals, non-avian reptiles, and birds adapted to a wide variety of primarily terrestrial habitats. Our most recent common amniote ancestor probably lived ~325 million years ago (Ma), and molecular data suggest that the major lineages of living reptiles arose in the Permian and Triassic (299–200 Ma), when land areas were coalesced into a single supercontinent, Pangaea. Conflicting morphological and molecular results for the origin of turtles has been particularly challenging to resolve, although most recent analyses place turtles with birds and crocodylians.**

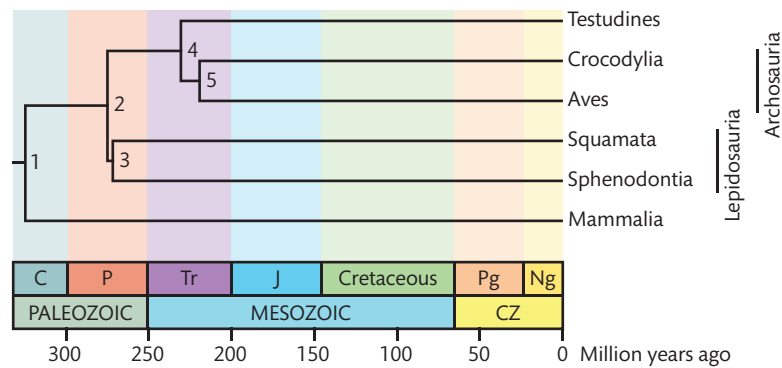
Amniota is a clade noted for the extraordinary ecological and taxonomic diversity of its more than 23,000 living species, comprising mammals and reptiles, including birds. The taxon is named for the characteristic egg structure in which the amnion membrane forms a fluid-filled cavity that surrounds the developing embryo. The amniote egg is considered one of the key adaptations in the evolution of vertebrate terrestrial life history (Fig. 1). This shared-derived character is generally accepted to be one of several key adaptations for enduring the terrestrial life adopted by our amniote ancestors, thereby facilitating the remarkable evolutionary success of the group. Considerable debate has been focused on precisely when our most recent common amniote ancestor lived (1). Tightly constrained radiometric analysis of fossils places the divergence between the first undisputed synapsids (mammals which have only one temporal fenestration in the skull) and diapsids (reptiles with two temporal fenestrations) at  $306.1 \pm 8.5$  Ma. This dating technique generally exhibits about 1% error (2). The upper bound of this narrow estimate for bird–mammal divergence

overlaps with a widely cited value of 310 Ma, based on the approximate methods of Benton (3) and employed by Kumar and Hedges (4) to calibrate a comprehensive molecular timescale for vertebrate evolution. The amniote timetree considered here includes six terminal taxa: Mammalia (mammals), Sphenodontia (the tuatara), Squamata (lizards and snakes), Testudines (turtles), Crocodylia (alligators and crocodiles), and Aves (birds). A combination of molecular and fossil evidence suggests that the major lineages of living reptiles likely originated in the Permian and Triassic (3–5) although exact divergence time estimates vary among the molecular studies used in the present synthesis.

Conflicting phylogenetic results from different morphological and molecular data sets have clouded the picture of amniote macroevolution and have slowed the process of establishing a clear consensus of views between paleontologists and molecular systematists. Synapsids (mammals) are accepted to be the closest relatives of diapsid reptiles. Turtles lack temporal holes in the skull, however, and have been viewed as the only surviving anapsid amniotes. Based on this and other characters, they



**Fig. 1** An amniote, the Rough Greensnake (*Opheodrys aestivus*), hatching from an egg. Photo credit: S. B. Hedges.



**Fig. 2** A timetree of amniotes. The divergence times are from Table 1. *Abbreviations:* C (Carboniferous), CZ (Cenozoic), J (Jurassic), Ng (Neogene), P (Permian), Pg (Paleogene), and Tr (Triassic).

are traditionally placed as the closest relatives of all other living reptiles (5, 7, 8). However, a recent morphological reevaluation of skull characters (9), and essentially all molecular genetic studies, indicates that turtles should be nested within diapsids despite their apparent anapsid skull morphology. Based on a growing body of genomic evidence (6, 10–14), it appears that turtles exhibit a secondary loss of skull fenestration or reversal to an ancestral condition.

Molecular data sets have not completely clarified the amniote picture, however, since different genes and sampling schemes have in some cases placed turtles within archosaurs, as the closest relative of crocodylians (6, 10, 13, 15), and in other cases, as the closest relative of an archosaur clade that includes crocodylians and birds (11, 12, 14, 16). Statistical evaluation of phylogenetic signal in these studies (10) has revealed that mitochondrial genome data have tended to separate turtles from archosaurs, whereas concatenated nuclear gene sequences under potentially strong selection such as globin genes, *lactate dehydrogenase (LDH)*, and ribosomal RNAs have tended to group turtles with crocodylians. The affinity of turtles and crocodylians has also been observed in evaluating short DNA word motifs embedded in more than 84 million basepairs (Mbp) of genomic sequence for amniotes (13). The earliest turtles appear ~223 Ma in the fossil record (3), which is within the interval of both older and younger molecular clock estimates for the time of their origin. The most recent comprehensive effort to resolve the relationships of the major groups of amniotes used more than 5100 amino acids from mostly single-copy nuclear *DPLA* and *GAG* genes within a maximum likelihood framework, and presented strong statistical support for a close

relationship of crocodylians and birds to the exclusion of turtles (11).

Despite considerable debate on the subject, few studies have presented average divergence time estimations across major branches of the amniote tree based on a calibrated clock analysis of numerous genes. Two highly visible studies within the last decade have set the foundation for an ongoing debate about integrating fossils and genes to estimate divergence times among major amniote clades. Kumar and Hedges (4) published their landmark comprehensive molecular timescale for vertebrate evolution based on protein clock calibrations of 658 nuclear genes and 207 vertebrate species. A point estimate of 310 Ma for the bird–mammal divergence, derived from radiometric dating of fossil evidence, was employed to externally calibrate the protein clock. Time estimates based on statistical tests of rate constancy (17, 18) were averaged across multiple genes and taxonomic groups and presented with 95% confidence intervals. Published dates relevant for the amniote timetree included a Permian average estimate for the origin of Lepidosauria at  $\sim 276 \pm 54.4$  Ma, and a bird–crocodylian split at  $\sim 222 \pm 52.5$  Ma.

Less than a year later, similar methods were focused specifically on the Reptilia. Hedges and Poling (6) analyzed combinations of 23 nuclear and two mitochondrial genes, including globulins, *LDH*, rRNAs, *alpha-crystalline*, *alpha-enolase*, and *cyt b*, to infer a molecular phylogeny of reptiles. As with previous analyses, the 310 synapsid–diapsid split date was used to anchor the molecular clock. The paper unconventionally joined turtles with crocodylians and suggested a more distant relationship between the tuatara and squamates based on a subset of amino acid data available for *Sphenodon*.

**Table 1.** Divergence times (Ma) and their confidence/credibility intervals (CI) among amniotes (Amniota).

Timetree		Estimates									
Node	Time	Refs. (4, 25)		Refs. (6, 27)		Refs. (12, 21, 23)		Ref. (15)		Ref. (20)(a)	
		Time	CI	Time	CI	Time	CI	Time	CI	Time	CI
1	324.5	-	-	323(27)	343-305	326(21)	354-311	-	-	-	-
2	274.9	276	383-169	245	269-221	285	-	250	267-233	285	296-274
3	271.5	-	-	-	-	-	-	-	-	268	280-256
4	230.7	225(25)	238-205	-	-	-	-	152	176-128	265	278-252
5	219.2	222	325-119	258(27)	279-238	196(23)	294-98	214	259-169	201	216-186

Timetree		Estimates (Continued)							
Node	Time	Ref. (20)(b)		Ref. (22)	Ref. (24)	Ref. (26)		Ref. (27)	
		Time	CI	Time	Time	Time	CI	Time	CI
1	324.5	-	-	-	-	-	-	-	-
2	274.9	289	302-276	-	294	-	-	-	-
3	271.5	275	292-258	-	-	-	-	-	-
4	230.7	273	291-255	191	278	-	-	-	-
5	219.2	194	218-170	175	254	259	282-236	258	279-238

Note: Node times in the timetree represent the mean of time estimates from different studies. Estimates are presented from an analysis of nine nuclear genes examined within a comprehensive analysis of 658 nuclear genes (4); 23 nuclear and nine mitochondrial protein-coding genes (6), 11 nuclear protein-coding genes (23); nuclear *LDH-A*, *LDH-B*, and *alpha-enolase* genes (15); 11 mitochondrial protein-coding genes (24); 12 protein-coding genes, two rRNA genes, and 19 tRNAs from mitochondria (25); 11 protein-coding genes and 19 tRNAs from mitochondria (12), 11 protein-coding genes and unspecified RNA genes from mitochondria (22); 325 protein-coding genes (21), mitochondrial genomes (26, 27), and the nucleotide (a) and amino acid sequences (b) of the nuclear *RAG1* gene (20).

Squamates were estimated to have diverged from other reptiles  $\sim 245 \pm 12.2$  Ma, birds from the crocodylian + turtle clade  $\sim 228 \pm 10.3$  Ma, and turtles from crocodylians  $\sim 207 \pm 20.5$  Ma. These results differ from previous average dates published for the vertebrate timescale (4) by suggesting a younger Triassic vs. Permian time frame for the origin of squamate lizards, and a slightly older time for the bird–crocodylian split. These divergence time estimates cannot be reconciled with fossil evidence that provides a minimum age estimate of at least  $\sim 223$  Ma for the oldest known turtles (3), but suggest that a number of key innovations during amniote evolution occurred within a roughly 40 million period of the Triassic. The study also posed a challenge for paleontologists to reconcile the derived position of turtles among amniotes and set a methodological framework for expanded application of molecular clock analyses to a variety of questions regarding vertebrate evolution.

Several additional studies warrant summary here for their contributions to establishing the amniote timetree. The first is specifically aimed at attempting to resolve

the phylogenetic position of turtles (15). The study adds considerable additional *LDH-A*, *LDH-B*, and *alpha-enolase* protein sequences to the available data matrix and employs the average distance methods and 310 Ma synapsid–diapsid clock calibration of Kumar and Hedges (4). Strong statistical support was provided for a turtle–crocodylian relationship to the exclusion of birds, with an exceptionally recent Upper Jurassic average divergence time estimate for turtles from crocodylians of only 152 Ma, some 71 million years younger than the oldest fossil turtle. No data were presented for *Sphenodon*, highlighting the overall lack of published divergence time estimates for this unique “living fossil.”

Rest *et al.* (12) examined 11 protein-coding genes and 19 tRNAs from the mitochondrial genomes of all major amniote lineages, emphasizing the importance of sampling the tuatara to accurately reconstructing reptile phylogeny. The study employed advances in Bayesian model-based methods of inference and resolved turtles as the sister group to archosaurs with 100% statistical support. Adopting a maximum likelihood approach to

estimating divergence times under a relaxed molecular clock assumption and multiple fossil calibration points (19), the authors reported a divergence time well into the Permian for the origin of Lepidosauria.

Most recently, Hugall *et al.* (20) analyzed the nuclear gene *RAG1* across 88 taxa spanning all major tetrapod clades. The study supported the close relationship of turtles to a monophyletic Archosauria and employed model-based rate-smoothing methods to estimate divergence times in amniotes. Results highlighted an ancient Permian origin for the tuatara estimated at ~268–275 Ma and revealed slower molecular evolutionary rates in archosaurs and especially turtles that tend to underestimate divergence times for these groups without using appropriate fossil calibration points. Comparison with other molecular clock studies underscored the bias toward inflated divergence time estimates created by saturated mitochondrial gene sequence data.

Seven other studies also present molecular time estimates for at least one node in the amniote tree of life (21–27). The genes examined in each of these studies are listed in the caption for Table 1 and the average time estimates from all 12 investigations considered here are reflected by the topology in Fig. 2. Based on this literature synthesis, the amniote common ancestor is dated at 325 Ma, with the origin of lepidosaurs taking place in the mid-Permian ~275 Ma, and the divergence of sphenodontids from squamates at 272 Ma, distinguishing the living tuatara as a remarkably ancient evolutionary relict. Turtles are estimated to have diverged from archosaurs in the early Triassic some 231 Ma, whereas crocodylians subsequently split from birds as recently as 219 Ma. It must be noted that confidence intervals on the estimates summarized in Table 1 are not available for several studies (12, 22, 24) or vary widely among studies depending on the number of genes investigated, the nature of the gene sequence data analyzed, and taxonomic sampling (e.g., 4 vs. 20). The results in Fig. 2 should therefore be interpreted cautiously within narrow time frames of amniote evolutionary history.

In summary, the amniote timetree paints a mixed portrait of organismal evolution among major tetrapod lineages that underwent a number of key vertebrate innovations adapted for the rigors of terrestrial life. The late Permian mass extinction was due presumably to widespread stressful environmental conditions produced by unfavorable ocean–atmosphere chemical interactions (28). Fossil diversity indicates a protracted biological recovery through the lower Triassic ~250 Ma (29) that falls between molecular time estimates for two major

periods of amniote cladogenesis: (1) the divergence and early diversification of lepidosaurs between ~270 and 275 Ma and (2) the origin of turtles and subsequent divergence of crocodylians and birds between roughly ~230 and 220 Ma. Young amniote lineages would have emerged into a relatively unconstrained evolutionary landscape before extensive Pangean supercontinental breakup. A combination of favorable environmental and biogeographic forces likely facilitated successful tetrapod invasion into a variety of open terrestrial niches during the Triassic, exemplified today by the extraordinary ecological, phenotypic, and taxonomic diversity of living amniotes.

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