Complete Mitochondrial DNA Sequences of the Green Turtle and Blue-Tailed Mole Skink: Statistical Evidence for Archosaurian Affinity of Turtles

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Turtles have highly specialized morphological characteristics, and their phylogenetic position has been under intensive debate. Previous molecular studies have not established a consistent and statistically well supported conclusion on this issue. In order to address this, complete mitochondrial DNA sequences were determined for the green turtle and the blue-tailed mole skink. These genomes possess an organization of genes which is typical of most other vertebrates, such as placental mammals, a frog, and bony fishes, but distinct from organizations of alligators and snakes. Molecular evolutionary rates of mitochondrial protein sequences appear to vary considerably among major reptilian lineages, with relatively rapid rates for snake and crocodilian lineages but slow rates for turtle and lizard lineages. In spite of this rate heterogeneity, phylogenetic analyses using amino acid sequences of 12 mitochondrial proteins reliably established the Archosauria (birds and crocodilians) and Lepidosauria (lizards and snakes) clades postulated from previous morphological studies. The phylogenetic analyses further suggested that turtles are a sister group of the archosaurs, and this untraditional relationship was provided with strong statistical evidence by both the bootstrap and the Kishino-Hasegawa tests. This is the first statistically significant molecular phylogeny on the placement of turtles relative to the archosaurs and lepidosaurs. It is therefore likely that turtles originated from a Permian–Triassic archosauromorph ancestor with two pairs of temporal fenestrae behind the skull orbit that were subsequently lost. The traditional classification of turtles in the Anapsida may thus need to be reconsidered.

Introduction

The position of turtles in vertebrate phylogeny remains a tangled problem with regard to how their lack of temporal fenestrae (openings behind the skull orbit involved in jaw muscle attachment) should be interpreted, i.e., whether it reflects an ancestral anaspid (holeless) condition of early reptiles or a state derived from the diapsid (two-hole) lineage leading to most extant reptiles, such as lizards, snakes, and crocodilians, as well as birds (Gauthier, Kluge, and Rowe 1988; Laurin and Reisz 1995; Lee 1995, 1997; Rieppel and deBraga 1996; Benton 1997, pp. 130–131; deBraga and Rieppel 1997; Platz and Conlon 1997; Wilkinson, Thorley, and Benton 1997). The most widely accepted view on the turtles' phylogeny since the late 1980s (fig. 1*A*) is that they are affiliated in the Anapsida as the sister clade to the Diapsida, in which archosaurs (e.g., birds, crocodilians, and dinosaurs) and lepidosaurs (e.g., lizards, snakes, and tuatara) are included (Gauthier, Kluge, and Rowe 1988; Laurin and Reisz 1995; Lee 1995, 1997; Benton 1997, pp. 130–131). This phylogeny depends largely on a traditional interpretation of turtles' lack of the temporal fenestration as the primitive characteristics common to extinct Paleozoic anapsids, among which the closest relatives of turtles have been proposed to be the captorhinids (Gauthier, Kluge, and Rowe 1988), procolophonids (Laurin and Reisz 1995), or pareiasaurs (Lee 1995, 1997).

Key words: green turtle, mole skink, reptiles, mtDNA, maximum-likelihood tree, molecular phylogeny.

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This view was recently challenged by Rieppel and deBraga (1996), who proposed the affinity of turtles to crown-group diapsids and, more specifically, to the lepidosaurs (fig. 1*B*). For this interpretation to hold, turtles must be lepidosauromorphs that have lost both the upper and the lower temporal fenestrae (Rieppel and deBraga 1996; deBraga and Rieppel 1997). More recently, however, the robustness of their conclusion, as well as the correctness of their morphological data set, has been questioned by other morphologists (Lee 1997; Wilkinson, Thorley, and Benton 1997). Because of the difficulty in interpreting the peculiar morphology of turtles, expectations have been placed on molecular phylogenetic approaches independent of morphological characteristics (Wilkinson, Thorley, and Benton 1997). Thus far, however, molecular studies have not established a consistent view on the turtles' phylogenetic placement, and no statistically well supported molecular phylogeny that can specify one of the alternative hypotheses of figure 1 has been obtained.

Except for Hedges, Moberg, and Maxson (1990), who used nuclear rRNA and protein genes, and Hedges (1994), who used mitochondrial rRNA and tRNA genes, the previous molecular studies used relatively short amino acid sequences for cytochrome *c* (Fitch and Margoliash 1967), crystallin (Caspers et al. 1996), hemoglobin (Fushitani et al. 1996), lactate dehydrogenase (Mannen et al. 1997), and pancreatic polypeptide (Platz and Conlon 1997). For example, Caspers et al. (1996) presented strong molecular evidence for a sister group relationship of birds and turtles relative to mammals, but the crystallin sequences alone did not clearly specify one of the three hypotheses of figure 1, among which a tree in figure 1*A* was favored. Although the pancreatic polypeptide data favored a tree in figure 1*C* (Platz and Conlon 1997), the other molecules supported either one of the

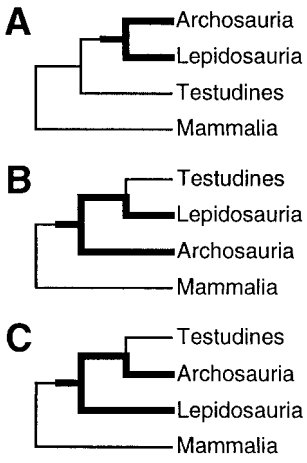

FIG. 1.—Phylogenetic hypotheses for the placement of turtles. *A,* Traditional placement of turtles as the sister clade to diapsids (archosaurs and lepidosaurs) (Gauthier, Kluge, and Rowe 1988; Laurin and Reisz 1995; Lee 1995, 1997; Benton 1997, pp. 130–131). *B,* Lepidosaurian affinity of turtles favored by some morphological studies (Rieppel and deBraga 1996; deBraga and Rieppel 1997). *C,* Archosaurian affinity of turtles concluded in the present study and supported by some other molecular (e.g., Platz and Conlon 1997; Zardoya and Meyer 1998) and morphological (e.g., de Beer 1937, pp. 462–463; Ax 1987, pp. 91–104) studies. Only the relationships among four clades with extant members are considered (with the exclusion of extinct groups): Archosauria (birds and crocodilians), Lepidosauria (lizards, snakes, and tuatara), Testudines (turtles), and Mammalia (mammals). Thick lines denote lineages that are likely to possess the diapsid condition, with the diapsid origin being placed in an arbitrary position on an ancestral lineage leading to archosaurs and lepidosaurs. In *B* and *C,* the anapsid condition of turtles must be a derived state from the diapsid condition.

trees in figure 1*A* and *B* or somewhat odd topologies in which, for example, birds and crocodilians did not immediately cluster with each other. In the most recent molecular study (Zardoya and Meyer 1998), the complete mitochondrial DNA (mtDNA) sequence for a side-necked turtle, *Pelomedusa subrufa,* was determined. Phylogenetic analyses of concatenated gene sequences clearly rejected the possibility of the turtles' earlier divergence than that between other reptiles and mammals (a popular view before the 1980s), and analyses of two rRNA gene sequences further suggested diapsid affinities of turtles and favored a sister group relationship of turtles with archosaurs (fig. 1*C*). However, statistical support for the latter claims still remained weak and turned out to be sensitive to ways of taxon representation (Zardoya and Meyer 1998). It was thus considered desirable to determine complete mtDNA sequences from representatives of lepidosaurs for further investigation of this issue.

We addressed this issue by independently sequencing complete mtDNA of the green turtle, in addition to that of a snake recently published by us (Kumazawa et al. 1998). However, presumably due to the unusually accelerated molecular evolutionary rates of snake mtDNAs (Kumazawa et al. 1998), the obtained sequence data did not serve to clearly specify one of the phylogenetic hypotheses of figure 1. We thus continued to sequence the complete mtDNA from a representative of the lizard group (blue-tailed mole skink), because our previous study using mitochondrial tRNA gene sequences (Kumazawa and Nishida 1995) suggested no rate acceleration in the lizard lineage. As expected, the skink became a precious short-brancher taxon from lepidosaurs in our phylogenetic analyses. This large molecular data set, combined with the reported complete mtDNA sequences for 16 other taxa, enabled us for the first time to provide a statistically significant conclusion on this issue. The resultant phylogenetic relationship (fig. 1*C*) is very different from the traditional view (fig. 1*A*).

Materials and Methods

Sequence Determination

DNA samples of the green turtle (*Chelonia mydas*) and blue-tailed mole skink (*Eumeces egregius lividus*) were the same as those used previously for sequencing mitochondrial tRNA genes (Kumazawa and Nishida 1995). They originated from frozen tissue collections of the Museum of Vertebrate Zoology of the University of California at Berkeley (collection numbers 13536 and 11013 for the turtle and skink, respectively). With total DNA extracted from these specimens, we initially amplified and sequenced the tRNA gene cluster regions and parts of the genes for cytochrome *b* (cytb) and 12S rRNA as previously described (Kocher et al. 1989; Kumazawa and Nishida 1993, 1995). Taxon-specific primers (see below) were synthesized on the basis of these partial sequences and used for long-and-accurate PCR (LA-PCR) in order to amplify spacing regions between these parts. The amplified products (2.0–10.5 kb; see fig. 2) were purified by agarose gel electrophoresis and used as templates either directly for the sequencing reaction or for additional short-sized amplification with dozens of universal primers synthesized on the basis of conserved sequences of each mitochondrial gene among known vertebrate mtDNA sequences (unpublished data). These amplified DNA fragments were sequenced for both strands with an Applied Biosystems 373A DNA sequencer using the primer-walking strategy. The complete mtDNA sequences were finally obtained by combining all the fragmentary sequences with special care with the identities in overlapping regions among them.

This rapid strategy for sequencing the entire mtDNA seems unlikely to be susceptible to errors caused by amplifying contaminating mtDNAs of other species or nuclear copies of mtDNA-like sequences. Either one or both of the paired primers used for the initial amplifications by LA-PCR were taxon-specific. Amplifications were carried out with different combinations of universal primers, and cross-checking of overlapping sequences among them was carefully done. The sizes of the products amplified by the taxon-specific primers (2.0–10.5 kb; see fig. 2) were larger than those of most known nuclear mtDNA copies. There was no evidence that determined sequences include pseudogenes. Since this strategy does not involve the cloning of amplified products, it is also unlikely to collect PCR errors.

Taxon-specific amplifications for the green turtle (see fig. 2) were carried out with the following combinations of primers (asterisks denote taxon-specific primers): L4437 in the IQM tRNA gene cluster region (TCA-

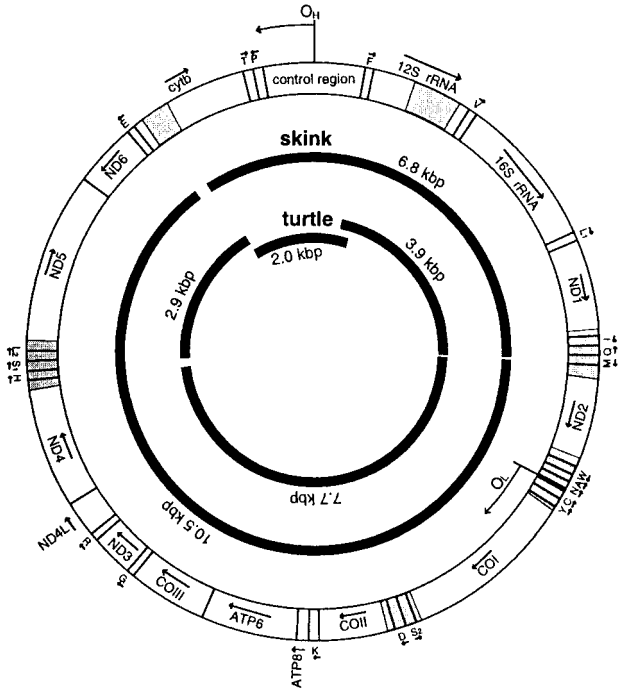

FIG. 2.—Amplification products by LA-PCR using taxon-specific primers. Gene organization of mtDNAs for the green turtle and blue-tailed mole skink is shown as a ring. The LA-PCR products for each species are shown as bold lines inside the ring. Shaded areas are the tRNA gene cluster regions and parts of cytb and 12S rRNA genes, which were initially amplified and sequenced in order to make taxon-specific primers for the LA-PCR amplification and/or for subsequent sequence determination (see *Materials and Methods* for more details).

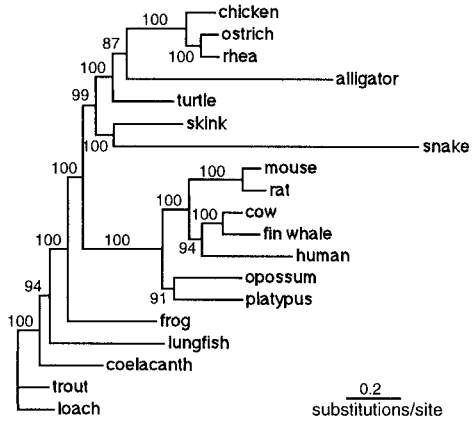

FIG. 3.—Maximum-likelihood tree among 19 vertebrates. Concatenated amino acid sequences of 12 light-strand mitochondrial protein genes were used (3,438 sites in total). The tree was built using PUZZLE, version 4.0 (Strimmer and von Haeseler 1996), with the mtREV24 substitution model (Adachi and Hasegawa 1996*b*) and the gamma-corrected rates (Ota and Nei 1994) as described in *Materials and Methods.* The estimated shape parameter for the gamma distribution (α) from the data set was 0.36. ML tree obtained with the protml program in MOLPHY (no gamma correction; Adachi and Hasegawa 1996*a*) showed an identical tree topology. Local bootstrap probabilities obtained by the RELL method of the protml program from 1,000 replications are shown as percentages along the corresponding branches. The names of the taxa used and their data sources are: mouse, accession number J01420 (Bibb et al. 1981); rat, X14848 (Gadaleta et al. 1989); human, J01415 (Anderson et al. 1981); cow, J01394 (Anderson et al. 1982); fin whale, X61145 (Arnason, Gullberg, and Widegren 1991); opossum, Z29573 (Janke et al. 1994); platypus, X83427 (Janke et al. 1996); chicken, X52392 (Desjardins and Morais 1990); ostrich, Y12025 (Härlid, Janke, and Arnason 1997); rhea, Y16884 (Härlid, Janke, and Arnason 1998); alligator, Y13113 (Janke and Arnason 1997); skink, AB016606 (this study); snake, AB008539 (Kumazawa et al. 1998); turtle, AB012104 (this study); frog, M10217 (Roe et al. 1985); lungfish, L42813 (Zardoya and Meyer 1996); coelacanth, U82228 (Zardoya and Meyer 1997); loach, M91245 (Tzeng et al. 1992); and trout, L29771 (Zardoya, Garrido-Pertierra, and Bautista 1995).

GCTAATTAAGCTTTCGGGCCCATACC) and CM-H1* in the HSL region (TAACCAAGCTTGAAG-GAGCCTCAGATTAGTTCTGGT) for 7.7 kb, CM-L1* in the HSL region (TTTCCGGATCCTAAAGGATA-GAAGTAATCCACTGGT) and H15149 in the cytb gene (Kocher et al. 1989) for 2.9 kb, CM-L3* (TAGGCTACGTCCTACCATGAGG) in the cytb gene and CM-H2* in the 12S rRNA gene (AGGGCGTTTT-CACTGGTGTGCA) for 2.0 kb, and L617m (AA-AGCATRGCACTGAAGATG) in the phenylalanine tRNA gene and H4408cm* (TATGGGCCCGAAAGCT-TAATTAGCTGAC) in the IQM region for 3.9 kb. Primers for taxon-specific amplifications for the blue-tailed mole skink are L4437 in the IQM region (see above) and EEL-H6* in the cytb gene (TAGCTAAGAA-TAGGCCG) for 10.5 kb and EEL-L4* in the cytb gene (CAACCGCATTCGTAGGCT) and H4433 in the IQM region (Kumazawa and Nishida 1995) for 6.8 kb.

Phylogenetic Analyses

Nineteen taxa were used in phylogenetic analyses of this study (see the legend of fig. 3 for their names and accession numbers in the DDBJ/EMBL/GenBank nucleotide sequence databases). We employed concatenated amino acid sequences of mitochondrial protein genes, which can provide one of the largest molecular data sets using clearly orthologous genes. The alignment of translated amino acid sequences of 12 light-strand mitochondrial protein genes was obtained with the aid of CLUSTAL W (Thompson, Higgins, and Gibson 1994) and, finally, inspected and corrected by eye. Ambiguous parts of the alignment were subsequently removed from the alignment. In the actual phylogenetic analyses, any gap sites among the taxa employed were additionally removed. It should be noted that a frameshift in the NADH dehydrogenase subunit 3 (ND3) gene (see, e.g., Härlid, Janke, and Arnason 1998; Mindell, Sorenson, and Dimcheff 1998) was corrected to restore the maximum matching of the alignment. The alignment is obtainable from Y.K. on request.

Because the data set of the mitochondrial proteins was found to have strong rate heterogeneities among both lineages and sites (see below), we primarily used maximum-likelihood (ML) analyses, which are considered to be robust against the among-lineage rate heterogeneity (Hasegawa, Kishino, and Saitou 1991; Li 1997, pp. 127–136), followed by confirmation by neighbor-joining analyses (Saitou and Nei 1987). The ML analyses were done using PUZZLE, version 4.0 (Strimmer and von Haeseler 1996), and MOLPHY, version 2.3 (Adachi and Hasegawa 1996*a*). PUZZLE implements the quartet puzzling (QP) algorithm, which searches for the ML tree within a relatively short computational time.

Table 1
Kishino-Hasegawa Tests for the Phylogenetic Position of Turtles

Tree No.	Sister Group of Testudines	Topological Hypothesis	ln*L*	Δln*L*	SE	Δln*L*/SE
18 taxa without the snake						
1	Archosauria	(outgroup, ((birds, alligator), turtle), skink)	−55,285.78			
2	Aves	(outgroup, ((birds, turtle), alligator), skink)	−55,305.82	20.04	8.76	2.29*
3	Crocodylia	(outgroup, (birds, (alligator, turtle)), skink)	−55,304.99	19.21	8.86	2.17*
4	Lepidosauria	(outgroup, (birds, alligator), (turtle, skink))	−55,336.56	50.77	15.82	3.21*
5	Diapsida	(outgroup, ((birds, alligator), skink), turtle)	−55,343.96	58.18	14.47	4.02*
19 taxa including the snake						
1′ . . .	Archosauria	(outgroup, ((birds, alligator), turtle), (skink, snake))	−59,231.24			
2′ . . .	Aves	(outgroup, ((birds, turtle), alligator), (skink, snake))	−59,246.75	15.51	8.46	1.83
3′ . . .	Crocodylia	(outgroup, (birds, (turtle, alligator)), (skink, snake))	−59,247.65	16.41	8.18	2.01*
4′ . . .	Lepidosauria	(outgroup, (birds, alligator), (turtle, (skink, snake)))	−59,284.39	53.15	14.58	3.65*
5′ . . .	Diapsida	(outgroup, ((birds, alligator), (skink, snake)), turtle)	−59,286.25	55.01	14.16	3.88*
6′ . . .	—	(outgroup, ((birds, (alligator, snake)), turtle), skink)	−59,239.66	8.42	18.48	0.46
7′ . . .	—	(outgroup, (((birds, alligator), turtle), snake), skink)	−59,238.27	7.03	8.62	0.82
8′ . . .	—	(outgroup, ((birds, turtle), (alligator, snake)), skink)	−59,247.05	15.80	16.79	0.94
9′ . . .	—	(outgroup, (((birds, alligator), snake), turtle), skink)	−59,246.56	15.32	15.89	0.96
10′ . . .	—	(outgroup, (((birds, snake), alligator), turtle), skink)	−59,248.79	17.55	17.75	0.99
11′ . . .	—	(outgroup, (birds, (turtle, (alligator, snake))), skink)	−59,258.37	27.13	18.38	1.48
12′ . . .	—	(outgroup, (((birds, turtle), alligator), snake), skink)	−59,252.01	20.77	12.07	1.72
13′ . . .	—	(outgroup, ((birds, (alligator, turtle)), snake), skink)	−59,254.20	22.96	11.83	1.94

NOTE.—The natural logarithm of the likelihood value and its standard error are indicated as ln*L* and SE, respectively. Δln*L* shows the difference in ln*L* from that of the ML tree topology. Values with an asterisk indicate that the corresponding hypothesis can be statistically rejected (5% significance) by the standard criterion Δln*L*/SE $>$ 1.96 (Kishino and Hasegawa 1989). All the possible unrooted tree topologies among the outgroup, birds, alligators, turtles, and skinks in the case of the 18 taxa (15 topologies) and among the outgroup, birds, alligators, turtles, skinks, and snakes in the case of the 19 taxa (105 topologies) were examined. Trees 2′ and 6′–13′ (except the ML tree topologies) are not statistically rejectable among them, although they are very unlikely (see text). Refer to figure 3 for in-group topologies for outgroup and birds.

PUZZLE has an advantage that the gamma correction for the rate heterogeneity among sites can be used conveniently in combination with an available amino acid substitution model, whereas the protml program in MOLPHY can provide local bootstrap probabilities (bootstrap values given to a node by fixing relationships in other parts of the tree) by the RELL method.

For both of the applications, the mtREV24 model, developed specifically for mitochondrial protein sequences (Adachi and Hasegawa 1996*b*), was used as an empirical model for amino acid substitutions, and parameters for amino acid frequency were estimated from the data set. In PUZZLE, among-site rate heterogeneity was corrected using the gamma-distributed model of substitution rates among sites with eight categories (Ota and Nei 1994), and the shape parameter of the gamma distribution was estimated from the data set. Kishino-Hasegawa tests or likelihood ratio tests (Kishino and Hasegawa 1989) were conducted using PUZZLE with the same method, models, and parameters as described above.

Phylogenetic analyses using PUZZLE were conducted by first establishing the ML tree (for both the topology and the branch lengths) and then evaluating by the Kishino-Hasegawa test whether alternative hypotheses are significantly worse than the ML tree topology. Because the QP algorithm in PUZZLE may not always provide the ML tree (Strimmer and von Haeseler 1996; Cao, Adachi, and Hasegawa 1998), it has been advised to conduct local rearrangements of the QP tree topology obtained by the QP algorithm and find the real ML tree with the highest likelihood value (Cao, Adachi, and Hasegawa 1998). In the present study, there was the same situation, in which the QP tree topology (tree 2′ in table 1) did not correspond to the ML tree topology (tree 1′ in table 1; see also fig. 3). To cope with this problem, we calculated likelihood values for all of 105 possible unrooted tree topologies among the outgroup, birds, alligator, turtle, skink, and snake, and the ML tree with the highest likelihood value among them (fig. 3) was determined. This procedure seems to have a practical meaning similar to that of the local rearrangements. It should be noted that in-group topologies within the outgroup (mammals, frog, and fishes) and within birds have been established by a number of previous studies using essentially the same data set of mitochondrial protein sequences (Janke et al. 1994, 1996; Zardoya and Meyer 1996, 1997, 1998; Härlid, Janke, and Arnason 1997, 1998; Janke and Arnason 1997; Kumazawa et al. 1998).

Results and Discussion

Characteristics of the Turtle and Skink mtDNAs

The complete mtDNA sequences of the green turtle and blue-tailed mole skink comprise of 16,497 and 17,407 bp, respectively. They share the same gene organization (fig. 2) that is typical of many other vertebrates, such as placental mammals (see, e.g., Anderson et al. 1981, 1982; Bibb et al. 1981; Gadaleta et al. 1989; Arnason, Gullberg, and Widegren 1991), frogs (Roe et al. 1985), the side-necked turtle (Zardoya and Meyer 1998), and bony fishes (Tzeng et al. 1992; Zardoya, Garrido-Pertierra, and Bautista 1995; Zardoya and Meyer 1996, 1997), but is distinct from the gene organizations

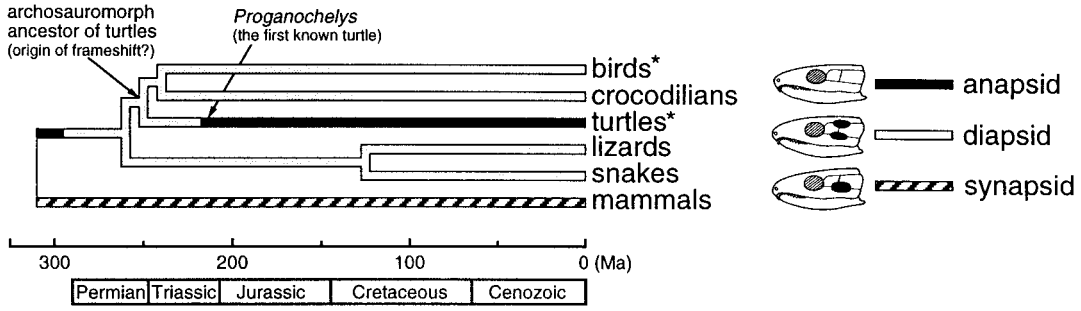

FIG. 4.—Evolution of turtles inferred from the molecular phylogeny of this study and paleontological records. The tree is based on the phylogenetic relationship shown in figure 3 and approximate divergence dates estimated from fossil records (Benton 1990): 310 MYA for synapsids versus sauropsids, 260 MYA for archosaurs versus lepidosaurs, 240 MYA for birds versus crocodilians, and 125 MYA for lizards versus snakes. Note that the divergence point of the turtles' lineage is placed in an arbitrary position on an appropriate internal branch suggested by our molecular phylogeny (see fig. 3). The inferred state of the temporal fenestrae based on the parsimony principle is shown along each lineage. The origin of the diapsid state was considered to correspond to the emergence of the late Carboniferous araeoscelidians (Benton 1997, pp. 130–131). An asterisks indicate taxa which were shown to include species having the ND3 gene frameshift (see text).

of crocodilians (Kumazawa and Nishida 1995; Quinn and Mindell 1996; Janke and Arnason 1997) and snakes (Kumazawa and Nishida 1995; Kumazawa et al. 1996, 1998) with respect to local arrangements of a few tRNA genes. Moreover, snake mtDNAs are known to maintain duplicate control regions (Kumazawa et al. 1996, 1998), a feature that is not found in the skink mtDNA, implying that duplication of the control region took place on a lineage leading to snakes after their divergence from the lizard.

Base compositions of the green turtle and skink mtDNAs are skewed in a similar way to those of other vertebrates, and the sizes of individual genes encoded in both the mtDNAs are similar to those of other vertebrates (data not shown). The slightly increased size of the skink mtDNA genome is due to three kinds of tandem repeats in the control region as well as a long intergenic spacer between genes for cytb and threonine tRNA created by a tandem duplication of an approximately 120-bp sequence (data not shown).

A characteristic stem-and-loop structure for the putative origin of light-strand replication inside the WANCY tRNA gene cluster, as well as conserved sequence blocks I–III inside the control region (reviewed in Clayton 1992), were found in both the green turtle and the skink mtDNAs (fig. 2 and data not shown), suggesting that the asymmetrical replication mechanism revealed for mammalian mtDNAs (Clayton 1992) also operates in these reptilian mtDNAs. The existence of the light-strand replication origin in the green turtle is consistent with its presence in mtDNAs of two other cryptodiran turtles (Seutin et al. 1994), but contrasts with its apparent disappearance from the WANCY region in the side-necked turtle, *P. subrufa* (Zardoya and Meyer 1998).

An apparent +1 frameshift at a specific position of the ND3 gene was reported to occur in some birds and turtles (Härlid, Janke, and Arnason 1997, 1998; Mindell, Sorenson, and Dimcheff 1998; Zardoya and Meyer 1998), but not in other birds (Mindell, Sorenson, and Dimcheff 1998), crocodilians (Janke and Arnason 1997), and snakes (Kumazawa et al. 1998). Avian taxa that have the frameshift and those that do not are not clearly separated from each other in well-accepted avian phylogenies (Mindell, Sorenson, and Dimcheff 1998). In this study, the same +1 frameshift of the ND3 gene was found for the green turtle but not for the skink. Occurrence of the very same type of frameshift in the diverse lineages favors the interpretation of their common origin and multiple losses, rather than their independent multiple originations in parallel. If the amniote phylogenetic relationship revealed in the present study is taken into consideration, a plausible explanation is that the frameshift originated on an ancestral lineage of turtles and archosaurs, and that it was lost on multiple lineages leading to crocodilians and some birds (fig. 4; see also Mindell, Sorenson, and Dimcheff 1998). More vigorous studies on this subject, including molecular mechanisms of the frameshift, are expected. Although Zardoya and Meyer (1998) reported another frameshift in the ND4L gene of the side-necked turtle, such a frameshift was not detected in the green turtle ND4L gene.

Molecular Phylogenetic Analyses

Figure 3 shows an ML tree obtained from the mitochondrial protein sequences of 19 taxa as described in *Materials and Methods.* The ML tree indicates the alliance between birds and crocodilians (the Archosauria clade) as well as between lizards and snakes (the Lepidosauria or Squamata clade). These clades have been supported by a number of morphological, paleontological, and molecular studies (see, e.g., Gauthier, Kluge, and Rowe 1988; Hedges 1994; Kumazawa and Nishida 1995; Benton 1997, pp. 133–156, 236–249; deBraga and Rieppel 1997; Kumazawa et al. 1998). The ML tree also points to the inclusion of turtles within the diapsid lineage rather than to them being an outgroup to the Diapsida. In addition, the ML tree indicates that turtles are more closely related to archosaurs than to lepidosaurs. Local bootstrap probability for this relationship obtained with MOLPHY was 100%. A nodal relationship among the turtle, birds, and alligator was provided with a strong bootstrap probability (87%) but it was slightly below the 95% confidence level. In order to make a thorough statistical evaluation of the turtles' position within Diap-

sida, the Kishino-Hasegawa tests (table 1) were further conducted by assuming and comparing several phylogenetic hypotheses. Because molecular evolutionary rates of snake mtDNAs are considerably higher than those of the other taxa (Kumazawa et al. 1998; see also below), inclusion of the snake in the analyses may have misleading effects on the accurate evaluation of relative likeliness among the hypotheses (Saitou and Imanishi 1989; Li 1997, pp. 127–136). The likelihood ratio tests were therefore conducted both with (19 taxa, 3,438 sites) and without (18 taxa, 3,465 sites) the snake.

In the case of 18 taxa without the snake, the ML tree topology (tree 1 of table 1) indicates that turtles cluster with archosaurian taxa (see also fig. 1*C*). The other four hypotheses shown (trees 2–5 of table 1) are significantly worse, with tree 5—which represents the traditional view of phylogenetic relationships (fig. 1*A*)—being the worst of all. Tree 4, which represents the lepidosaurian affinity of turtles (fig. 1*B*), is also statistically rejectable. We calculated likelihood values for all of the 15 possible unrooted tree topologies among the outgroup, birds, alligator, turtle, and skink and found that all of them except tree 1 are significantly worse than tree 1 (data not shown). These results provide strong statistical evidence for the archosaurian affinity of turtles.

This conclusion was further reinforced by analyses that included the snake sequence (table 1). The ML tree topology (tree 1′) supports the archosaurian affinity of turtles. Hypotheses for the lepidosaurian affinity of turtles (tree 4′) and for the sister group status of turtles to the Diapsida (tree 5′) are both significantly worse than tree 1′. Among the 105 possible unrooted tree topologies, except tree 1′, 9 topologies (trees 2′ and 6′–13′) could not be rejected by the standard criterion $\Delta \ln L/\mathrm{SE} > 1.96$ (Kishino and Hasegawa 1989). However, trees 6′–13′ are unlikely, because the monophyly of a well-established clade, Squamata (lizards and snakes), is violated. Furthermore, in trees 6′, 8′, 10′, and 11′, even archosaurs (birds and the alligator in this case) do not cluster with each other relative to the squamates. The Squamata and Archosauria clades have been established by various morphological and molecular studies, as described above. Only tree 2′ remains a possible hypothesis, but we consider it also unlikely. The difference of its log-likelihood value from that of tree 1′ is close to the significant level, and a similar hypothesis in the case of 18 taxa (tree 2) was statistically rejected.

Rate Heterogeneity

Simulation studies have shown that correct phylogenetic reconstruction may be hampered by the heterogeneity of molecular evolutionary rates among both lineages and sites, but that, except in extreme cases, it should be achievable by appropriate selection of methods, models, and parameters (see, e.g., Felsenstein 1988; Saitou and Imanishi 1989; Hasegawa, Kishino, and Saitou 1991; Li 1997, pp. 127–136). Branch lengths in figure 3 suggest strong rate heterogeneity among reptilian lineages. As previously described (Kumazawa and Nishida 1995; Janke and Arnason 1997; Kumazawa et al. 1998), the snake and crocodilian sequences appear to have evolved rapidly. The reason for the rate acceleration in serpentine and crocodilian mtDNAs remains unclear, although the instability of mtDNA gene arrangement features in these animals (Kumazawa and Nishida 1995; Kumazawa et al. 1996, 1998; Quinn and Mindell 1996; Janke and Arnason 1997) may be associated with it. On the other hand, much slower evolutionary rates for mtDNAs of turtles than for those of mammals have been shown at the DNA sequence level (Bowen, Nelson, and Avise 1993). Consistent with this finding, a terminal branch leading to the turtle is relatively short (fig. 3), indicating a similar rate reduction in turtles at the amino acid sequence level. The skink sequences also appear to have evolved slowly (fig. 3).

However, we do not believe that the among-lineage rate heterogeneity erroneously led to our phylogenetic conclusion for the archosaurian affinity of turtles. First, exclusion of a fast-evolving taxon (the snake) made this conclusion clearer (table 1). Second, an infamous pattern of errors in analyzing data with unequal rates among lineages is that fast-evolving taxa tend to be clustered (see, e.g., Penny, Hendy, and Henderson 1987; Felsenstein 1988; Li 1997, pp. 127–136). The ML tree (fig. 3) does not show such a pattern, but fast-evolving taxa (the snake and the alligator) and slow-evolving taxa (the turtle and the skink) are dispersed in two basal reptilian + avian clades. Third, simulation studies (Hasegawa, Kishino, and Saitou 1991; Li 1997, pp. 127–136) have shown that the ML method, unlike the maximum-parsimony method, is quite robust against the effects of unequal rates among lineages (even against a ninefold rate difference). The neighbor-joining method is also considered robust against the unequal rates, if the distances are estimated accurately (Felsenstein 1988; Saitou and Imanishi 1989; Li 1997, pp. 127–136). The neighbor-joining tree constructed from the ML distance estimates (data not shown) showed the same topology as and similar branch lengths to those obtained for the ML tree of figure 3, further supporting the robustness of our phylogenetic conclusion.

We also do not believe that possible rate heterogeneity among sites has misled our conclusion. This type of rate heterogeneity was explicitly corrected using the gamma-distributed substitution rates, because the gamma parameter estimated from the data set ($\alpha = 0.36$) pointed to the strong among-site rate heterogeneity that needs to be corrected. When uniform rates among sites were assumed, the same ML tree topology as in figure 3 was obtained (data not shown). However, the neighbor-joining tree based on the ML distance estimates with site-homogeneity showed a quite unusual topology, (fishes, frog, (skink, ((mammals, (alligator, snake)), (birds, turtle)))), presumably because of inaccurate distance estimation resulting from the inappropriate assumption of the uniform rates (Felsenstein 1988; Li 1997, pp. 127–136). This further validates our use of the gamma-corrected rates.

Phylogenetic Resolution

Previous molecular studies relevant to the phylogenetic position of turtles provided neither a consistent

view on the issue nor significant statistical support for one of the alternative hypotheses of figure 1 (see *Introduction*). The lack of well-supported resolution in the previous studies may be primarily due to insufficient phylogenetic information, obtained from shorter sequences than those used in this study. When the 12 protein sequences of our data set were analyzed individually, none of them, by itself, could resolve the phylogenetic placement of turtles with strong statistical evidence. In the ML analyses using the 18 taxa, 7 of 12 protein sequences (cytb, ND 2, 3, and 4, cytochrome oxidase subunits I and III, ATPase subunit 6) supported the archosaurian affinity of turtles (tree 1 of table 1), but without statistically rejecting many of 14 alternative hypotheses (data not shown). The topology supported by the other 5 proteins differed from protein to protein, and their log-likelihood values were only marginally better than those of tree 1 (data not shown).

We believe that the increase of statistical support for the archosaurian affinity of turtles by concatenation of the protein sequences resulted from amplification of phylogenetic signals relative to stochastic errors, rather than from that of positively false signals in the data. The inconsistency in phylogenetic estimation could, in principle, arise not only from the temporal (as discussed above), but also from the modal heterogeneity of sequence evolution (Li 1997, pp. 127–136). In order to examine this possibility, we conducted an ML analysis by deleting 6 taxa (mouse, opossum, rhea, snake, trout, and loach) which were shown to have significantly (5% chi-square test with PUZZLE) different amino acid frequencies from the average values among the 19 taxa, and we confirmed the robustness of our phylogenetic conclusion (data not shown). Furthermore, some amino acids with aliphatic side chains were reported to be responsible for a notorious inconsistency example in analyzing a distantly related metazoan phylogeny (Naylor and Brown 1997). Our conclusion was also unaffected (data not shown) when the data were analyzed after converting all isoleucines and valines to leucines, as done by Cao et al. (1998).

Evolution of Turtles

The present study provided strong statistical support by both the bootstrap (fig. 3) and the Kishino-Hasegawa (table 1) tests for the turtles' origination from an archosauromorph ancestor with two pairs of temporal fenestrae that were subsequently lost (figs. 1*C* and 4). This implies that key characters on the number and style of the temporal fenestra, on which the traditional classification of reptiles has depended, are more unstable and variable than previously thought, requiring reconsideration of the traditional classification of turtles in the Anapsida (see Rieppel and deBraga [1996] and deBraga and Rieppel [1997] for more detailed discussion on this character). There is no strong reason to preclude the possibility of the secondary loss of temporal fenestrae (deBraga and Rieppel 1997). Rather, currently available morphological evidence shows other examples in which the secondary loss of the lower temporal fenestrae should be postulated (e.g., extinct euryapsids such as the nothosaurs, plesiosaurs, and ichthyosaurs, extinct dinosaurian ankylosaurids, and extinct archosauromorph trilophosaurids) (Rieppel and deBraga 1996; Benton 1997, pp. 109, 110, 144–152, 213, 214; deBraga and Rieppel 1997). It is noteworthy that the trilophosaurids are affiliated in the Archosauromorph (Benton 1997, pp. 144–145), which is now proposed to have phylogenetic relatedness to turtles.

The archosaurian affinity of turtles was once supported from morphological standpoints (see, e.g., de Beer 1937, pp. 462–463; Ax 1987, pp. 91–104). To the best of our knowledge, however, no recent study based on morphological evidence has reached such a conclusion, including that by Rieppel and deBraga (1996), who deduced that turtles have an affinity to lepidosaurian diapsids (fig. 1*B*). If our molecular phylogeny really is the case, this discrepancy implies the existence of considerable homoplasy in the morphological data so far reported, which may have been caused, at least in part, by the peculiar morphology of turtles. Then, how could one find the closest relative of turtles among the Permian–Triassic archosauromorphs (Benton 1997, pp. 144–148)? We suggest two lines of investigation with regard to this question.

One line of investigation would be to reanalyze the morphological data under the topological constraints proposed herein. Preliminary inspection of the reported morphological data matrix (Rieppel and deBraga 1996; deBraga and Rieppel 1997; Lee 1997) under a topological constraint, (Synapsida, (Lepidosauriformes, (Archosauromorpha and Testudines))), pointed to close association between turtles and some archosauromorphs (Rhynchosauria and *Trilophosaurus*) with regard to a few characters that have been interpreted to have been acquired independently by these groups, e.g., loss of premaxillary teeth and loss of the femoral fourth trochanter. Moreover, as described above, turtles and trilophosaurids share the characteristic of lost lower temporal fenestrae. It thus seems intriguing to test whether these characters represent synapomorphies supporting the phylogenetic affinity between turtles and the above-mentioned archosauromorph groups by more intensive morphological analyses in the future.

Another line of approach would be to estimate the divergence time of the turtles' lineage from the major archosaurian lineage leading to extant birds and crocodilians. The present data set based on 12 mitochondrial proteins appears to include taxa with increased molecular evolutionary rates (snakes and crocodilians; see fig. 3), and it thus seems difficult to assume the general molecular clock for all the taxa sampled. However, our molecular phylogeny (fig. 3) clearly suggests that the origin of the turtles' lineage preceded the divergence between Aves and Crocodylia, which has been estimated to be at least 240 MYA from paleontological records (Benton 1990; see also fig. 4). The molecular phylogeny also suggests that origination of the turtles' lineage was preceded by separation between archosauromorph and lepidosauromorph lineages, which has been roughly estimated to be 260 MYA or earlier (Benton 1990). Thus, it is likely that the divergence of turtles from the major

archosaurian lineage took place within a time range from the Permian to the early Triassic, somewhat earlier than the emergence of the oldest known turtle, *Proganochelys*, in the Late Triassic (Benton 1997, pp. 233–236; see also fig. 4).

Recent intensive debate on the phylogenetic position of turtles has raised the more general and fundamental question of how evolutionary processes of morphologically specialized organisms like turtles can best be understood (see, e.g., Rieppel and deBraga 1996). We consider that such a question can be fruitfully approached by effective interactions and complementation between morphological (or paleontological) and molecular evolutionary studies.

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