

The Complete Mitochondrial Genome of *Alligator mississippiensis* and the Separation Between Recent Archosauria (Birds and Crocodiles)

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The complete mitochondrial genome of the alligator, *Alligator mississippiensis*, was sequenced. The size of the molecule is 16,642 nucleotides. Previously reported rearrangements of tRNAs in crocodile mitochondrial genomes were confirmed and, relative to mammals, no other deviations of gene order were observed. The analysis of protein-coding genes of the alligator showed an evolutionary rate that is roughly the same as in mammals. Thus, the evolutionary rate in the alligator is faster than that in birds as well as that in cold-blooded vertebrates. This contradicts hypotheses of constant body temperatures or high metabolic rate being correlated with elevated molecular evolutionary rates. It is commonly acknowledged that birds are the closest living relatives to crocodiles. Birds and crocodiles represent the only archosaurian survivors of the mass extinction at the Cretaceous/Tertiary boundary. On the basis of mitochondrial protein-coding genes, the Haemothermia hypothesis, which defines birds and mammals as sister groups and thus challenges the traditional view, could be rejected. Maximum-likelihood branch length data of amino acid sequences suggest that the divergence between the avian and crocodilian lineages took place at ≈ 254 MYA.

Introduction

The origin of the Crocodylia can be traced back paleontologically to the Middle Triassic, ≈ 240 MYA. Since the late Triassic/early Jurassic, 180–200 MYA, crocodilian fossils have maintained the general skeletal features characterizing recent crocodilians, showing that their morphological evolution has been strikingly conservative (Carroll 1988). The 22 species of recent crocodilians are organized in three families, Alligatoridae, Crocodylidae, and Gavialidae.

Although similar in appearance, crocodilians are only distantly related to lizards. The birds are generally regarded as the closest extant relatives of the crocodilians, owing to some unique archosaurian features characterizing both birds and crocodilians (Carroll 1988). A sister group relationship between birds and reptiles, notably crocodilians, has been questioned by the "haemothermia" hypothesis according to which, on the basis of both morphological and molecular data, a sister group relationship between birds and mammals has been advocated (Dene et al. 1982; Gardiner 1982; Løvtrup 1985; Hedges, Moberg, and Maxson 1990). These conclusions have been challenged, however, by other studies (Benton 1985; Kemp 1988; Marshall 1992) which have favored the traditional view of monophyly of Diapsids (birds, lizards, snakes, dinosaurs, crocodiles). Studies of α -crystallin A (de Jong et al. 1985), α -hemoglobin (Perutz et al. 1981), mitochondrial tRNA genes (Kumazawa and Nishida 1995), and mitochondrial 12S and 16S rRNA genes (Hedges, Moberg, and Maxson 1994) have also supported the traditional amniote relationship with lizards, crocodiles, and birds as the sister group of mammals. Due to the somewhat limited amount of molecular data, the support for the conclusions was not

particularly strong, however, and the rRNA study reconstructed questionable relationships among mammals.

Two rearrangements of tRNAs have been described in the crocodilian mitochondrial genome, making it unique among vertebrates (Kumazawa and Nishida 1995; Quinn and Mindell 1996). In the present study, we describe the complete mitochondrial genome of an alligator, *Alligator mississippiensis*, thereby making it possible to examine all potential structural rearrangements in the genome. In addition to this, we examine the phylogenetic relationships among mammals, birds, and crocodilians and provide a molecular dating of the divergence between birds and crocodilians.

Materials and Methods

Mitochondrial (mt) DNA of the alligator was isolated from frozen liver tissue following the procedure described by Arnason, Gullberg, and Widegren (1991). The sample was kindly provided by Dr. Ruth Elsey, Rockefeller Wildlife Refuge, Louisiana.

The mtDNA fraction was digested separately or in combination with *Bln* I, *Spe* I, and *Hind* III, and the fragments were ligated into M13 mp18/mp19 and cloned in *E. coli* strain DH5- α . Sequencing was performed manually by primer walking applying the dideoxy termination technique with [35 S]-dATP (Sanger 1981). The sequence of the whole molecule was determined on the basis of natural (not PCR) clones, except for one portion extending from position $\approx 11,000$ to position $\approx 13,500$. This region, as well as ≈ 400 nucleotides (nt) around cloning sites, were PCR amplified and sequenced by cycle sequencing on ABI prism 310.

The phylogenetic analyses of concatenated sequences of mitochondrial protein-coding genes were performed using the PHYLIP (Felsenstein 1991, v. 3.5c), MOLPHY (Adachi and Hasegawa 1996a), and PUZZLE (Strimmer and von Haeseler 1996, v. 3.0) packages. Gaps and ambiguous alignments adjacent to gaps were excluded from the phylogenetic analyses. The analyses were performed by amino acid (aa) sequences as well as nt analysis of second codon position sequenc-

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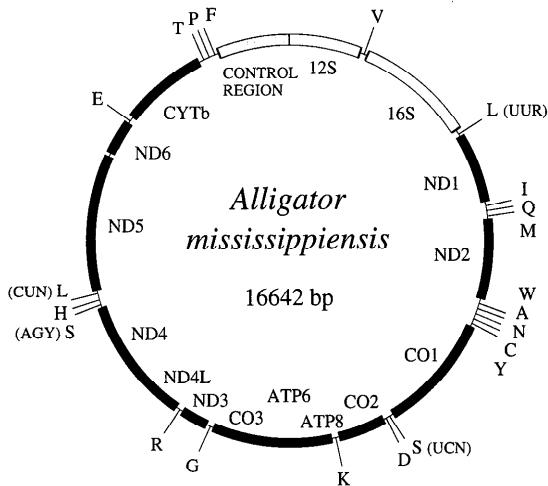


FIG. 1.—Genetic map of the *Alligator mississippiensis* mitochondrial genome. The tRNAs are identified by one-letter aa code and, in the case of tRNA^{Ser} and tRNA^{Leu}, by codon family.

es. 12S and 16S rDNA sequence data as well as large proportions of the tRNA genes of the alligator were phylogenetically studied earlier (Hedges 1994; Kumazawa and Nishida 1995) and were not reanalyzed here. The protein-coding genes of the following species were included in the alignment and analysis of alligator data: carp, X61010 (Chang, Huang, and Lo 1994); loach, M91245 (Tzeng et al. 1992); *Xenopus*, X02890 (Roe et al. 1985); chicken, P18946 (Desjardins and Morais 1990); ostrich, Y12025 (Härlid, Janke, and Arnason 1997); platypus, X83427 (Janke et al. 1996); wallaroo, Y10524 (Janke, Xu, and Arnason 1997); opossum, Z29573 (Janke et al. 1994); hedgehog, X88898 (Krettek, Gullberg, and Arnason 1995); rat, P00159 (Gadaleta et al. 1989); mouse, J01420 (Bibb et al. 1981); armadillo, Y11832 (Arnason, Gullberg, and Janke 1997); harbor seal, X63726 (Arnason and Johnsson 1992); horse, X79547 (Xu and Arnason 1994); Indian rhinoceros, X97336 (Xu, Janke, and Arnason 1996); cow, V00654 (Anderson et al. 1982); fin whale, X61145 (Arnason, Gullberg, and Widegren 1991); blue whale, X72204 (Arnason and Gullberg 1993); gibbon, X99256 (Arnason, Gullberg, and Xu 1996); human, X93334 (Arnason, Xu, and Gullberg 1996).

The sequence of the mtDNA of the alligator has been deposited at the EMBL database with accession number Y13113. Users of the sequence are kindly requested to refer to the present paper and not only to the accession number.

Results

The mitochondrial genome of the alligator is 16,642 nt long and codes for 22 tRNAs, 13 protein-coding genes, and 2 rRNAs, as is characteristic for mitochondrial genomes of other metazoans. The organization of the genome is shown in figure 1. The arrangement of some tRNA genes differs from those of other vertebrates, but the order of the protein-coding genes is the same as in mammals, amphibians, and fishes. Thus, the alligator does not share the NADH6/Cyt *b* rearrange-

ment occurring in the avian lineage. The alligator is unique among hitherto described vertebrates in having the control region immediately adjacent to the 12S RNA gene. As a result, the tRNA for phenylalanine forms a cluster with the tRNAs for threonine and proline (fig. 1). Furthermore, the tRNAs for serine (AGY) and histidine have, compared to their arrangement in other vertebrates, changed their positions to now form the cluster tRNA^{Ser}(AGY), tRNA^{His}, tRNA^{Leu}(CUN).

The lengths of noncoding regions between tRNA genes and protein-coding genes of the alligator differ considerably from the corresponding lengths in mammals, amphibians, and fishes. It is probable that these deviations are related to the rearrangement process of some tRNA clusters in the alligator. Some noncoding regions are longer than those in other mitochondrial genomes which have maintained the ancestral gene order described in fishes, *Xenopus*, and eutherians. The noncoding region between Cyt *b* and tRNA^{Thr} is 31 nt long. This is probably a result of the relocation of the tRNA for phenylalanine. The origin of L-strand replication, which in vertebrates is usually located between tRNA^{Ala} and tRNA^{Asp}, is missing in the mtDNA of the alligator. Thus, the absence of ori-L appears to be a common characteristic of both birds and crocodylians.

Cao et al. (1994) have shown that large data sets, such as concatenated sequences of single genes, give more robust results than analyses performed on individual genes. Therefore, the present phylogenetic analyses were based on the concatenated sequences of 11 protein-coding genes, excluding NADH3 and NADH6 from the analysis. The length of NADH3 in the ostrich (Härlid, Janke, and Arnason 1997) deviates radically from the length of the same gene in other vertebrates due to an AGA termination codon after 207 nt. NADH6 was excluded, because it is encoded by a different strand (the L-strand) than are other mtDNA protein-coding genes and thus differs distinctly in nt and aa composition from the other protein-coding genes. After removing gaps and ambiguous sites around gaps, 9,168 nt sites remained for phylogenetic analysis. Table 1 shows the nt composition in the 11 concatenated protein-coding genes with respect to codon position as well as in the control region of the mtDNA of vertebrates that are included in the analysis.

As is evident from table 1, the mitochondrial protein-coding genes of different vertebrates included in the analysis differ considerably in nt composition. However, according to a pairwise 5% level χ^2 test of base composition at second codon position as calculated by the PUZZLE program, only the hedgehog differs from homogenous composition ($P = 0.017$). The fishes and the hedgehog deviate with respect to aa composition, but it is unlikely that the inclusion of these taxa as outgroup and/or as one among various other mammalian taxa influences the tree reconstruction.

In order to avoid the potential risks and inconsistencies of a single method or data set, the phylogenetic reconstructions were based on different approaches, maximum likelihood (ML) (Felsenstein 1981), neighbor-joining (NJ) (Saitou and Nei 1987), and maximum par-

Table 1
Nucleotide Composition of the Control Region and 11 Protein-Coding Genes (NADH6 and NADH3 excluded)

	CONTROL REGION				FIRST CODON POSITION				SECOND CODON POSITION				THIRD CODON POSITION			
	A	G	C	T	A	G	C	T	A	G	C	T	A	G	C	T
Alligator	30.6	11.3	21.4	36.6	29.7	21.5	27.0	21.8	17.8	12.3	28.8	41.1	40.6	5.0	36.5	17.9
Birds ^a	27.7	13.9	26.5	32.0	29.0	22.0	28.3	20.7	18.0	13.1	29.0	40.0	38.6	5.0	41.8	14.7
Mammals ^a	33.0	13.7	24.0	29.3	30.9	21.3	25.3	22.6	18.8	12.7	26.5	42.0	42.4	4.2	32.5	20.9
<i>Xenopus</i>	39.3	9.4	17.9	33.4	28.6	23.1	22.7	25.6	18.3	13.6	27.7	40.4	43.9	3.6	22.9	29.6
Fishes ^a	33.5	14.6	19.6	32.4	26.6	26.0	27.2	20.4	18.3	13.9	27.2	40.6	40.6	6.6	34.6	18.1

^a Mean values for species in figure 2.

simony (MP) (Fitch 1971), applied to aa sequences as well as nt analysis of second codon position. The mtREV-24 (Adachi and Hasegawa 1996b) was applied to aa sequences, and the Tamura-Nei (TN) model of sequence evolution (Tamura and Nei 1993) was applied to nt sequences. Figure 2 shows an ML tree based on aa sequence data as constructed by quartet puzzling (QP) of the PUZZLE program. The lengths of various branches of the tree, designated a-r, are given in table 2. The bootstrap support for branch b, the bird/alligator lineage, was 94%, 95%, and 100%, respectively, in MP, NJ, and

QP analyses of the aa sequences. The corresponding values for second codon position were 62%, 57%, and 83%, respectively.

The avian/crocodylian relationship was further investigated by a likelihood ratio test of alternative tree

Table 2
Maximum-Likelihood (ML) Branch Lengths

	Length	SE
External		
Loach	0.057	0.005
Carp	0.044	0.004
<i>Xenopus</i>	0.132	0.008
Alligator	0.304	0.013
Ostrich	0.069	0.006
Chicken	0.059	0.005
Wallaroo	0.071	0.006
Opossum	0.091	0.006
Platypus	0.141	0.008
Hedgehog	0.186	0.009
Mouse	0.046	0.004
Rat	0.042	0.004
Gibbon	0.056	0.005
<i>Homo</i>	0.045	0.004
Armadillo	0.096	0.006
Cow	0.047	0.004
Blue whale	0.013	0.002
Fin whale	0.012	0.002
Harbor seal	0.067	0.005
Indian rhinoceros	0.038	0.004
Horse	0.038	0.004
Internal		
a	0.091	0.007
b	0.050	0.006
c	0.113	0.008
d	0.056	0.006
e	0.020	0.004
f	0.058	0.005
g	0.117	0.008
h	0.102	0.007
i	0.039	0.005
j	0.031	0.004
k	0.132	0.007
l	0.024	0.004
m	0.020	0.004
n	0.020	0.003
o	0.072	0.005
p	0.031	0.004
q	0.014	0.003
r	0.018	0.003

NOTE.—Calculations of ML branch lengths and standard errors (SE) were based on the tree in figure 2. The tree was established by analysis (mtREV-24 model) of the concatenated aa sequences of 11 protein-coding mitochondrial genes.

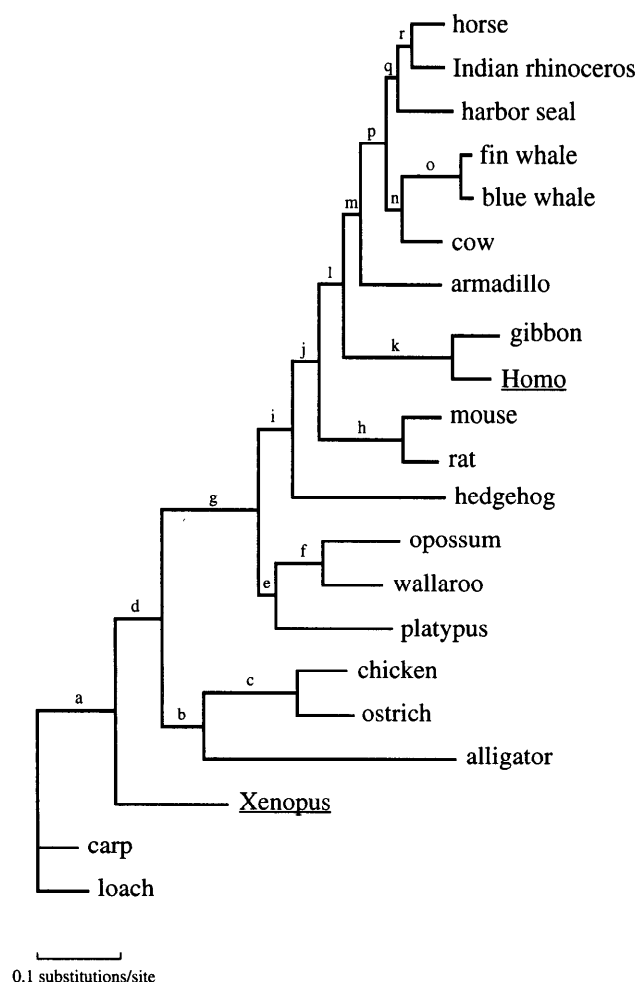


FIG. 2.—Maximum-likelihood tree based on aa sequences of 11 protein-coding genes as reconstructed by the PUZZLE program. Maximum-likelihood branch lengths for the external and internal branches (a-r) are given in table 2.

Table 3
Phylogenetic Analysis for Different Positions of Birds Among Tetrapods

TREE	AMINO ACID			NUCLEOTIDE		
	$\Delta\ln L$	SE	pBoot	$\Delta\ln L$	SE	pBoot
(OG, (mammals, (birds, crocodiles)))	(49,205.8)		95.2	(18,143.9)		93.1
(OG, (crocodiles, (birds, mammals)))	-67.0	± 20.7	<0.0	-29.4	± 16.8	3.3
(OG, (birds, (mammals, crocodiles)))	-38.7	± 23.3	4.8	-27.3	± 16.7	3.6

NOTE.—The log-likelihood value of the best topology is shown in angle brackets. The differences of log-likelihood values of alternative trees from that of the best tree ($\Delta\ln L$) as well as the standard error (SE) are shown (Kishino and Hasegawa 1989). pBoot indicates the estimated bootstrap probability (Kishino, Miyata, and Hasegawa 1990). OG: outgroup species as in figure 2.

topologies (table 3). Applying the test to nt or aa sequences, the two relationships alternative to that of joining birds and crocodilians were rejected near or above the 95% confidence level. Likelihood mapping (Strimmer and von Haeseler 1997), a maximum-likelihood analysis of all possible quartets for an internal branch based on aa sequences, strongly supported the sister group relationship of birds and crocodilians depicted in figure 2. This relationship was recognized by 83 (92.2%) of the 90 possible quartets, whereas 6 quartets (6.7%) supported an alligator/mammal relationship. No quartet recognized a bird/mammal relationship, and one quartet remained ambiguous.

The ML branch lengths of table 2 were used to estimate divergence times between and among different groups, notably between birds and crocodilians. The estimates were performed by applying two references: the paleontologically defined divergence between mammals (Synapsida) and birds plus crocodiles (Diapsida) at 310 MYA (Benton 1990) and a mammalian calibration point (A/C-60), the separation of artiodactyla (cow) and cetaceans at 60 MYA (Arnason and Gullberg 1996). Taking the evolutionary rate of the common alligator/bird lineage (branch b) as the same as in the alligator, the divergence time between alligator and birds can be expressed as: alligator branch \times 310/alligator branch + branch b, where 310 represents the time, 310 Myr, after the separation between Synapsida and Diapsida. This calculation yields a divergence time of ≈ 266 MYA for the avian/crocodilian split. However, the difference in branch lengths between alligator and birds indicates that their evolutionary rates are quite different, the avian rate being markedly slower than that of the alligator. Therefore, assuming the same rate in branch b as in the birds, the avian/crocodilian divergence is dated at ≈ 242 MYA.

A/C-60 has been shown to provide realistic datings for various mammalian divergences (Arnason et al. 1996; Xu, Janke, and Arnason 1996; Janke, Xu, and Arnason 1997; Arnason, Gullberg, and Janke 1997), but due to differences in evolutionary rates, the superimposition of A/C-60 on nonmammalian vertebrate classes needs careful calibration (Härlid, Janke, and Arnason 1997). Therefore, the reference should be applied with caution to such a deep divergence as that between birds and crocodilians, because similar evolutionary rates and processes over a long period of time have to be postulated. The alligator evolves, however, at a rate similar to that of an average mammalian lineage and application of A/C-60 places the avian/crocodilian split at ≈ 276

MYA, close to the dating (266 MYA) based on ML branch lengths and assuming the same evolutionary rate in branch 'b' as in that leading to the alligator. Applying as a reference the avian/crocodilian divergence at a mean value of 254 MYA, the divergence between the palaeognath ostrich and the neognath chicken is placed at ≈ 92 MYA.

The topology of the mammalian part of the phylogenetic tree in figure 2 is consistent with previous studies based on complete mtDNAs (Janke et al. 1996; Xu, Janke, and Arnason 1996; Arnason, Gullberg, and Janke 1997; Janke, Xu, and Arnason 1997), but in the present data set with the addition of the alligator, the monotreme/marsupial (Marsupionta) relationship has become less stable. Quartet puzzling and NJ reconstruct the relationship shown in figure 2, but with less support than in a previous study without the alligator (Janke, Xu, and Arnason 1997). In the present data set, the bootstrap/QP support values for second codon position and aa data varied between 42% and 94%. Maximum likelihood identified monotremes and eutherians as sister groups, while MP supported the traditionally acknowledged phylogeny with marsupials and eutherians as sister groups; neither relationship was significantly supported, however. Maximum likelihood analysis (likelihood mapping) of all 144 possible quartets involving branch e recognized the Marsupionta relationship in 109 (75.7%) quartets and a monotreme/eutherian sister group relationship in 27 (18.9%) quartets. The traditional tree, a eutherian/marsupial sister group relationship, was favored in only 4 (2.8%) instances. The remaining four quartets remained ambiguous; three showed least support for the traditional tree and one had least support for the monotreme/eutherian relationship. No quartet remained completely unresolved.

Discussion

Morphological evidence as well as analyses of molecular sequences have hitherto yielded somewhat equivocal answers regarding the relationship between birds and other tetrapods. The "Haemothermia" hypothesis (Gardiner 1982; Løvtrup 1985) challenged the traditional view by advocating a sister group relationship between birds and mammals. Molecular data have given somewhat inconclusive results, although a combined analysis of different nuclear encoded protein-coding genes has favored the avian/crocodilian relationship (Larhammar and Milner 1989). Studies of mitochondrial

12S and 16S rRNA genes (Hedges 1994) and 11 mitochondrial tRNA sequences (Kumazawa and Nishida 1995) have also supported an avian/crocodylian clade. The present analysis of concatenated protein-coding sequences of complete mitochondrial genomes showed significant support for an avian/crocodylian clade, and the alternative haemothermia hypothesis could be statistically rejected.

Depending on the approach, estimates of the time of divergence between birds and crocodylians yielded three datings, 242, 266, and 276 MYA. The 242- and 266-MYA datings were based on different assumptions with respect to the rate of evolution of the common avian/crocodylian branch (branch b in fig. 2 and table 2).

According to Benton (1990), *Stagonosuchus* and other related fossils from the Anisian (240 MYA) are the oldest fossils representing the Crocodylotarsi, whereas *Lagosuchus* from the Carnian (227 MYA) is the oldest fossil representing the Ornithosuchia. This leads to the conclusion, that, based on the fossil record, a minimum divergence time for the two lineages is at least 245 MYA (Benton 1990). Even though the 242-MYA dating of the avian/crocodylian divergence is not directly rejectable by the fossil record, it is very close to the age, 240 Myr, of diversified Crocodylotarsi fossils. The 242-MYA dating may therefore constitute an underestimate. When the evolutionary rate of the alligator was allocated to branch b (the common avian/crocodylian lineage), the divergence of crocodiles and birds was dated at 266 MYA. The application of the newly established molecular reference A/C-60, the artiodactylan/cetacean divergence set at 60 MYA (Arnason and Gullberg 1996), produced a dating of 276 MYA for the same divergence. Taking into account that unexplorable deviations from constant evolutionary rates may affect the outcome, the difference between the 266-MYA and 276-MYA datings is not unexpected. The similarity between the two outcomes is underlined by the fact that a postulate of an artiodactyl/cetacean split at 58 MYA (rather than 60 MYA) would yield identical outcomes in the two calculations.

The age of the Archosauromorpha fossil (*Protorosaurus*) from the Kazanian is 255 Myr (Benton 1990). Considering the fact that molecular divergences must, for natural reasons, precede the possible morphological identification of these divergences (Martin 1993; Arnason et al. 1996), the *Protorosaurus* fossils do not exclude a somewhat earlier dating than 255 MYA for the avian/crocodylian divergence. However, rather than to favor either the 242- or the 266-MYA dating, we tentatively place the dating of the avian/crocodylian divergence at 254 MYA, i.e., the mean of the two datings. This dating is not in direct conflict with the age of *Protorosaurus* and, furthermore, allows reasonably well for the crocodylotarsian diversification recorded at 240 MYA.

Relative to an avian/crocodylian divergence at 254 MYA, the divergence between paleognathous (ostrich) and neognathous (chicken) birds is estimated at \approx 92 MYA, slightly earlier than the previously proposed dating, 80–90 MYA (Härlid, Janke, and Arnason 1997).

The dating proposed by Härlid, Janke, and Arnason (1997) was based on a divergence of synapsida and diapsida at 300 MYA and an avian rate of evolution on the whole branch. The present dating (92 MYA) of the paleognathous/neognathous split is somewhat lower than expected for a basal position of paleognathous birds, as the deepest divergences among birds have been estimated at \approx 130 MYA (Cooper and Penny 1997). The more recent dating of the origin of paleognathous birds yields additional support to the finding of a nonbasal position of paleognathous birds (Härlid, Janke, and Arnason 1997), as there seem to be older divergences among birds than those involving the paleognathes.

As is evident from the branch lengths, the evolutionary rate of the mitochondrial DNA of cold-blooded alligator is similar to that of mammals and considerably faster than that of birds. Thus, the evolutionary rate of mitochondrial DNA is independent of metabolic rate and/or body temperature, in spite of contrary proposals. Thomas and Beckenbach (1989) have suggested that a constant body temperature would result in less constraints on protein function, thus allowing for higher evolutionary rates. In a similar context, Martin and Palumbi (1993) have argued that oxygen radicals produced by high metabolic rates would result in greater stress on DNA, leading to a higher evolutionary rate in warm-blooded animals. Neither of these explanations appears consistent with the fact that the cold-blooded alligator exhibits a rate higher than those of other cold-blooded vertebrates, and even higher than those of some mammals. A similar observation has been made in a study of some mitochondrial tRNAs (Kumazawa and Nishida 1995). Thus, it is questionable whether there are general explanations for the differences in evolutionary rates observed among vertebrates.

A recent study applying *Xenopus* as outgroup provided strong support for the Marsupionta hypothesis, i.e., the existence of a common monotreme/marsupial clade as a sister group to eutherians (Janke, Xu, and Arnason 1997). In the present study, with the inclusion of the alligator and two avian sequences, the stability of the monotreme/marsupial relationship was reduced. This is not entirely unexpected, since the previous study showed that the inclusion of the chicken as the only outgroup reduced support for the monotreme/marsupial relationship. Additional data of monotremes and marsupials are therefore needed to minimize or exclude the effects of long-branch attraction and/or destabilization in tree reconstruction due to biases with respect to nt or aa composition undetected by the χ^2 test for compositional biases.

On the basis of 109 morphological characters but excluding fossil evidence, Gauthier, Kluge, and Rowe (1988) found support for a (mammalian/crocodylian, avian) clade with a basal position of lizards, snakes, and turtles. This topology disrupts the monophyly of the Diapsida (lizards, snakes, crocodiles, birds), which was strongly supported when paleontological data were included in the comparison. The present analysis is inconsistent with the haemothermia hypothesis, but additional sequence data representing turtle and/or lizard and snake

will be needed for exploring the monophyly of the Diapsida on the molecular level.

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