Tetrapod Phylogeny Inferred from 18S and 28S Ribosomal RNA Sequences and a Review of the Evidence for Amniote Relationships

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The 18S ribosomal RNAs of 21 tetrapods were sequenced and aligned with five published tetrapod sequences. When the coelacanth was used as an outgroup, Lissamphibia (living amphibians) and Amniota (amniotes) were found to be statistically significant monophyletic groups. Although little resolution was obtained among the lissamphibian taxa, the amniote sequences support a sister-group relationship between birds and mammals. Portions of the 28S ribosomal RNA (rRNA) molecule in 11 tetrapods also were sequenced, although the phylogenetic results were inconclusive. In contrast to previous studies, deletion or down-weighting of base-paired sites were found to have little effect on phylogenetic relationships. Molecular evidence for amniote relationships is reviewed, showing that three genes (beta-hemoglobin, myoglobin, and 18S rRNA) unambiguously support a bird-mammal relationship, compared with one gene (histone H2B) that favors a bird-crocodilian clade. Separate analyses of four other genes (alpha-crystallin A, alpha-hemoglobin, insulin, and 28S rRNA) and a combined analysis of all sequence data are inconclusive, in that different groups are defined in different analyses and none are strongly supported. It is suggested that until sequences become available from a broader array of taxa, the molecular evidence is best evaluated at the level of individual genes, with emphasis placed on those studies with the greatest number of taxa and sites. When this is done, a bird-mammal relationship is most strongly supported. When regarded in combination with the morphological evidence for this association, it must be considered at least as plausible as a bird-crocodilian relationship.

Introduction

The classical view of tetrapod relationships (fig. 1) is based largely on fossil evidence (Romer 1966; Carroll 1988; Gauthier et al. 1988). However, morphological and molecular data from living species recently have challenged some long-held beliefs. The most controversial suggestion is that mammals, not crocodilians, may be the closest relatives of birds (Gardiner 1982; Lovtrup 1985). Moreover, the single origin of the Lissamphibia (frogs, salamanders, and caecilians) never has been firmly established (Parsons and Williams 1963; Jarvik 1968, 1980; Lovtrup 1985; Milner 1988; Panchen and Smithson 1988). To address these questions concerning tetrapod phylogeny, we have examined the phylogenetic relationships of 26 tetrapods representing.

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Fig. 1.—Currently recognized relationships of the major tetrapod groups, based largely on the fossil record (Romer 1966; Carroll 1988). Solid lines indicate continuous fossil record; dashed lines denote either the absence of fossils (of the major groups) or inferred transitions.

nearly all major lineages, by using nucleotide sequences of the slow-evolving 18S ribosomal RNA (rRNA) molecules. Additional sequence data also were obtained from portions of the 28S rRNA subunit in 11 tetrapods.

Methods

We obtained sequences from 15 species of amphibians representing 14 families (seven frogs, four salamanders, and four caecilians), a turtle, a crocodilian, two squamates, and two birds by direct sequencing of nuclear 18S rRNA. The anuran taxa are Bufonidac (*Bufo valliceps*), Discoglossidac (*Discoglossus pictus*), Hyliidac (*Hyla cinerea*), Leptodactylidac (*Eleutherodactylus cuneatus*), Microhylidac (*Gastrophynge carolinensis*), Pelobatidac (*Scaphiopus holbrooki*), and Sooglossidac (*Nesomantis thomasseti*). The caecilians are Caeciliaidac-1 (*Grandisonia alternans*), Caeciliaidac-2 (*Hypogeophis rostratus*), Ichthyophiidac (*Ichthyophis bannanicus*), and Typhlonectidac (*Typhlonectes natans*). The salamanders are Ambystomatidac (*Ambystoma mexicanum*), Amphiumidac (*Amphiuma tridactylum*), Plethodontidac (*Plethodon yonahlossee*), and Sirenidac (*Siren intermedia*). The amniotes are a turtle (*Pseudemys scripta*), an alligator (*Alligator mississippiensis*), a lizard (*Sceloporus undulatus*), a snake (*Heterodon platyrhinos*), and two birds [Galliformes (*Gallus*) and Passeriformes (*Turdus migratorius*)]. These were compared with published sequences of a pipid frog (*Xenopus laevis*; Salim and Mayden 1981) and of four mammals: *Oryctolagus cuniculus* (Rairkar et al. 1988), *Rattus norvegicus* (Torczynski et al. 1983; Chan et al. 1984), *Mus musculus* (Raynal et al. 1984), and *Homo sapiens* (Torczynski et al. 1985). This broad sampling of taxonomic diversity within each of the three orders of amphibians permits the distinction between those sites that are unique to a single lineage and those more informative sites that are representative of an entire group. The sequence from the only living actinistian fish, the coelacanth (Stock et al., accepted), was used to provide an outgroup. Some of the same taxa (and samples) used to obtain 18S rRNA sequences also were sequenced at the 28S rRNA gene. They include *Bufo*, *Discoglossus*, *Hyla*, *Typhlonectes*, *Ambystoma*, *Pseudemys*, *Sceloporus*, *Heterodon*, *Alligator*, *Turdus*, and *Gallus*. Published 28S rRNA sequences of *Xenopus* (Ware et al. 1983), *Mus* (Hassouna et al. 1984), and *Homo* (Gonzalez et al. 1985) were used for comparison. Sequenced were three regions of the 28S rRNA corresponding to the following sites in *Mus* (Hassouna et al. 1984): 1–355, 2157–2611, and 4277–4611.

rRNA was isolated from ~1g of liver (or, if available, 1g of ova). The tissue was homogenized in a buffer [10 mM Tris hydroxymethyl amino methane–HCl pH 8.0,
1 mM ethylene diamine tetra-acetate pH 8.0, 2% (w/v) sodium dodecyl sulfate, 5% (w/v) sodium tri-isopropyl naphthalene-sulfonate] by using a Brinkman Polytron. The homogenate was extracted twice with phenol, once with phenol/chloroform, and once with chloroform, followed by ethanol precipitation and resuspension of the pellet in diethylpyrocarbonate (DEPC)-treated water.

Direct sequencing of the extracted rRNA (by using reverse transcriptase) followed the procedure of Lane et al. (1985), although the chase step was not found to be useful and thus was omitted. A combination of 17 oligonucleotide primers allowed us to generate (a) nearly uninterrupted sequences from the entire 18S rRNA subunit (~1,900 bp) in each of the species and (b) ~400 bp from portions of the 28S molecule. The 18S primers used and their starting positions in Mus (Raynal et al. 1984) are CTAGAATT(AG)CCACAGTTATCC (145), TACCATCGAAGTTGATAGGC-AGA (354), ACCGGCGGC1GCTG1G (614), GTCATTCCATTATTCC (860), CGG(AG)TCCAGAATTTCACCTCT (956), GCCCTTCCGTCAATTCTTTAAGTTTCACGC (1185), GTCAATTAAGCCGC (1233), AAGAAGCCTGACTGCACCCACC (1324), TCAAGGCGCATCACAGCCTGTTATTG (1482), ACGGGCGGTG1GC (1693), and GTTACCTGTGGCCAC (1824). The 28S primers and their starting position in Mus (Hassouna et al. 1984) are GTTGGTTTCTTTCCT (56), TTTGGGCTGCATTCCA (291), CCTTATCCCGAGTTTTCA (2619), and CAGGTCGCTCAGGAT (4613).

Alignments initially were done with Intelligenetics software, although refinement of the entire alignment was made by eye, in order to increase sequence similarity. Regions that could not be aligned, because of length or sequence variability, were omitted from the analyses. Ambiguities (multiple bands at the same site on a gel) were scored as “N” and were treated in the parsimony analyses as missing data and were not used in the calculation of pairwise distances for the neighbor-joining (NJ) analyses. The informative sites (those with at least two bases, each occurring in more than one taxon) were analyzed with the maximum-parsimony (MP) method in PAUP (Phylogenetic Analysis Using Parsimony, version 3.0). Values for consistency index (CI) presented do not include sites with unique variants. The NJ method (Saitou and Nei 1987), version 2.0, was used to generate trees from distance matrices (corrected for multiple substitutions). The statistical significance of groups in both the MP and NJ analyses was evaluated by the bootstrap method (Felsenstein 1985), with 1,000 iterations.

Amino acid sequences of seven genes relevant to amniote relationships were obtained from the National Biomedical Research Foundation Protein Identification Resource (PIR) data bank (version 21), from the Swiss Protein data bank (version 11), and from Lance et al. (1984). The data-base locus names for the sequences used in the combined analysis (see below) are as follows (names with “$” are from Swiss Protein; all others are from PIR): alpha-crystallin A (CYHUAA, CYCHAA, CYAQAA, CYLZAA, CYFGAA, and CYFGA2), alpha-hemoglobin (HAHU, HACH2, HAAQ, HASNV, S00525, and HAXL1), beta-hemoglobin (HBHU, HBCH, HBAQ, S00538, and HBXL), cytochrome c (CCHU, CCCH, CCRS, CCST, and CCFG), histone H2B (HSB022, HSCCH22, HSAK22, and HSXLB1), insulin (INS$HUMAN, INS$CHICK, INRS, and INCD), and myoglobin (MYHU, MYCH, MYAQ, MYLZM, MYTTM, and MYCA).
Results
Phylogeny Estimation

The aligned 18S rRNA sequences are shown in appendix A (fig. A1). Of the 1,746 alignable sites, 217 are variable and 123 of those are phylogenetically informative for the MP analysis. Twelve most-parsimonious trees were found, each with a length of 230 and a CI of 0.63. They differed only in the relationships of some lissamphibian groups. In a bootstrap analysis (fig. 2), Lissamphibia, Amniota, Aves, and Mammalia each are statistically significant monophyletic groups (>95% of bootstrap trees), although the taxonomic diversity of the two latter groups is not well represented. The most interesting finding is that birds + mammals, not birds + crocodilian, form a group (88%). Lissamphibia is supported by nine fully consistent (no homoplasy) sites, Amniota by 11 sites, Aves by nine sites, and Mammalia by 15 sites. The group containing birds and mammals is supported by four fully consistent sites. None of the 123 informative sites unambiguously supports a bird-crocodilian grouping. When only divariant sites (Nanney et al. 1989) are used, the same groups are defined in a majority-rule bootstrap MP analysis (not shown).

Pairwise estimates of sequence divergence were obtained using the formula of Tajima and Nei (1984), although those values are very close to estimates based simply on the number of differences divided by the total number of sites. These estimates ranged from 0.1% (Grandisonia vs. Hypogeophis) to 7.0% (Oryctolagus vs. Ichthyophis) and were used to construct an NJ tree (fig. 2). The groups defined in that tree are very similar to those defined in the majority-rule (>50%) bootstrap MP analysis, although the alligator and turtle now cluster.

The 28S rRNA data set (appendix B, fig. B1) is smaller, totaling 374 aligned sites. Because of difficulties in obtaining complete sequence from many of the taxa,
only regions yielding relatively continuous sequence across all taxa (i.e., regions corresponding to sites 220–339, 2325–2532, and 4579–4602 in *Mus*) are shown in appendix B and were used in the analyses. Two most-parsimonious trees were found, each with a length of 48 and a CI of 0.63. Bootstrap analyses of these data show support for a monophyletic Anura, Aves, Squamata, and Mammalia (fig. 3). However, the turtle and salamander now cluster, and only one group (Mammalia) is strongly supported. Moreover, no site unambiguously supports a bird-mammal or bird-crocodilian grouping. Such inconclusive results probably reflect the small size (35 variable sites) of the 28S data set.

There are two other phylogenetic studies of 28S rRNA that involve some tetrapods. Larson and Wilson (1989) sequenced 13 salamander species and one caecilian species at four regions spanning ~900 bp of the 28S molecule. Hillis and Dixon (1989) sequenced about one-half (2,100 bp) of the 28S gene from four species of vertebrates, including two amniotes (bird and squamate). There is little overlap between those two studies and our 28S data set, in terms of sites sequenced and taxa examined. Nonetheless, it is possible to examine the relationships of the amniotes and the three orders of lissamphibians further by using selected taxa from those two studies and our rRNA data. When such an examination is made, it must be assumed that the specific taxa used in each study are representative of the more inclusive taxon (e.g., birds, mammals, and crocodilians). If rates of substitution vary greatly among lineages within each group, then such an assumption may not be justified.

For the lissamphibian analysis, we used the published sequences of *Mus* (Hassouna et al. 1984; Raynal et al. 1984) as the outgroup. *Xenopus* (Salim and Maden 1981; Ware et al. 1983) was used as the representative frog, *Typhlonectes* as the caecilian, and *Ambystoma* as the salamander. MP and NJ analyses (fig. 4) both support a salamander-caecilian clade (length of most-parsimonious tree = 211; CI = 0.69), as was found by Larson and Wilson (1989). The tree with caecilian and frog as sister groups is four steps longer, whereas the third alternative (frog + salamander) is not well supported, requiring 10 additional steps.

An amniote analysis of all 18S and 28S rRNA data, including those of Hillis and Dixon (1989) for a squamate and bird (except in regions of overlap with our data), was performed. Of the groups with sequences from multiple species (mammals, birds, and squamates), a single representative was chosen, depending on the availability and completeness of the sequence data: *Gallus, Homo,* and *Sceloporus* for 18S rRNA and

![Diagram](image-url)

**Fig. 3.**—Tetrapod relationships inferred by maximum-parsimony and neighbor-joining analyses of 28S rRNA sequence data (appendix B). The root was placed between the majority of lissamphibians (caecilian + frogs) and the amniotes.
FIG. 4. Lissamphibian relationships inferred by maximum-parsimony and neighbor-joining analyses of 18S and 28S rRNA sequence data. Both trees are rooted with the mammal.

_Turdus_, _Homo_, and _Heterodon_ for our 28S data. No crocodilian or turtle sequences were available for the Hillis and Dixon data set. When _Xenopus_ was used as the outgroup, a bird-mammal relationship was supported in the MP analysis (length of most parsimonious tree = 222; CI = 0.64), whereas a bird-crocodilian clade was supported in the NJ analysis (fig. 5). In the NJ analysis, there was strong statistical support for a group containing bird, mammal, and crocodilian.

It has been suggested that nucleotide substitutions in the base-paired regions of rRNA evolve in tandem and that those sites should be either assigned one-half weight or deleted entirely from the analysis (Wheeler and Honeycutt 1988). We investigated both alternatives with our 18S rRNA data set, using the secondary structure of rat 18S rRNA (Chan et al. 1984) to determine paired sites. Of the informative sites, 50 are in the base-paired region, 52 are unpaired, and 21 are indeterminate (i.e., region is unknown). With one minor exception involving the cluster of frog families, either weighting paired sites one-half or deleting them from the MP analysis each resulted in the same groups defined in the majority-rule (50%) bootstrap MP analysis of the entire data set (fig. 2)—although with slightly lower bootstrap confidence limits at some nodes, probably because of the reduction in the number of characters.

Only two regions of the 18S molecule exhibit major length variation among the tetrapods. They represent two of the four divergent tracts noted by Raynal et al. (1984) in a comparison of _Xenopus_ and _Mus_. One, beginning at position 197, involves both the insertion of 6–11 bases in the four mammal species and the insertion of two bases in one amphibian (_Plethodon_). The second region (beginning at position 241) is the longest, varying from 14 to 108 bases, and is for the most part unalignable. The length variation mostly involves some long insertions in four taxa of frogs (_Nesomantis_, _Eleutherodactylus_, _Bufo_, and _Hyla_). This lends support to recent classifications placing the families that they represent (Sooglossidae, Leptodactylidae, Bufonidae, and Hylidae) in the superfamily Bufonoidea (Laurent 1979; Dubois 1983). A fifth taxon, _Gastrophryne_ (Microhylidae), has the next longest insertion. The four mammal species also have moderately long insertions in this variable region.

Other occurrences of length variation involve differences of only one to three bases and are mostly in the unalignable regions. Of the 1,746 alignable sites in the 18S data set, 23 exhibited insertions or deletions of only one base (in 12 of those sites, the change was restricted to one taxon), one exhibited insertions or deletions of two bases, and one exhibited insertions or deletions of three bases. Among the relatively few (13) insertion/deletion events that were phylogenetically informative, the mammals were supported by insertions at two sites (863 and 1513), and the birds were supported by insertions at one site (1640). Other patterns appeared to be homoplasious with respect to the relationships obtained with base substitutions (fig. 2), and none supported a crocodilian-bird grouping. Many of the short insertions in both data sets involve the frog _Xenopus_ and the four mammalian species. These are the only taxa that were cloned and sequenced rather than sequenced directly with reverse transcriptase, suggesting a possible sequencing artifact. Comparison of (a) rRNA sequence
FIG. 5.—Amniote relationships inferred by maximum-parsimony and neighbor-joining analyses of 18S and 28S rRNA sequence data. Both trees are rooted with the outgroup (frog or fish).

obtained directly in the present study with (b) DNA sequence from the same taxa obtained via the polymerase chain reaction (in progress) will help to verify those insertion/deletion events. Because of the possibility that some may represent artifacts, insertion/deletion data were not used in the phylogenetic analyses.

Molecular Evolution

Transitions are the dominant type of base substitutions, accounting for 189/328 (58%) of the inferred changes in the most parsimonious arrangements of 18S rRNA. They make up a smaller proportion among the sites with unique variants (38%; \( N = 98 \)), compared with the proportion among informative sites (66%; \( N = 230 \)). When only the informative sites are considered, the proportion of transitions is nearly identical in the Lissamphibia (68/105 = 65%) and the Amniota (75/113 = 66%). Likewise, there is little difference in the proportion of transitions among sites in base-paired regions (78/133 = 59%) versus unpaired regions (74/140 = 53%).

Discussion

rRNA Evolution

The finding that transitions are much less frequent at unique-variant sites (38%) than at informative sites (66%) suggests that selection, probably over a long period of time (millions of years), is involved in producing the transition bias observed in rRNA. The unique-variant sites, in general, represent base substitutions that have occurred more recently than those informative differences which are shared by more than one taxon. However, the mechanism suggested by Brown (1985; pp. 109-110) for mitochondrial rRNA—i.e., that uncompensated transitions occurring in regions of base pairing are more stable than uncompensated transversions—is not supported by the finding of similar frequencies of transitions at paired versus unpaired sites. His model would predict that transitions are considerably more frequent at base-paired sites. Thus, although selection against transversions appears to be operating in rRNA, it is still unclear what mechanism is responsible for the selection.

Ochman and Wilson (1987) estimated that the 16S/18S rRNA base substitution rate is \( \sim0.8\%\), \( \sim1.0\%\), and \( \sim2.0\%/50\) Myr (\( = 8, 10, \text{and } 20 \times 10^{-11}/\text{site/year/lineage} \)) in vertebrates, bacteria, and flowering plants, respectively. The 21 additional vertebrate sequences reported herein now allow a refinement for the vertebrate substitution rate. Perhaps the best estimate of this can be obtained by using the lissamphibian/amniote divergence, believed to have occurred \( \sim350\) Mya—but no later than 338 Mya, when the two groups appear together in the fossil record (Carroll 1988; Smithson 1989). The mean sequence divergence between these two groups is 4.4%, resulting in a substitution rate of \( 6.3 \times 10^{-11}/\text{site/year/lineage} \), only slightly lower than Ochman and Wilson’s estimate of \( 8 \times 10^{-11}/\text{site/year/lineage} \) (an estimate that is based on a small number of vertebrate sequences). Two other estimates, involving
fewer taxa, are $3.4 \times 10^{-11}$ for the divergence of lizards and snakes (150–200 Mya; Carroll 1988) and $4.3 \times 10^{-11}$/site/year/lineage for the Mus-Rattus divergence (15–25 Mya; Sarich 1985; Jaeger et al. 1986). Further estimates based on the amniote fossil record are questionable, because they presuppose the classical relationships challenged here.

**Base Pairing and Phylogeny**

Our results with down-weighting or deleting base-paired sites do not agree with the conclusions of Wheeler and Honeycutt (1988)—i.e., that base-paired sites have a significant and detrimental effect on estimates of phylogenetic relationships. Their conclusion was based on an analysis of some 5.8S rRNA sequence data from insects, where they obtained different phylogenies after partitioning the data into paired and unpaired sites.

Smith (1989) recently came to the opposite conclusion of Wheeler and Honeycutt, with a reanalysis of some 18S rRNA data from echinoderms. He also partitioned the data into paired and unpaired sites, and he found that, while both types of data gave similar results for taxa with close evolutionary relationships, paired sites gave “better” results in comparisons between more distantly related taxa. As in the Wheeler and Honeycutt study, a morphology-based tree was considered the correct phylogeny against which to judge the molecular phylogeny. Smith concluded that “analysis of 18S rRNA total sequences will only produce reliable results if the species diverged within the last 100 Ma or so.”

These two studies illustrate several points that need to be addressed. First, both studies considered the phylogeny based on morphology to be “correct.” We are not aware of any group that has a known phylogeny, regardless of the extent of its fossil record, and therefore such an initial bias is not justified. If the morphological phylogenies considered correct by Wheeler and Honeycutt (1988) and Smith (1989) are not, then their conclusions are severely affected. Second, the reason each study found that subsets of the complete data set generated different phylogenies may be attributed to the size of the data set, not to the type of characters (paired vs. unpaired). Just as phylogenetic inference improves with the addition of characters, fewer characters will result in a less stable phylogeny. It is possible that the subsets of sequence data used in those two studies were too small to answer the questions posed. Finally, the generalizations made in those two studies not only conflict with each other but do not agree with the results of the present study, where there was no significant effect of base pairing on phylogenetic inference. It is clear that more analyses (and possibly simulations), taking the above factors into consideration, are needed before generalizations regarding the effect of base pairing can be drawn.

**Amphibian Phylogeny**

Our results with 18S rRNA provide clear statistical support for the monophyly of the Lissamphibia, rejecting hypotheses of polyphyly and paraphyly (Jarvik 1968 1980; Lovtrup 1985). The grouping of salamanders and caecilians is strongly supported (81% and 94%; fig. 4), agreeing with the conclusions of Larson and Wilson (1989) but not with a recent morphological analysis (Milner 1988) joining salamanders and frogs. However, most of the morphological characters supporting a salamander-frog clade are absences (Milner 1988, p. 71), thus not providing strong evidence; the frogs-salamander clade was the least supported of the three alternatives considered in the present study, requiring 10 additional steps.
Among the amphibians, the two salamander genera *Ambystoma* and *Siren* formed the best-supported group (fig. 2). A relationship between the Ambystomatidae and Sirenidae has not been suggested previously, and the Sirenidae were not represented in the study using rRNA sequence data from portions of the 28S molecule (Larson and Wilson 1989). The group containing four families of bufonoid frogs and *Gastrophryne* in figure 2, also supported by insertion/deletion data not used in the phylogenetic analysis (see above), adds some support to this higher-level grouping of frogs. However, more sequence data from the 28S rRNA gene are needed to test the significance of these amphibian groupings which are associated with low confidence limits.

**Amniote Phylogeny: Molecular Evidence**

While the monophyly of the amniotes generally is accepted (Romer 1966; Carroll 1988), it now has strong statistical support from a large molecular data set. Within the amniotes, our sequence data support (at the 88% significance level) the highly controversial grouping of birds and mammals. In addition to 18S rRNA, amino acid sequences from two other genes (beta-hemoglobin and myoglobin) provide unambiguous evidence for this grouping (Dene et al. 1980; Goodman et al. 1987; Bishop and Friday 1988). Alpha-hemoglobin sequences supported a bird-crocodilian grouping in an MP analysis (Goodman et al. 1987), although an analysis using maximum likelihood supports a bird-mammal relationship (Bishop and Friday 1988). Sequences of alpha-crystallin A support either birds + crocodilians (de Jong et al. 1985) or birds + lizards (Bishop and Friday 1988), depending on the method of analysis. Cytochrome *c* sequences join birds with squamates (lizards and snakes), not with mammals (Goodman et al. 1982). However, contrary to the conclusions of de Jong et al. (1985, p. 491) and Benton and Clark (1988, p. 298), cytochrome *c* sequences cannot support a bird-crocodilian relationship—because no crocodilian sequences from this gene are yet available! The two earlier studies that were cited as sources, Maeda and Fitch (1981) and Goodman et al. (1982), do not present or discuss cytochrome *c* sequences from any crocodilian.

Recently, Larhammar and Milner (1989) cited amino acid sequences of two additional genes as evidence pertaining to the relationships of birds, mammals, and crocodilians. Sequences from pancreatic polypeptide and insulin were shown to have more unique sites in the mammal sequence than in the bird or crocodilian sequences. Larhammar and Milner suggested that this provided support for a bird-crocodilian relationship. However, this conclusion, which has been noted elsewhere (Lance et al. 1984), requires that a constant rate of substitution be invoked, a controversial topic not addressed in their paper. For pancreatic polypeptide, there are other mammal and bird sequences available, but additional tetrapods will need to be sequenced before phylogenetic interpretations can be made other than by invoking the constant-rate model.

Insulin was one of the first proteins to be sequenced, and there are many sequences available besides the three used by Larhammar and Milner. An alignment of all 35 different insulin sequences (A and B chains) available, representing 42 vertebrate taxa, is given in appendix C. An MP analysis of those aligned sequences resulted in >1,000 most-parsimonious trees of length 148 (CI = 0.72). In a bootstrap MP analysis, all 25 mammals form a group, which clusters with three birds (chicken, ostrich, and turkey) and not with the alligator (which clusters with rattlesnake and ratfish), although two other birds (goose and duck) are placed farther back in the tree. The majority-rule tree (fig. 6) of that analysis shows slightly less resolution because some of those
groups were weakly supported. The NJ analysis gives a different result: birds cluster more closely with the group containing ratfish, alligator, and rattlesnake (fig. 6). The fact that birds are not monophyletic in either analysis suggests that more data, especially from additional birds and crocodilians, are needed before conclusions can be drawn. On the other hand, small molecules such as insulin will always be limited in their ability to generate statistically significant results in phylogenetic analyses, because of the small number of supporting sites possible.

Sequences are available for two other proteins, histone H2B and immunoglobin heavy chain (v-region), from birds, mammals, crocodilians, and other tetrapods. Neither has been mentioned previously in discussions of crocodilian-bird-mammal relationships. Histone H2B is a highly conserved protein, with only four variable sites among one crocodilian (Crocodylus), one bird (Gallus), three mammals (Bos, Homo, and Mus), a frog (Xenopus), and a fish (Salmo). The sequences for the crocodilian and chicken are incomplete, but there are 57 sites in common among the seven vertebrate taxa. Mammals have the derived residue alanine at site 21 (in the complete human sequence), while the other vertebrates have threonine. The frog has valine at site 69, while the others have an isoleucine. The only two informative sites are 25 and 26. At site 25, glycine is present in the fish, bird, and crocodilian, whereas aspartic
acids are present in the mammals and frog. Site 26 shows unambiguous support for a
bird-crocodilian grouping (glycine in fish, frog, and mammals and aspartic acid in
bird and crocodilian).

More than 100 amino acid sequences are available for the immunoglobulin heavy-
chain (variable-region) genes. Most are from humans and mice, but there are also
sequences of a crocodilian (Caiman), a bird (Gallus), and several other tetrapods.
Unfortunately, two features make them poor candidates for the analysis of amniote
relationships: (1) there are hundreds of separate genes (Alt et al. 1987; Riblet et al.
1987; Tutter and Riblet 1989), the orthologies of which are poorly understood, and
(2) differences among sequences are great. The first feature is a significant problem,
because many of the gene duplications appear to have predated amniote evolution,
as suggested by the fact that the mouse alone has representatives of all nine gene
families (Riblet et al. 1987). Nonetheless, we aligned 31 sequences, including human
(10), mouse (10), rat (one), dog (two), rabbit (three), chicken (one), caiman (three),
and Xenopus (one). When the last sequence was used as outgroup, an MP analysis
(not shown) placed the chicken with one group of placental mammals and placed the
caiman with another. These results confirm that the immunoglobulin heavy-chain ν-
region genes will be of limited use for phylogenetic analysis until orthologies are better
understood.

An eye-lens protein originally called c-crystallin, present only in birds and croco-
dilians, has been cited as molecular evidence for a bird-crocodilian relationship (de
Jong et al. 1985; Stapel et al. 1985; Benton and Clark 1988). However, this lens
protein has been identified as lactate dehydrogenase, which is recruited in the lens,
possibly to serve the function of reducing glare in aquatic birds and crocodilians (Wis-
tow et al. 1987). It does not represent a separate gene or even a duplication of lactate
dehydrogenase, and therefore it provides no evidence for amniote relationships.

To summarize the molecular evidence for amniote relationships, one gene sup-
ports a bird-crocodilian relationship, three genes support a bird-mammal relationship,
and four genes support different relationships depending on the type of analysis (table
1). Only three analyses support a relationship between birds and some other amniote
group besides mammals or crocodilians, and a mammal-crocodilian grouping is not
supported by any of the genes. This indicates that the sequence data bearing on amniote
relationships do not support a random array of phylogenetic hypotheses as inferred
by Benton and Clark (1988, p. 298) and Benton (1990, p. 418) but do favor primarily
two alternative arrangements: birds + crocodilians or birds + mammals, with the
latter group more strongly favored. It also suggests that these three groups may form
a monophyletic group of amniotes, as indicated by morphological analyses of nonfossil
data (Gardiner 1982; Gauthier et al. 1988), although few of the analyses of individual
genes place birds, mammals, and crocodilians together (figs. 2 and 3; also see Goodman
et al. 1987; Bishop and Friday 1988). The analyses of individual genes of course
cannot be weighed equally, because they are based on different numbers of taxa and
sites (table 1). For example, the sequence data from the globin genes and 18S rRNA
are considerably more substantial than the histone H2B and insulin data sets.

One approach for analyzing molecular data from different genes is to combine
the sequences in one analysis. Goodman et al. (1987) did this with some of the amino
acid sequence data available for vertebrates and obtained a tree close to the classical
phylogeny (fig. 1). This can be a very useful method of analysis when the individual
gene sequences are too short to provide statistical significance and when the taxa
represented at each gene are similar. Unfortunately, the taxonomic representation of
Table 1
Molecular Evidence for Amniote Relationships

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of Taxa</th>
<th>No. of Sites</th>
<th>Amino Acid</th>
<th>Nucleic Acid</th>
<th>Relationship Supported</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha crystallin A</td>
<td>31</td>
<td>173</td>
<td>X</td>
<td>X</td>
<td>Birds + Birds + Other</td>
<td>de Jong et al. 1985; Bishop and Friday 1988</td>
</tr>
<tr>
<td>Alpha hemoglobin</td>
<td>44</td>
<td>142</td>
<td>X</td>
<td>X</td>
<td>Birds + Mammals</td>
<td>Goodman et al. 1987; Bishop and Friday 1988</td>
</tr>
<tr>
<td>Beta hemoglobin</td>
<td>44</td>
<td>146</td>
<td>X</td>
<td>X</td>
<td>Birds + Other</td>
<td>Goodman et al. 1987; Bishop and Friday 1988</td>
</tr>
<tr>
<td>Histone H2B</td>
<td>7</td>
<td>57</td>
<td>X</td>
<td>X</td>
<td>Birds + Mammals</td>
<td>Present study</td>
</tr>
<tr>
<td>Insulin</td>
<td>43</td>
<td>55</td>
<td>X</td>
<td>X</td>
<td>Birds + Other</td>
<td>Present study</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>28</td>
<td>154</td>
<td>X</td>
<td>X</td>
<td>Birds + Other</td>
<td>Goodman et al. 1987; Bishop and Friday 1988</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>26</td>
<td>1746</td>
<td>X</td>
<td>X</td>
<td>Birds + Other</td>
<td>Present study</td>
</tr>
<tr>
<td>28S rRNA</td>
<td>14</td>
<td>374</td>
<td>X</td>
<td>X</td>
<td>Birds + Other</td>
<td>Present study</td>
</tr>
</tbody>
</table>

Note.—Only genes with sequences from at least one representative bird, mammal, crocodilian, and nonamniote outgroup taxon are listed. All groups supported by different analyses are listed. Birds do not form a monophyletic group in the insulin analysis.
the amniote sequence data presently available is unequal, limiting the value of a combined sequence analysis.

Nonetheless, we have assembled all available protein and nucleotide sequence data pertaining to amniote relationships and have performed that analysis, using one representative of each major group. The protein data include sequences of alpha-crystallin A, alpha-hemoglobin, beta-hemoglobin, cytochrome c, histone H2B, insulin, and myoglobin from representative amniotes (see Methods). The frog *Xenopus* was used as the outgroup, although *Rana* had to be used with alpha-crystallin A and carp was used with insulin. The rRNA sequence data were the same used in figure 5.

Both analyses (MP and NJ) of the seven protein sequences (fig. 7) join the mammal and squamate together, but the relationships of the turtle, bird, and crocodilian do not agree, and the confidence levels are relatively low. When all available protein data (831 aligned amino acid sites) and nucleotide sequence data (3,305 aligned nucleotide sites) are analyzed, the only groups formed in the MP analysis are bird + mammal and turtle + crocodilian. However, the NJ analysis provides yet another arrangement, placing squamate + mammal and bird + crocodilian together (fig. 7).

One possible reason for the inconclusive results of the combined sequence analysis is the lack of agreement (regarding relationships) of the individual genes. Where conflicts occur, a consensus often results in weakened support for one of the alternatives, obscuring the original differences (Maeda and Fitch 1981). Such an analysis is more useful when homoplasy is randomly distributed, in which case increasing the size of the data set may increase the significance of the relationships. Another problem involves the poor taxonomic representation in the combined analysis. Although some of the genes have excellent coverage for certain groups (e.g., mammals and birds for the globins, amphibians for the 18s rRNA), there is insufficient overlap to use most of the taxa in the combined analysis. When only one representative of a group is chosen, chance convergence can pose a problem, especially in nucleotide sequence data, where only four states are possible. Also, the choice of taxa can have a significant effect on phylogenetic inferences in amniotes (Bishop and Friday 1988; Gauthier et al. 1988). At present, only a single species each of turtle and crocodilian are represented among the rRNA sequence data, a situation that almost certainly underrepresents the sequence diversity present in living representatives of those two old groups. Confidence in their phylogenetic position among the amniotes would be much greater if sequences were available for additional turtles and crocodilians. Likewise, the confidence in a bird-
mammal clade in the 18S rRNA data is good because it is based on two birds and four mammals, but it would be better if a monotreme and marsupial were among the mammals and if a ratite was among the birds. Until sequences become available from a broader spectrum of taxa, we believe that the molecular data are best interpreted at the level of individual genes, where strengths and weaknesses can be evaluated. A similar opinion was expressed by Maeda and Fitch (1981).

Several mechanisms other than phylogeny have been invoked to explain the association of birds and mammals by molecular data. One argument is that selection acting on the oxygen-binding abilities of globin proteins has led to molecular convergence in these endothermic groups (Dickerson and Geis 1983). Such an argument is unlikely, though, with the rRNA genes, which show functional constancy throughout eukaryotes (Woese 1987). Another mechanism has been proposed by Bishop and Friday (1988), who suggested that the unusual allosteric properties of crocodilian beta-hemoglobin (Perutz 1983) may have helped to “obscure a sister-group relationship to birds.” However, no more than three amino acid replacements are involved (Perutz 1983), and thus it would seem improbable that all crocodilian sites carrying a record of ancestry with birds would (by chance) be obliterated by those three replacements. Yet another hypothesis centers on the observation that birds and mammals have relatively high GC content in their genomes. It has been suggested by Bernardi et al. (1985) that this may be a means of protecting genes from heightened mutability as well as maintaining stability of chromosome structure at high body temperatures. But, as noted by Bishop and Friday (1988), the higher GC content in birds and mammals is accomplished not by a shift toward amino acids coded for mainly by G’s and C’s but by an increase in G’s and C’s at synonymous sites and in noncoding regions (Bernardi et al. 1985).

It is possible that an accelerated substitution rate in the bird and mammal lineages has led to chance convergence. If this has occurred, a parsimony analysis would incorrectly join those two rapidly evolving lineages (Felsenstein 1988). In the present study, the branches leading to birds and mammals are relatively long (fig. 2), and therefore chance convergence of this type might occur. However, if multiple substitutions were common, then it should be reflected in a relatively large number of saturated sites (i.e., those with all four bases present). In the 18S rRNA analysis, 1,527 (87%) of the alignable sites are unvaried, 198 (11.3%) are divariant, 18 (1.0%) are trivariant, and only 3 (0.2%) are fully saturated. This suggests that chance convergence is probably not a significant factor. Also, Felsenstein’s model involves only four taxa in an unrooted network. It is likely that additional taxa (such as those cited in the present study) will reduce the probability of chance convergence. A thorough test of this hypothesis will be possible when more birds and mammals are examined at 18S rRNA.

Amniote Phylogeny: Morphological Evidence

The proposal by Gardiner (1982) and Løvtrup (1985)—i.e., that birds and mammals share a common ancestry, a conclusion based on morphology—has not been widely accepted. However, it has evoked a critical reanalysis of the morphological characters bearing on amniote relationships, from both extant and fossil forms (Gauthier et al. 1988). Although both analyses performed by Gardiner et al. (1988) supported a bird-crocodilian relationship, mammals joined those two groups in the analysis of nonfossil data, thus supporting Gardiner’s group Thecodontia (birds-mammals-crocodilians). Gauthier et al. discovered that the data critical to placing mammals at
the base of the amniote tree (i.e., fig. 1) resided in certain synapsid fossils. When those fossils were removed from the analysis, the remaining fossil data and recent data supported Gardiner’s bird-mammal-crocodilian group. It is misleading to state that “the addition of data on 207 skeletal characters from 29 diverse groups of well-preserved fossil amniotes restored a more traditional cladogram” in the Gauthier et al. study (Benton 1990, p. 413). Gauthier et al. (1988, p. 185) are careful to point out that it was the addition of some intermediate synapsid fossils—not the addition of early or late synapsids, Archaeopteryx, or any other amniote fossils—that was responsible.

Although Gauthier et al. (1988) stressed the “importance of fossils,” another message from their analysis is that the morphological data are homoplasious and that the homoplasy is nonrandom. Indeed, if the critical synapsid fossils are later found to be responsible for the homoplasy, then they could be considered “important” in obscuring—not in retrieving—amniote phylogeny. Although Hillis and Dixon (1989) recently stated that molecular studies “cannot yet challenge most of the framework of higher vertebrate phylogeny,” Gauthier et al. in fact have shown that the morphology-based framework is weak. The molecular data also show homoplasy, but they nonetheless pose a serious challenge to the classical relationships of amniotes.

A valid point has been made elsewhere (Maeda and Fitch 1981; Bishop and Friday 1988)—i.e., that the period during which all of the major amniote groups diverged was relatively short compared with the total elapsed time (fig. 1)—suggesting that there was little time for diagnostic sequence differences to evolve. An analogous situation involves the human-chimpanzee-gorilla relationship, where the divergences probably were close in time and have been difficult to distinguish. Nevertheless, a recent analysis of primate relationships has shown that a large amount of sequence data can resolve even these closely spaced divergences (Koop et al. 1989). In the same light, we anticipate that amniote relationships eventually will be resolved by a large body of sequence data with broad taxonomic representation.

Acknowledgments

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**Fig. A1.**—Aligned 18S rRNA sequences of 26 tetrapods. Shown are entire 18S rRNA sequences, with gaps (•) inserted to increase sequence similarity. Nucleotide position is indicated on the scale below the alignment. Dashes (−) denote areas not sequenced, a “v” above the alignment indicates a variable site, and a solid line above the sequences indicates a region not used in the phylogenetic analysis because of questionable alignment. Coela = coelacanth; Disco = Discoglossidae; Pipid = Pipidae; Pelob = Pelobatidae; Soogid = Sooglossidae; Lepto = Leptodactylidae; Bufon = Bufonidae; Hylid = Hylidae; Micro = Microhylidae; Siren = Sirenidae; Amphi = Amphiumidae; Amby = Ambystomatidae; Peth = Pseudophryniidae; Icht = Ichthyophididae; Cae1 = Caeciliidae; Grand = Grandisoniidae; Cae2 = Caeciliidae; Hyp = Hypogeophis; Typh = Typhlonectidae; Turt = turtle; Lizar = lizard; Croco = crocodylians; Passe = Passeriformes; Gall = Galliformes; Rabbi = rabbit. The sequences of Coela, Pipid, Mouse, Rat, Human, and Rabbi are from the literature, all others are new (deposited in GenBank under accession numbers M36342–M36362).
<table>
<thead>
<tr>
<th>Species</th>
<th>Length</th>
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</thead>
<tbody>
<tr>
<td>Ceola</td>
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</tr>
<tr>
<td>Disco</td>
<td>950</td>
</tr>
<tr>
<td>Pipid</td>
<td>950</td>
</tr>
<tr>
<td>Pelob</td>
<td>950</td>
</tr>
<tr>
<td>Soogl</td>
<td>950</td>
</tr>
<tr>
<td>Eleut</td>
<td>950</td>
</tr>
<tr>
<td>Hyld</td>
<td>950</td>
</tr>
<tr>
<td>Micro</td>
<td>950</td>
</tr>
<tr>
<td>Siren</td>
<td>950</td>
</tr>
<tr>
<td>Amphi</td>
<td>950</td>
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<td>Ambys</td>
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<tr>
<td>Pleth</td>
<td>950</td>
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<td>Ichth</td>
<td>950</td>
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<tr>
<td>Csec1</td>
<td>950</td>
</tr>
<tr>
<td>Csec2</td>
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<td>Tylph</td>
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<td>Turtl</td>
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<td>Snake</td>
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<td>Passe</td>
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<td>Rat</td>
<td>950</td>
</tr>
<tr>
<td>Human</td>
<td>950</td>
</tr>
<tr>
<td>Rabbi</td>
<td>950</td>
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</tbody>
</table>
APPENDIX B

FIG. B1.—Aligned 28S rRNA sequences of 14 tetrapods. Shown are combined sequences from three regions of the 28S rRNA molecule corresponding to the positions in mouse (Hassouna et al. 1984) indicated in boldface numbers on the scale below the alignment. Other symbols and abbreviations are as in appendix A. The sequences of Pipidae, mouse, and human are from the literature; all others are new (deposited in GenBank under accession numbers M36363–M36373.)
APPENDIX C

<table>
<thead>
<tr>
<th>Human</th>
<th><em>FV</em>NGLCGSHLVEALYLVGGERFFYTP<em>KGGVEGCCTSICSLYGLENYCN</em></th>
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</thead>
<tbody>
<tr>
<td>Macaque</td>
<td><em>FV</em>NGLCGSHLVEALYLVGGERFFYTP<em>KGGVEGCCTSICSLYGLENYCN</em></td>
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<td>Monkey</td>
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<td>COW</td>
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<tr>
<td>PIG/DOG/GOAT</td>
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</tr>
<tr>
<td>Camel</td>
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<tr>
<td>Horse</td>
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</tr>
<tr>
<td>Cat</td>
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<tr>
<td>Rabbit</td>
<td><em>FV</em>NGLCGSHLVEALYLVGGERFFYTP<em>KGGVEGCCTSICSLYGLENYCN</em></td>
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<tr>
<td>SPINY MOUSE</td>
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<td>MOUSE</td>
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<td>Guinea Pig</td>
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</tr>
<tr>
<td>Chimpanzee</td>
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<tr>
<td>Porcupine</td>
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</tr>
<tr>
<td>Nutria</td>
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<td>Elephant</td>
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<td>COD</td>
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<td>CARP</td>
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**FIG. C1.**—Aligned insulin amino acid sequences of 42 vertebrates. Shown are combined sequences of the A and B chains of insulin, from the National Biomedical Research Foundation Protein Identification Resource and Swiss Protein data banks and from Lance et al. (1984). Gaps are indicated by an asterisk (*).

**LITERATURE CITED**


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