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VARIABILITY IN TRANSCRIBED REGIONS OF RIBOSOMAL DNA AND EARLY DIVERGENCES IN BIRDS

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ABSTRACT.—We mapped ribosomal DNA (rDNA) restriction sites and length differences in 10 species from 8 orders and *Alligator mississippiensis* to assess the level of variation and to determine phylogenetic relationships. We found 19 phylogenetically informative characters, including 15 restriction site variants and 4 length differences, in coding and noncoding transcribed regions. The nontranscribed region sites mapped were unique to each taxon. Anseriformes and Galliformes are early divergents along the avian lineage, and only tinamous and ratites (paleognaths) are as old or older. Galliformes is more closely related to a clade of seven neognath orders and families than is Anseriformes, and one apparent synapomorphy involving length variation unites Anseriformes and Tinamiformes. In comparisons of 19 rDNA transcribed region restriction sites conserved among birds and *Alligator mississippiensis* with published sequences, all are present in *Mus musculus*, and only three (16%) are absent from *Xenopus laevis*. Nucleotide sequence data from variable rDNA regions, particularly internal transcribed spacer (ITS) and external transcribed spacer (ETS) regions, will provide greater resolution of relationships among more recent avian orders. *Received 20 January 1989, accepted 3 June 1989.*

BIRDS first diverged from vertebrate ancestors in the late Jurassic approximately 150 million years ago. Earliest divergences among extant forms appear to be approximately 80 million years old (Cracraft 1986), and extant orders are often believed to have originated concomitantly during the late Cretaceous and early Tertiary periods. Although many details are known regarding species-level relationships of birds, few derived characters have been recognized that unite particular avian orders and families in sister-group relationships. Fossils have revealed few such characters, and the activity of flight restricts the degree of divergence possible in major skeletal elements. Thus, the notion of concomitant origins for many orders is based more on an absence of knowledge of relationships than on evidence, and is symptomatic of a shortage of informative characters.

Ribosomal DNA (rDNA) provides a valuable tool to assess phylogenetic relationships among higher level avian taxa. This gene system consists of a complex of coding and noncoding sequences which evolve at different rates. Coding regions (28S, 18S, 5.8S genes) tend to be conserved across higher taxa, including kingdoms and phyla (Goldman et al. 1983, Elwood et al. 1985, Apples and Honeycutt 1986). Transcribed spacer regions are less conserved than coding regions, and contain phylogenetically informative characters for primates (Wilson et al. 1984) and for frogs in the genus *Rana* (Hillis and Davis 1986). The nontranscribed spacer is the most variable rDNA region, and populational differences have been found within some species (e.g. Suzuki et al. 1986, 1987).

We mapped both restriction-site and length variation for rDNA transcribed regions in 10 avian taxa and one crocodilian. We found rDNA

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transcribed regions to be phylogenetically informative with respect to earliest diverging extant bird forms, although many characters are conserved across all taxa studied. Based on our rDNA characters, Anseriformes and Galliformes are early divergents within birds and second in age to the paleognaths (ratites and tinamous). Galliformes appears more closely related to the other neognaths than Anseriformes based on two shared restriction sites; and Ciconiiformes, Accipitridae, and Cathartidae are sister taxa relative to the other study groups.

METHODS

We selected 10 representative species from 8 different avian orders. Most of the orders have been proposed, at one time or another (e.g. Huxley 1867, Mayr and Amadon 1951, Sibley et al. 1988), as being relatively basal in phylogenetic position among extant birds. We also chose a phylogenetically diverse group of study taxa in light of the slow pace of nuclear rDNA change, and our desire to maximize resolution of relationships. We analyzed: *Crypturellus undulatus* (Undulated Tinamous), *Cygnus buccinator* (Trumpeter Swan), *Gallus gallus* (domestic chicken), *Passer domesticus* (House Sparrow), *Falco tinnunculus* (Eurasian Kestrel), *Bubo bubo* (Great Eagle-Owl), *Chordeiles minor* (Common Nighthawk), *Buteo buteo* (Common Buzzard), *Cathartes melambrotus* (Greater Yellow-headed Vulture), and *Mycteria americana* (Wood Stork).

We isolated high molecular weight nuclear DNA from muscle, liver or blood samples following the techniques of Bingham et al. (1981). Genomic DNA was subjected to single and double digests involving 20 different restriction endonucleases (*Alu*I, *Bam*HI, *Bcl*I, *Bgl*II *Bst*EII, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Nco*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Stu*I, *Xba*I, *Xho*I, *Xmn*I). Digestions were routinely done with 3–10 units of enzyme per μg DNA and for 3–16 hours. Digested fragments were separated on 0.8–2.0% agarose TAE (0.4 M Tris, 0.01 M 2Na EDTA, 0.05 M sodium acetate) gels run at ca. 35 mA for 16 h and stained with ethidium bromide. Higher percent agarose gels were used to improve resolution of fragments shorter than 400 base pairs (bp).

Separated DNA fragments were denatured in the gel by soaking in a 0.6 M NaCl, 0.4 M NaOH transfer solution for 30 min. Single stranded fragments were transferred from the gel to a nylon membrane (Gene Screen Plus®; DuPont) following Southern (1975) with modification for alkaline transfer (Chomczynski and Qasba 1984). Nylon membranes were then washed in prehybridization solution (1% sodium dodecyl sulfate, 50% formamide, 5% Denhardt's solution, 1.5% denatured salmon sperm DNA) at 37°C for 40 min.

Ribosomal DNA fragments on nylon membranes were detected by hybridization with radiolabeled rDNA clones of 18S (p2546) and 28S (pI19) genes from *Mus musculus* (Arnheim 1979). Cloned rDNA genes were labeled in a random priming reaction (Feinburg and Vogelstein 1984), ethanol precipitated, suspended in 1 × TE, and spun in a Damon/IES HN-SII tabletop centrifuge for 4 min at 2,500 rpm through a sephadex G-50 column to remove unincorporated nucleotides. The labeled probe was denatured, combined with prehybridization solution (1×10^6 dpm/ml) and allowed to hybridize with nylon membranes for 10 h or longer at 37°C. Nylon membranes were washed three times for 10 min in a 2X SSC, 0.1% SDS solution, and twice for 15 min in a 0.1% SSC, 0.1% SDS solution at 55°C with agitation, and blotted dry with toweling. Membranes were exposed to X-ray film in cassettes with intensifying screens at −70°C for 8–24 hours.

A restriction site map was first determined for *Gallus gallus* using single and double digests, and oriented to the conserved (Cortadas and Pavon 1982) *Eco*RI site at the 3' ends of both the 18S and 28S genes. Relative placement for each restriction site was determined by three or more double digests with different pairs of enzymes. Maps were then determined for the 10 other taxa, based on side-by-side comparisons for all taxa on a single gel, and using the *Gallus gallus* map as a guide. To help map the length variation in the 28S gene, we used a partial digestion strategy in which a complete digestion with *Eco*RI was digested with 0.375 units *Alu*I per μg DNA for 30 min. This provided a series of partially digested 28S *Eco*RI-18S *Eco*RI fragments for comparison of length differences across taxa, when hybridized with a shortened (1.1 kilobase [kb], *Eco*RI-*Pvu*II fragment) 28S probe. Length variation involving additions and deletions were placed within the smallest fragment known to contain them.

Phylogenetic hypotheses are based on the criterion of parsimony using shared derived characters (*synapomorphies*) (Henning 1966, Gaffney 1979), with variable restriction sites coded as present or absent. Polarity of character-state changes for birds were established using *Alligator mississippiensis* as an outgroup. Differences in sequence length cannot be mapped as precisely as restriction-site differences and could potentially be due to different combinations of additions/deletions in different taxa. For this reason, we are more cautious in calling them synapomorphies, and we conducted separate phylogenetic analyses with and without length-variation characters. Phylogenetic analyses were conducted using D. L. Swofford's PAUP program (version 2.4), and the branch and bound algorithm following Hendy and Penny (1982).

RESULTS

As in other organisms, the rDNA repeat unit in birds is organized into three coding regions,

the 18S, 5.8S, and 28S genes, with internal transcribed spacer regions between the 18S and 5.8S genes (ITS1) and between the 5.8S and 28S genes (ITS2). The intergenic spacer that separates tandemly arrayed repeat units consists of an external transcribed spacer (ETS) adjacent to the 18S gene and a larger nontranscribed spacer (NTS) (Fig. 1).

The overall rDNA repeat length (all coding and spacer regions) varied among taxa, and *Gallus gallus* had a repeat length of ca. 28 kb (McClements and Skalka 1977). Repeat unit lengths were not determined for all taxa because of the difficulty in sizing the large NTS fragment without a specific probe for that region. However, we measured length variation in the 18S *Eco*RI to 28S *Eco*RI fragment and found the following lengths (in kb): *Crypturellus* 5.6; *Alligator* 6.7; *Cygnus* and *Chordeiles* 7.5; *Gallus* and *Buteo* 7.8; *Bubo, Cathartes* and *Mycteria* 8.1; *Passer* and *Falco* 8.9. Because we found no length variation within the 18S gene, and relatively minor length variation (<500 bp) within the 28S gene, most length variation occurred in spacer regions (Fig. 1).

Within rDNA coding regions, we found 19 *sympleisiomorphic* (shared primitive) and 12 *synapomorphic* (shared derived) restriction site characters (Fig. 1). We found one synapomorphic and one sympleisiomorphic character in the ETS, and single synapomorphic characters in ITS1 and ITS2. Three synapomorphic sequence additions were located in the 28S gene, and one synapomorphic deletion was found in the ITS2 region. Because we lacked partial digestion comparisons or a probe covering part of the ETS/NTS region, we could only compare among taxa the first site occurring upstream of the 18S probe. All such sites mapped to the NTS were *autapomorphous* (unique) and are not included in Fig. 1, as they provide no information relating to phylogeny. The high degree of divergence for NTS sites indicates that they may be useful in phylogenetic studies of more closely related species.

Not all restriction sites for the 20 enzymes that occur within ITS regions are mapped. Only those that could be readily examined using the 18S and 28S probes were plotted. Presence of multiple ITS sites for any enzyme means that some will be undetected using single and double digests hybridized with two probes. Greater effort (more digests) was made to map any ITS sites that appeared phylogenetically informative. All restriction sites within the 18S and 28S genes have been mapped, with the exception of *Alu*I in which only the variable site in 28S is plotted. *Alu*I was used to map length variation in the 28S gene and was not examined with the 18S probe.

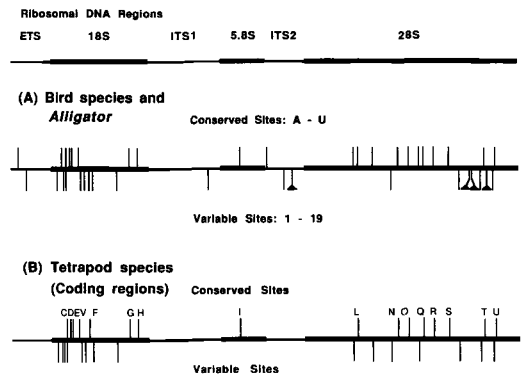

Fig. 1. Conserved and variable endonuclease restriction sites and insertions/deletions (triangles) in transcribed rDNA regions. (A) Composite map for 10 avian species and *Alligator mississippiensis;* characters unique to a single species excluded. (B) Composite map for sites only from (A) as found in *Xenopus laevis, Mus musculus,* and *Alligator* and avian species from this study (*Cla*I excluded); sites unique to a single class included. Conserved sites A–V, beginning with A in the ETS, are identified as follows: A = *Cla*I, B = *Sma*I, C = *Xba*I, D = *Nco*I, E = *Bst*EII, F = *Sma*I, G = *Bst*EII, H = *Eco*RI, I = *Bcl*I, J = *Sac*I, K = *Stu*I, L = *Bam*HI, M = *Sac*I, N = *Nco*I, O = *Bgl*II, P = *Bam*HI, Q = *Sac*II, R = *Sma*I, S = *Pvu*II, T = *Sac*I, U = *Eco*RI, V = *Pst*I. Variable sites and length changes are as identified in Table 1, beginning with 1 in the ETS for (A).

These restriction-site and sequence-length differences provide a total of 19 phylogenetically informative characters (Table 1). For example, an *Xba*I restriction site 2.0 kb upstream of the 28S *Eco*RI site unites *Gallus, Passer, Buteo, Falco, Bubo, Chordeiles, Cathartes,* and *Mycteria* relative to *Crypturellus, Cygnus,* and *Alligator.* However, synapomorphic gain of one 18S *Pst*I site and loss of another 18S *Pst*I site unite *Passer, Buteo, Falco, Bubo, Chordeiles, Cathartes,* and *Mycteria* (Fig. 2). Gain of a third *Pst*I site 1.2 kb upstream of the 18S *Eco*RI site unites *Buteo, Cathartes,* and *Mycteria.* Analysis of restriction-site and length-variation characters together (Table 1) resulted in a single most parsimonious tree with two unresolved trichotomies. The tree has a *consistency index* (minimum number of steps possible divided by number of steps required)

TABLE 1. Phylogenetically informative characters from a composite rDNA restriction map (Fig. 1), used to determine a cladogram (Fig. 3). 1 = present, 0 = absent. Species are coded as follows: (1) *Alligator mississippiensis*, (2) *Crypturellus undulatus*, (3) *Cygnus buccinator*, (4) *Gallus gallus*, (5) *Passer domesticus*, (6) *Falco tinnunculus*, (7) *Bubo bubo*, (8) *Chordeiles minor*, (9) *Buteo buteo*, (10) *Cathartes melambrotus*, and (11) *Mycteria americana*.

	Species										
	1	2	3	4	5	6	7	8	9	10	11
External transcribed spacer											
1 *PstI*[1]	0	0	0	0	0	0	0	0	1	1	1
18S gene											
2 *Hin*dIII	0	0	0	0	1	1	1	1	1	1	1
3 *Xho*I	0	1	1	1	1	1	1	1	1	1	1
4 *Sac*II[1]	0	0	0	0	1	1	1	1	1	1	1
5 *Bam*HI*	0	1	1	1	1	1	1	1	1	1	1
6 *PstI*[2]	1	1	1	1	0	0	0	0	0	0	0
7 *PstI*[3]	0	0	0	0	1	1	1	1	1	1	1
8 *Sac*II[2]*	0	1	1	1	1	1	1	1	1	1	1
9 *Sac*I	0	1	1	1	1	1	1	1	1	1	1
Internal transcribed spacer											
10 *Stu*I (ITS1)	0	0	0	0	0	0	0	0	1	1	1
11 *Kpn*I (ITS2)	0	1	1	1	1	1	1	1	1	1	1
12 −340 bp (ITS2)	0	0	0	0	0	0	0	1	1	1	1
28S gene											
13 *Xba*I	0	0	0	1	1	1	1	1	1	1	1
14 *Sac*II[3]	0	0	0	1	1	1	1	1	1	1	1
15 +80 bp	0	0	0	0	1	1	1	1	1	1	1
16 +100 bp	0	1	1	0	0	0	0	0	0	0	0
17 *Alu*I*	0	1	1	1	1	1	1	1	1	1	1
18 +70 bp	0	0	0	0	1	1	1	1	1	1	1
19 *Sac*II[4]	1	1	1	0	0	1	1	1	1	1	1

* These sites are present in *Xenopus laevis*, *Mus musculus*, or both (Fig. 1), and are not plotted in Fig. 3 as they are not unique in birds.

of 0.94 based on 16 informative characters unique to birds, and 17 postulated character changes (Fig. 3), indicating high consistency within the data set. The only *homoplasious* (inconsistent) character in the cladogram is shared loss of a *Sac*II site in the 28S gene by *Gallus* and *Passer*. Restriction-site losses are more likely to be homoplasious than shared gains, as site loss can result from change in any one of the bases in a recognition sequence, whereas site gain cannot happen as often with a single base change (DeBry and Slade 1985).

DISCUSSION

Crocodylia is the sister taxon to Aves, and together they constitute the Archosauria (Gauthier 1986, Goodman et al. 1987, Gauthier et al. 1988). As a representative of Crocodylia, *Alligator* is a suitable outgroup for analyses of avian relationships. As expected, the 10 avian taxa are shown to be a monophyletic group relative to *Alligator*, based on three shared derived characters (Fig. 3). Although six characters appear to unite the bird taxa relative to *Alligator* based on restriction maps (Table 1), three of these sites (*Alu*I 28S, *Bam*HI 18S, *Sac*II[2] 18S) are present in *Xenopus laevis* and *Mus musculus*, and the three sites are not unique in birds.

Two basic groups of extant birds are generally recognized. The ratites—large flightless birds such as the Ostrich (*Struthio camelus*), Emu (*Dromaius novaehollandia*), cassowarys (*Casuarius*), rheas (*Rhea*), and kiwis (*Apteryx*)—and tinamous make up one group; and all other extant birds are included in the second (Paleognathae and Neognathae, respectively). Paleognath monophyly has been one of the most controversial issues in avian systematics (summarized in Sibley and Ahlquist 1972, 1981; Olson 1985). Although a consensus has emerged supporting monophyly, based on osteology (Bock 1963, Parkes and Clark 1966, Cracraft 1974, Houde 1988), immunological distances (Prager et al. 1976), DNA-hybridization (Sibley and Ahlquist 1981), and alpha-crystallin A amino acid sequences (Stapel et al. 1984), some questions re-

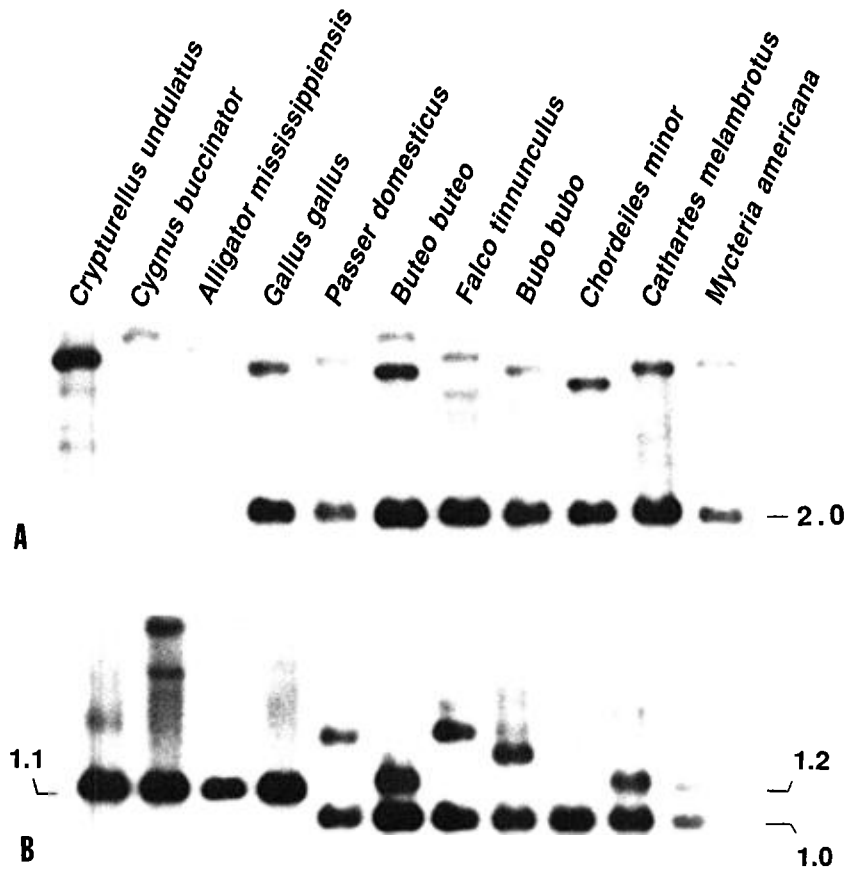

Fig. 2. Restriction-site variation in rDNA coding regions from individuals of 11 species. (A) *Eco*RI-*Xba*I double digest with fragment indicating shared derived gain of an *Xba*I site 2.0 kb upstream of the 28S *Eco*RI site for 8 taxa. (B) *Eco*RI-*Pst*I double digest indicating (1) *Pst*I site 1.1 kb upstream of 18S *Eco*RI site in 4 taxa including the outgroup, *Alligator* (shared derived loss of this site in 7 taxa was determined based on separate partial digestions), (2) shared derived gain of *Pst*I site 1.0 kb upstream of the 18S *Eco*RI site for 7 taxa, and (3) shared derived gain of *Pst*I site 1.2 kb upstream of the 18S *Eco*RI site for 3 taxa. Numbers indicate fragment length. Fragments without length measurements given represent the upstream portions of cleaved sequences and results of partial digestions.

main. Dissenters stress morphological differences within paleognath species and the possibility that diagnostic traits are primitive and, hence, not phylogenetically informative (e.g. Gingerich 1973). Tinamous are clearly distinct from ratites, but they have been included in Paleognathae based largely upon the shared palatal condition (Pycraft 1900). Tinamous have been linked to Galliformes by many workers (e.g. Seebohm 1888; Beddard 1898, 1911; Chandler 1916; Banks 1970; Sibley and Frelin 1972, Houde 1988) based on osteology, morphology of intestines, pterylography, molt, and protein isozymes. Their true affinity remains uncertain.

A related issue is whether the orders Galliformes and Anseriformes are more closely related to paleognaths or to neognaths, and whether these two orders are sister taxa. Anseriformes and Galliformes were considered non-sister neognath taxa by Olson and Feduccia (1980). Cracraft and Sibley et al. recognized them as sister taxa and placed them within the Neognathae (Cracraft 1988) and Paleognathae (Sibley et al. 1988). The phylogeny inferred from transferrin immunological distances also depicts Anseriformes and Galliformes as sister groups (Ho et al. 1976). A reanalysis of these data yielded two equally parsimonious, shorter trees (Cracraft and Mindell in press), and one of these, allowing negative branch lengths as

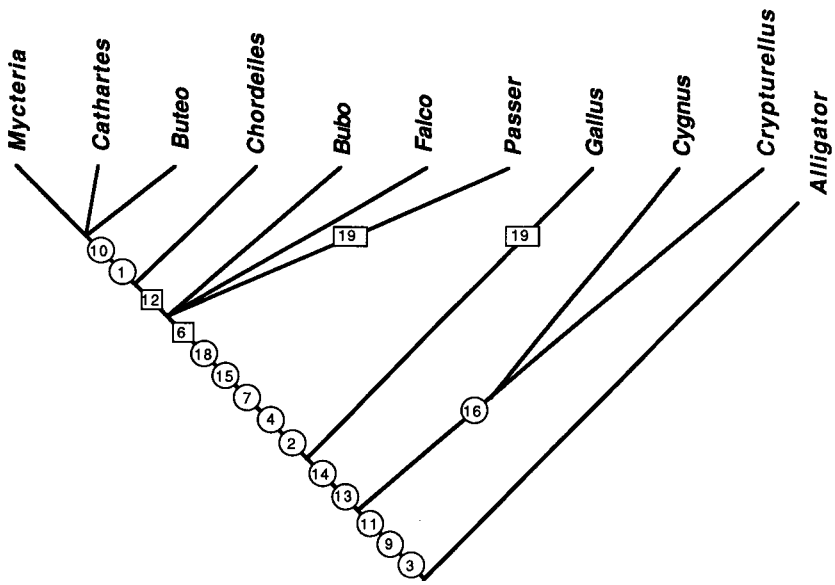

Fig. 3. Most parsimonious phylogenetic tree for 10 avian species with *Alligator* as an outgroup, based on 16 rDNA restriction-site or length variation characters. This tree requires 17 character changes and has a consistency index of 0.94. Circles represent shared derived gains; squares represent shared derived losses; rectangles represent homoplasious losses. Species and character numbers are as specified in Table 1.

in the published tree, separated Anseriformes and Galliformes. In both trees, Galliformes and Anseriformes are phenetically closer to neognaths. Alternatively, Olson (1985) placed Anseriformes as sister to Charadriiformes based on fossils purportedly linking the two groups (e.g. *Presbyornis* of the early Eocene, *"Telmatornis" rex* dating from the Cretaceous) and suggested that inquiry into a possible relationship between Galliformes and Columbiformes is warranted.

Six rDNA synapomorphies (four coding region restriction-site differences and two sequence additions in the 28S gene) separate Anseriformes (*Cygnus*) and Galliformes (*Gallus*) from the other neognaths (*Passer, Falco, Bubo, Chordeiles, Buteo, Cathartes,* and *Mycteria*), making this the most strongly supported node on the tree (Fig. 3). In additional phylogenetic analyses, the shortest trees ($n = 2$) that break the clade of these seven "other" neognaths required 22 steps, 5 steps more than the most parsimonious tree. In the most parsimonious tree (Fig. 3), two 28S restriction site synapomorphies unite Galliformes (*Gallus*) with other neognath representatives, and one sequence addition in 28S unites Anseriformes (*Cygnus*) with Tinamiformes (*Crypturellus*), a paleognath. Anseriformes and Galliformes are clearly distant from the seven other neognaths, but no data directly support their status as sister taxa. The shortest tree found that unites Anseriformes (*Cygnus*) and Galliformes (*Gallus*) as sisters is three steps longer than the most parsimonious tree. Thus, the nature of Anseriformes and Galliformes as relatively early divergents within or from neognaths is corroborated by all five data sets available (see above). Sister status for them is not supported, although closest relatives for these orders remain uncertain.

Another longstanding controversy involves placement of Cathartidae as closer to Ciconiiformes or to Accipitridae. A consensus to unite Cathartidae with Ciconiiformes (Ligon 1967, Rea 1983, Olson 1985, Sibley et al. 1988) has developed, and although the rDNA data do not resolve this, two synapomorphic site gains in transcribed spacer regions unite Ciconiiformes, Cathartidae, and Accipitridae indicating their close relationship relative to the other taxa. Exclusion of *Falco* from this clade suggests that the order Falconiformes is polyphyletic, as both Accipitridae and Falconidae are generally included in that order. At least three additional steps would be required in the phylogenetic tree (Fig. 3) to maintain *Buteo, Falco,* and *Cathartes* as a monophyletic group. Monophyly of Falconiformes has long been questioned, and although data presented here do not rule out

monophyly, the possibility of polyphyly deserves further consideration.

In phylogenetic analysis with the four length variation characters excluded, a most parsimonious tree (not shown) was determined with 13 steps, and consistency index of 0.92. Differences from the cladogram based on all site and length variation (Fig. 3) are that Anseriformes and Tinamiformes are not sister taxa but are joined in an unresolved trichotomy, and the clade of Caprimulgiformes, Accipitridae, Cathartidae, and Ciconiiformes is not distinguished. Thus, these two clades (supported only by length variation) are tentative, whereas the other relationships are well supported by restriction-site differences.

The 28S gene includes two types of sequences. "Expansion" segments have diverged and evolved as a unit within the 28S gene, whereas "core" segments show no similarity among themselves. Divergence among taxa in expansion segments is greater than in core segments, accounting for nearly all of the differences in length of 28S/26S rDNA genes found among eukaryotic species (Clark et al. 1984, Tautz et al. 1988). Length variation in the 28S gene that we found among birds is probably due to expansion segment differences as well. The rate of divergence in these expansion segments is such that they can be phylogenetically informative for higher level avian taxa. Additions of 70 bp and 80 bp in the 28S gene are synapomorphies for the clade of seven neognath species and are corroborated by four restriction-site synapomorphies. The third 28S length variation (+100 bp) is neither directly corroborated by, nor in conflict with, any other characters (Fig. 3). Our findings are similar to apparent expansion segment synapomorphies for species of *Rana* reported by Hillis and Davis (1987).

Intrataxon variation in rDNA sequences could have large effects on phylogenetic analyses involving small samples, as in our study. However, rDNA sequences in transcribed regions, and particularly coding regions, tend to be conserved across higher taxa. To examine this proposition in more detail, we compared presence/absence of restriction sites from published *Mus musculus* (Goldman et al. 1983, Walker et al. 1983, Hassouna et al. 1984, Raynal et al. 1984) and *Xenopus laevis* (Fedoroff 1979, Maden et al. 1982, Stewart et al. 1983, Ware et al. 1983) rDNA sequences with 31 of our mapped restriction sites in birds and *Alligator*. We included 12 variable and 19 sympleisiomorphic (conserved) coding region sites for birds and *Alligator*. Restriction maps were determined by computer (Pustell and Kafatos 1982) and aligned with our own restriction maps by hand. All of the 19 conserved sites are present in *Mus musculus*, and only 3 (16%) of those 19 sites are absent from *Xenopus laevis*. Hillis and Dixon (in press) found that discrete nucleotide sequence data for a 2.5 kb portion of the 28S gene did not resolve sister relationships among the classes Reptilia, Mammalia, and Aves, as is the case for our restriction-site data. We found no restriction-site variability in transcribed rDNA regions for 32 House Sparrows based on mapping for eight enzymes (unpubl. data). Most intraspecific rDNA variation reported to date occurs within nontranscribed spacer regions (e.g. Schafer and Kunz 1985; Suzuki et al. 1986, 1987; Schaal et al. 1987), which was excluded from the present analysis. Thus, we feel the restriction map characters from our representative species are informative. The degree of conservation found among classes and within a species supports the use of small samples for investigation of higher level relationships within classes. However, actual conservatism of these characters within avian orders and families can be determined only by more extensive sampling studies.

Of 21 informative restriction sites for species in the genus *Rana*, only 2 (9.5%) were mapped to coding regions (Hillis and Davis 1986), whereas 7 of 19 (36.8%) coding region sites were informative within birds. If divergence rates are similar in *Rana* and birds, then the greater percentage of variable coding region sites in birds suggests that divergences among Tinamiformes, Anseriformes, Galliformes, and the seven neognaths as a group are as old as, or older than, the early *Rana* divergence (ca. 50 million years). Three of four synapomorphies in birds that involve change in rDNA transcribed spacer regions occurred within the clade of seven neognaths, indicating more rapid divergence in spacer as compared with coding regions.

Characters from the restriction-site maps of rDNA transcribed regions provide phylogenetic information concerning some of the earliest divergences within extant birds. Based on analysis of representative species, Galliformes and Anseriformes are clearly early divergents along the avian lineage. Galliformes appears more closely related to the other neognaths than

Anseriformes, and one 28S length character unites Anseriformes and Tinamiformes. However, further evidence is required to resolve their sister relationships (or sister status) and relative affinity to neognaths or paleognaths. Restriction-site maps provide a gross-level entry to the phylogenetic information in rDNA sequences. Resolution of more recent avian orders may be possible with sequence data from variable rDNA regions determined here, especially ITS and ETS regions showing synapomorphies within the clade of seven neognaths (Fig. 3).

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100 Years Ago in The Auk

Excerpts from "The present status of the wild pigeon (*Ectopistes migratorius*) as a bird of the United States, with some notes on its habits" by William Brewster (1889, Auk 6: 285-291)

[In 1889 Brewster went to Michigan in what turned out to be an unsuccessful attempt to find nesting birds. A flock "that covered at least eight acres" was reported, but not actually seen. In this article he discusses some other reports of birds in the area including that "The last nesting in Michigan of any importance was in 1881, a few miles west of Grand Traverse. It was of only moderate size,—perhaps eight miles long." Larger nesting events which extended "for twenty-eight miles, averaging three or four miles wide," were reported in the 1870s.

After discussing nesting behavior, all of which was acquired secondhand, he discusses the clutch size. They did manage to find a nest or two on this trip. He concludes:]

"All the netters with whom we talked believe firmly that there are just as many Pigeons in the West as there ever were. They say the birds have been driven from Michigan and the adjoining States partly by persecution, and partly by the destruction of the forests, and have retreated to uninhabited regions, perhaps north of the Great Lakes in British North America. Doubtless there is some truth in this theory; for, that the Pigeon is not, as has been asserted so often recently, on the verge of extinction, is shown by the flight which passed through Michigan in the spring of 1888. This flight, according to the testimony of many reliable observers, was a large one, and the birds must have formed a nesting of considerable extent in some region so remote that no news of its presence reached the ears of the vigilant netters. Thus it is probable that enough Pigeons are left to re-stock the West, provided that laws, sufficiently stringent to give them fair protection, be at once enacted. The present laws of Michigan and Wisconsin are simply worse than useless, for, while they prohibit disturbing the birds *within* the nesting, they allow unlimited netting only a few miles beyond its outskirts *during the entire breeding season*. The theory is that the birds are so infinitely numerous that their ranks are not seriously thinned by catching a few million of breeding birds in a summer, and that the only danger to be guarded against is that of frightening them away by the use of guns or nets in the woods where their nests are placed. The absurdity of such reasoning is self-evident but, singularly enough, the netters, many of whom struck me as intelligent and honest men, seem really to believe in it. As they have more or less local influence, and, in addition, the powerful backing of the large game dealers in the cities, it is not likely that any really effectual laws can be passed until the last of our Passenger Pigeons are preparing to follow the Great Auk and the American Bison."