

Alignment and Phylogenetic Analysis of β -Fibrinogen Intron 7 Sequences Among Avian Orders Reveal Conserved Regions Within the Intron

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We sequenced β -fibrinogen intron 7 (β -*fibint* 7) from 28 species of birds, representing 18 families in nine orders. Although the antiquity of the avian orders is estimated to be 55 to 90 Myr, and numerous indels have accrued among diverging lineages, the intron sequences were not difficult to align. However, alignment of avian sequences with mammal or snake sequences was difficult, and the residual phylogenetic signal was weak. β -*fibint* 7 is an AT-rich intron, and its base composition varies little over the diversity of birds represented by our sample. Alignment of these anciently diverged sequences reveals at least five clusters of conserved nucleotides; at least two clusters appear to be in excess of the minimal set usually associated with intron excision, but their functions are unknown. Two equally most-parsimonious (MP) trees were found when indels were not included in the phylogenetic analysis, and six such trees were found when indels were included. The Neighbor-Joining and maximum-likelihood trees were identical to each other and to one of the MP trees in each MP analysis. Indels, as well as nucleotide substitutions, are phylogenetically informative, and bootstrap support exceeded 90% for 21 of 24 inferred nodes when indels were included in the MP analysis. All traditional orders represented by two or more species appear monophyletic. Relationships among avian orders are strongly supported with the exception of an inferred sister-group relationship between Caprimulgiformes and Columbiformes. A relatively close relationship between Piciformes and Passeriformes is inferred, at odds with earlier DNA-DNA hybridization studies but consistent with traditional classifications. Among Passeriformes, the traditional perspective of a sister-group relationship of suboscines and oscines is supported, as is the subsequent split of the oscines into a lineage representative of the Corvidae before the diversification of the Passerida. The four species of owls divide into two strongly supported clades, corresponding to the widely accepted bifurcation of owls into two families, Tytonidae and Strigidae. A sister-group relationship between gallinaceous birds and waterfowl, the Galloanserae, is also strongly supported.

Introduction

Introns of nuclear genes have proved useful for phylogenetic analysis of recently evolved vertebrates (Koop et al. 1989; Slade, Moritz, and Heideman 1994; Miller and Withler 1996; Schneider et al. 1996; Prychitko and Moore 1997, 2000; Johnson and Clayton 2000; Walton, Nedbal, and Honeycutt 2000; Deinard and Smith 2001; Giannasi, Malhorta, and Thorpe 2001; Shapiro and Dumbacher 2001). Introns have several properties that make them useful for phylogenetic analyses. They are easy to amplify by PCR for a broad range of taxa because they are flanked by conserved exons, which provide sites for PCR primers. They evolve more slowly than mtDNA (Prychitko and Moore 1997, 2000; Johnson and Clayton 2000) but more rapidly than nuclear exon sequences (overall) and approach the rates of pseudogenes and third positions of exons (Li 1997, fig. 7.2; Hughes and Yeager 1997). This high substitution rate, along with the frequent occurrence of indels (insertions and deletions), implies that much intron sequence evolution is adaptively neutral, or nearly so. Moreover, to the extent that intron 7 of the β -fibrinogen gene (β -*fibint* 7) is typical, intron evolution has a lower transition: transversion ratio than mtDNA and the substitution matrix (Yang 1994), which characterizes rates of specific nucleotide substitutions (A \rightarrow T, A \rightarrow G, etc.), is more homogeneous (i.e., the rates among different types of substitutions are more uniform). Consequently, homoplasy is lower in introns than mtDNA (Prychitko and Moore 2000).

Although valuable for resolving phylogenies of recently evolved groups, it is unclear how far back in evolutionary time intron sequences might give phylogenetic resolution. Computer simulations (Moore, Smith, and Prychitko 1999) suggest that introns do not begin to saturate significantly in avian species until they have diverged for 50 Myr, and it is likely that they retain phylogenetic information of greater antiquity. Diverging introns also accumulate indels of various lengths, which have the potential of being useful characters in establishing evolutionary relationships, but severe alignment problems may occur when more anciently diverged sequences are aligned. Prychitko and Moore (1997, 2000) demonstrated the ability of β -*fibint* 7 to provide a well-resolved gene tree for woodpeckers, a subfamily of birds that appears to be approximately 7 to 8 Myr old (Moore, Smith, and Prychitko 1999). Although indels occurred in the β -*fibint* 7 sequences of the woodpeckers, they did not pose serious difficulties with alignment.

The phylogenetic relationships among avian orders and among families within most avian orders are poorly understood because of the apparent rapid radiation of modern birds (Sibley and Ahlquist 1990; Hedges 1994; Feduccia 1996; Cooper and Penny 1997; Groth and Barrowclough 1999; Mindell et al. 1999). This explosive period of evolution, which occurred some time between 55 and more than 90 MYA (reviewed by Waddell et al. 1999; Mindell et al. 1999; van Tuinen and Hedges 2001), appears to have generated all of the modern orders of birds in approximately 5 to 10 Myr (Feduccia 1995). Because modern avian orders diverged within a short span of time relatively long ago, the number of synapomorphies that delineate basal relationships is limited. Morphological studies such as those by Cracraft (1988) and Cracraft and Mindell (1989) had difficulty resolving relationships

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among particular avian orders because of a lack of sufficient synapomorphies. Sibley and Ahlquist's (1990) extensive DNA-DNA hybridization analysis has provided a good beginning for DNA-based inference of avian phylogeny but has been criticized from a number of perspectives (Lanyon 1992; Mindell 1992; Harshman 1994). At the very least, the phylogeny hypothesized by Sibley and Ahlquist (1990) needs to be corroborated.

Several recent studies have explored the value of DNA sequence data from various genes for resolving relationships among major avian lineages (Caspers et al. 1997; Harlid, Janke, and Arnason 1997, 1998; Mindell et al. 1997, 1999; Groth and Barrowclough 1999; Garcia-Moreno and Mindell 2000; van Tuinen, Sibley, and Hedges 2000). All of these should be considered pilot studies because they were based on limited taxic samples and/or genes for which it was unclear whether they would retain phylogenetic signal sufficient to resolve relationships of this antiquity. All of these studies have produced some resolution but have produced some unsatisfactory results as well. Phylogenies based on mtDNA and a small taxic sample root the avian tree in a way that is at odds with traditional concepts of avian classification (Mindell et al. 1997, 1999; van Tuinen, Sibley, and Hedges 2000; Johnson 2001). Increasing the size of the taxic sample appeared to "solve" the rooting problem in one study (van Tuinen, Sibley, and Hedges 2000) but not in another (Johnson 2001), and Braun and Kimball (2002) recovered the traditional root in reanalyses of the mtDNA data if the analysis was restricted to transversion parsimony or maximum likelihood based on complex models. Moreover, the studies of Mindell et al. (1999) and Johnson (2001) inferred other relationships at odds with more traditional classifications, which were strongly supported by morphological characters (e.g., the suboscine and oscine passerines not being sister taxa). The study of van Tuinen et al. (2000), based on the 12S and 16S rRNA and tRNA-Valine genes from the mt-genome and the nuclear 18S rRNA gene, found strong statistical support for five basal nodes (Aves, Paleognathae, Neognathae, Galloanserae, and Neoaves) but insignificant support for any lower level relationships. A phylogenetic analysis of exon sequence from the nuclear encoded RAG-1 gene provides strong statistical support for the traditional root, splitting the ancestor of modern birds into paleognath and neognath lineages, but provides low resolution among some major lineages (Groth and Barrowclough 1999).

Although, all DNA sequence-based studies to date have been based on very limited numbers of taxa, the lack of stable resolution may result from the rates at which the various genes used in the studies are evolving as much as, or more than, the limited taxic sample. Specifically, mtDNA may be evolving too fast and nuclear gene exons evolving too slow to be optimal for resolving relationships among avian orders. A reasonable hypothesis is that nuclear gene introns, which evolve more rapidly than exons but more slowly than mitochondrial-encoded genes may be evolving at rates suitable for resolving relationships among taxa of the antiquity of avian orders and families.

The high substitution rate in introns and the frequent occurrence of indels suggest that their nucleotide sequence

is adaptively neutral, although their existence must be perpetuated by natural selection because the intron/exon structure of genes is identical among distantly related taxa (Lewin 1997). β -*fibint* 7, for example, has been amplified by PCR from birds (Prychitko and Moore 1997, 2000; Johnson and Clayton 2000), reptiles (Giannasi, Malhotra, and Thorpe 2001), and mammals (C. Krajewski, personal communication) utilizing the same exon-based primers. Although the extent of functional sequences within introns is not known, it is known that introns contain conserved sequences for correct splicing (Keller and Noon 1985; Mount et al. 1992). Conserved sequences that function as alternative splice sites (Leicht et al. 1993), as regulatory elements (Gasch, Hinz, and Renkowitz-Pohl 1989; Schultz et al. 1991), and in maintaining secondary structure of transcripts (Kirby, Muse, and Stephan 1995) have also been identified in some introns.

In this study, β -*fibint* 7 was sequenced for 28 species of birds, representing 18 families in nine avian orders, for the following purposes: (1) to assess problems of sequence alignment among these relatively old taxa, (2) to determine if an intron is able to resolve relationships at these taxonomic levels, (3) to examine the conservation of nucleotide sequence and composition (GC content), and (4) to examine patterns of insertions and deletions (indels) over the course of evolutionary history represented by these species. Our taxon sample is very limited, but we include species that probably represent the full temporal extent of neornithine evolution (Mindell et al. 1999; van Tuinen, Sibley, and Hedges 2000). β -*fibint* 7 sequences have lower levels of homoplasy and experience fewer multiple substitutions than mitochondrial-encoded protein genes (Prychitko and Moore 1997, 2000); therefore, they may retain sufficient phylogenetic signal to resolve relationships among these most ancient of extant avian lineages.

Materials and Methods

DNA Preparation, PCR Amplification, and Direct Sequencing

Although the availability of specimens was limited, we assembled a series of birds that represent distinct genera within families, families within orders and different avian orders. Specimens are tabulated under *Supplementary Materials* (see below).

Methods of DNA isolation, PCR amplification, PCR-product cleaning, and DNA quantitation used in this study are those described by Prychitko and Moore (1997). We had access to different automated DNA sequencing instruments during the course of the study: ABI 377, ABI 3700 (Applied Biosystems), and Pharmacia ALF. For the ABI sequencers, reactions were done using the *Taq* Big Dye primer cycle sequencing kit (Applied Biosystems) according to the manufacturer's specifications. For the ALF sequencer, reactions were done using the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham) according to the protocols described by Prychitko and Moore (1997) and Prychitko, Ries, and Moore (1998).

Sequence Construction and Alignment

The entire β -*fibint 7* sequence for each specimen was reconstructed by linking together overlapping fragments generated using primers FIB-B17U, FIB-B17L, FIB-B17U2, FIBL210, FIBU330, and FIB-B17L2 (Prychitko and Moore 1997). These primer combinations result in approximately 70% overlap in sequence from the sense and nonsense strands. Two or more DNA isolations were made for each specimen, and in several cases, an isolation was made from a second specimen representing the same species. β -*fibint 7* was amplified and sequenced for these additional isolations and used to verify the sequences reported in this study.

Sequences were aligned with ClustalX (Thompson et al. 1997; Hall 2001). To explore the range of plausible alignments and the consistency of alignment procedures, we initiated several alignments (see *Results and Discussion*), following a different strategy in each. The alignment presented in the *Supplementary Materials* and used in further analyses was obtained as follows: Members of the order Piciformes were aligned first, beginning with the infraorder Picidae (northern flicker, bar-breasted piculet, Eurasian wryneck, and scaly-throated honeyguide), because we are relatively confident of their relationships and had previously aligned these sequences (Prychitko and Moore 2000). The black-spotted barbet and ivory-billed aracari (infraorder Ramphastides) sequences were aligned separately and then added to the overall Piciformes alignment as a profile (Thompson et al. 1997), because both sequences contain sets of three large, identical deletions totaling 303 nucleotides, and New World barbets and toucans (aracari) are thought to be sister taxa (Lanyon and Hall 1994). Individual sequences and profiles representing other avian orders were subsequently added to the alignment in random order. When an avian order was represented by more than one species, sequences were aligned within the order, before adding that order to the overall alignment. When a new avian order was added to the alignment, a "profile" alignment was performed whereby the sequences to be added created a profile that was aligned to the profile comprising the overall alignment.

Phylogenetic Analysis

Phylogenetic analyses were performed with PAUP* version 4.0b5 (Swofford 1998). Details of each analysis are given in the *Results* section.

Results

Insertions/Deletions and Alignment

There is considerable length variation among the β -*fibint 7* sequences examined. The average sequence length is 867 bp and varies from 1,012 bp in mourning dove to 619 bp in the black-spotted barbet. The aligned sequences consist of 1,155 sites. The sequences contain numerous indels, which involve both single and multiple nucleotides. Most indels appear to have resulted from single evolutionary events (i.e., insertion or deletion of a single nucleotide or string of nucleotides). As examples, a single

nucleotide insertion at site 839 is uniquely shared by the western bluebird and hermit thrush, the two representatives of the passerine family Muscicapidae, and the three representatives of the Picidae (Piciformes), the wryneck, piculet, and flicker, uniquely share a 14-bp deletion (sites 1071 to 1084). There are three exceptionally long deletions, two of which are shared by both the black-spotted barbet and the ivory-billed aracari (nucleotide sites 188 to 256 and 746 to 954) and one that is unique to the tawny frogmouth (nucleotide sites 179 to 363). Consequently, these three species have the shortest β -*fibint 7* sequences. The longest apparent insertion occurs in both the mourning dove and the rock dove and spans nucleotide sites 25 to 101. Some regions of the intron appear to have sustained two or more "overlapping" indel events as the lineages diverged; the region from sites 100 to 136 is an example. In most cases, however, these appeared to be reasonably interpreted as independent events. For example, the long deletion (sites 188 to 256) shared by the barbet and toucan "overlaps" apparent indels in other species, but it is reasonable to interpret these as independent characters. In these cases, however, the states of the shorter indels obscured by the longer indels could not be determined and were scored as unknown. In a few instances, there appeared to be complex indels in a region when the alignment as a whole was considered, but the region was identical for subsets of taxa. We considered these regions as single characters with multiple states (e.g., 0 to 5), where each state corresponds to a unique configuration for that region, as opposed to (0,1) for simple indels where the nucleotides are either present (1) or absent (0). The multiple-state indels are given in conjunction with the alignment in the *Supplementary Materials*.

Based on our alignment, we judge that there is a total of 125 indels in the data set.

Base Composition

β -*fibint 7* is AT-rich in woodpeckers (Prychitko and Moore 1997, 2000) and doves (Johnson and Clayton 2000). In this analysis involving avian species representing nine orders, the β -*fibint 7* sequences again consisted of a high percentage of A and T (see table 1). For the 28 species sequenced in this study, the β -*fibint 7* sequences on average consisted of almost 65% A and T, and there is little variance in the base composition among orders (see table 1).

Pattern of Nucleotide Substitution

Prychitko and Moore (2000) reported that homologous nucleotides that differ by transitions (ts) versus transversions (tv) in β -*fibint 7* of woodpeckers were in a ratio of approximately 1:1. In this analysis, ts/tv ratios for β -*fibint 7* were calculated between each pair of species and then plotted as a function of the corresponding genetic distance (uncorrected p-distance) between each species pair (fig. 1). The pairwise distances are not independent because of varying degrees of shared ancestry, so we have not attempted a statistical analysis. Nevertheless, the plot does suggest a decline in transitions relative to transversions as genetic distance increases (least-squares fitted

Table 1
Nucleotide Base Composition of β -Fibrinogen Intron 7 Among Avian Orders

Order	A	C	G	T
Piciformes	31.8 (± 1.38) ^a	17.1 (± 0.51)	18.5 (± 0.65)	32.6 (± 1.77)
Passeriformes	32.0 (± 0.84)	17.2 (± 0.25)	18.1 (± 0.48)	32.7 (± 0.70)
Strigiformes	31.5 (± 0.10)	16.6 (± 0.69)	18.5 (± 0.26)	33.5 (± 0.95)
Caprimulgiformes	31.5	17.6	17.6	33.2
Columbiformes	30.4 (± 0.42)	18.0 (± 0.14)	17.7 (± 1.06)	33.9 (± 0.57)
Gruiformes	30.1 (± 0.26)	17.5 (± 0.46)	18.9 (± 0.15)	33.5 (± 0.58)
Struthioniformes	30.4	15.8	18.8	35.1
Galliformes	32.1	16.8	18.2	33.0
Anseriformes	32.8 (± 0.10)	17.6 (± 0.06)	17.3 (± 0.42)	32.3 (± 0.23)

^a Mean percent (\pm SD).

line, slope = -0.69). The Y-intercept suggests a native ratio of 1.59 transitions to 1 transversion.

Conserved Sites

The aligned sequences contain 46 nucleotide sites (of 1,155) that are conserved across all 28 species. The positions of the conserved sites are mapped onto the intron in figure 2. It is apparent that the conserved sites are clustered as opposed to being randomly distributed. To test this hypothesis statistically against the alternative that they are randomly distributed, we did a χ^2 goodness-of-fit test comparing the distribution of conserved sites to a Poisson distribution (Prychitko and Moore 2000). The hypothesis of a random distribution is rejected (intron divided into 57 blocks of 20 nucleotides and one block of 15, X = number of conserved sites per block, $m = 0.793$, $P \ll 0.001$; 8 df). The variance of X (2.66) is greater than the mean (0.793), indicating that substitutions are clustered (Bliss and Fisher 1953).

It is difficult to delimit precisely the clusters without knowledge of the function of the conserved regions. However, figure 2 suggests that there are at least five clusters of conserved sites: one each at the 5' end and the 3' end of the intron and one in each of the regions between sites 139 and 176, 571 and 732, and 958 and 1060. Moreover, it is apparent that conserved sites are even more clustered within these broadly inclusive regions (e.g., nine

of 13 nucleotides in the span 654 to 666 are conserved across all species).

Phylogenetic Analysis

We performed a total of four phylogenetic analyses, three based on nucleotide substitutions only (maximum parsimony [MP], Neighbor-Joining [NJ] and maximum likelihood [ML]) and one based on nucleotide substitutions plus indels (MP).

All MP searches were TBR (tree bisection-reconnection) heuristic searches with 10 replicates of randomly added OTUs conducted with characters unordered and equally weighted. The nucleotide-only MP search found two equally parsimonious topologies (1,155 characters, length = 1,669 steps, CI [consistency index] excluding uninformative characters = 0.61); the two topologies differ with regard to where the ovenbird joins the tree. One of the two MP topologies is shown in figure 3.

Fifty-three of the 125 indels are phylogenetically informative (i.e., occurred in 2 to 26 of the 28 species). A matrix of the 125 indels was constructed and added to the nucleotide substitution matrix, giving a total of 1,280 characters for the nucleotide-plus-indel MP analysis. This analysis found six equally parsimonious trees (length = 1,821 steps, CI excluding uninformative characters = 0.63). Two of the six nucleotide-plus-indel topologies are identical to the two nucleotide-only MP topologies. The six topologies result from the two ovenbird topologies in combination with three variations on the position of the tawny frogmouth. The phylogenetically informative indels were mapped onto the phylogeny using a MP criterion. Thirty-five of these, which were unambiguous (neither homoplastic nor involving multiple, overlapping insertions or deletions), are indicated in figure 3 with their sizes along the internodes.

We used the Tajima-Nei distance estimator to compute the Neighbor-Joining tree because of the apparent ts/tv ratio close to 1 and a base composition that differs substantially from 25% representation by each nucleotide. Tajima-Nei is therefore appropriate because it is the simplest model that includes the essential nucleotide substitution properties (Kumar, Tamura, and Nei 1993). The NJ tree topology computed is identical to that of the MP tree illustrated in figure 3.

The computational demands of ML estimation precluded an exhaustive search based on this many taxa

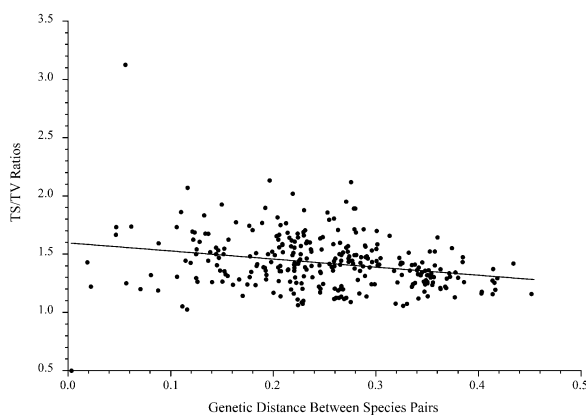


FIG. 1.—Transition to transversion substitution ratio of β -fibrinogen intron 7 as a function of genetic distance among avian species pairs. The line is a least-squares fitted regression line.

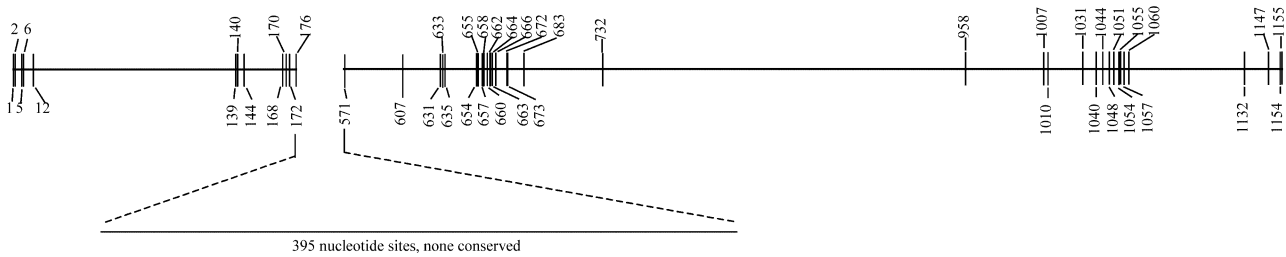


FIG. 2.—Conserved nucleotide sites in β -fibrinogen intron 7. Numbered tick marks represent conserved sites. Numbering is from the 5' end of the sense strand.

and characters. As an alternative, we did a partial search based on a stepwise strategy simplified from Frati et al. (1997). We estimated the model parameters for the GTR (general time reversible) model, gamma corrected (four rate categories) with a proportion of invariant sites by ML based on the topology of the NJ tree. This gives good approximations because the parameter estimates are not strongly dependent on the topology (Yang, Goldman, and Friday 1995). The estimated parameter values were then fixed, and the ML topology and branch lengths were estimated. We then specified this topology as a user tree to refine the ML estimates of the model parameters and branch lengths. The topology illustrated in figure 3 proved to be the ML tree, as estimated by this procedure, as well as the NJ tree and one of the MP trees.

One thousand bootstrap replicates were generated for the MP analysis with indels; these are presented in figure 3.

Discussion

Alignment Limitations

An objective in aligning DNA sequences is to identify the insertions and deletions that actually occurred as the sequences diverged from a common ancestor. This is relatively easy when closely related species are compared but becomes increasingly more difficult for more distantly related species.

Although programs such as ClustalX facilitate alignment of multiple sequences, there is an element of subjectivity in judging which alignment best reflects the evolutionary history of indels. The several alignment procedures we undertook differed in the order in which taxa were entered into the alignment, in gap-opening penalties (GOP) and gap-extension penalties (GEP), and in the extent to which profiles and the final alignment were fine tuned by ClustalX tools or manually. Nevertheless, all of the resultant alignments were very similar and produced only minor differences in subsequent phylogenetic analysis. With regard to aligning intron sequences such as β -*fibint* 7, with large as well as small indels, it was our experience that specifying high GOP (40+) and low GEP (0.05) were important to identify large indels but that subsequent fine tuning of small, restricted regions was facilitated by reducing the GOP (<10).

Alignment of multiple DNA sequences for subsequent phylogenetic analysis is fraught with a potential

circularity: Better alignment is achieved if sequences are entered in order of phylogenetic relatedness, but knowledge of relatedness is based on the alignment. To facilitate alignment, we first aligned sequences from species traditionally assigned to the same order (Piciformes, Strigiformes, etc. [see fig. 3]). However, we assumed no knowledge of relationships beyond this point, as sequences and profiles representing distinct orders were entered into the growing alignment in random order. The assignment of species in our study to orders is not contentious; therefore the tree we inferred (fig. 3) should not be an artifact of circular reasoning.

In sum, alignment of this intron among species representing distinct avian families and orders is time consuming, tedious, and uncertain in restricted regions for some species, but it is not an insurmountable or even difficult task. However, adding reptilian or mammalian sequences to the analysis manifested problems both with alignment and with detection of phylogenetic signal. We attempted to amplify and sequence β -*fibint* 7 from a crocodilian species (*Alligator mississippiensis*), intending to use it as an outgroup, but our amplifications were unsuccessful. Although we believe β -*fibint* 7 from the alligator could be amplified and sequenced, we abandoned the effort when we obtained snake (Giannasi, Malhotra, and Thorpe 2001) and mammal sequences (C. Krajewski, personal communication). We were unable to align the snake and mammal sequences with our avian sequences using ClustalX, but we were able to align them using the computer program T-Coffee (C. Notredame, <http://www.ch.embnet.org/software/TCoffee.html>). However, the reptile and mammal sequences were so disparate relative to the avian sequences that they did not provide a strong, statistically supported root for the phylogeny. This appears to establish a limit for the use of β -*fibint* 7 in antiquity. The lineages leading to mammals and snakes are thought to have diverged from that leading to birds at least 310 and 260 MYA, respectively (Benton 1990). On the other hand, β -*fibint* 7 is readily aligned among the modern avian orders represented in this study, which are thought to have diverged from 55 to more than 90 MYA, and it retains significant information on relationships among the orders and families, as well as the lower taxa. Thus, it appears that β -*fibint* 7 would certainly be useful in phylogenetic studies of groups as old as 55 Myr, probably as old as 90 Myr, but not as old as 260 Myr. At the other extreme, β -*fibint* 7 has the potential to resolve relations among species of birds that diverged as recently as 2 to 5 MYA

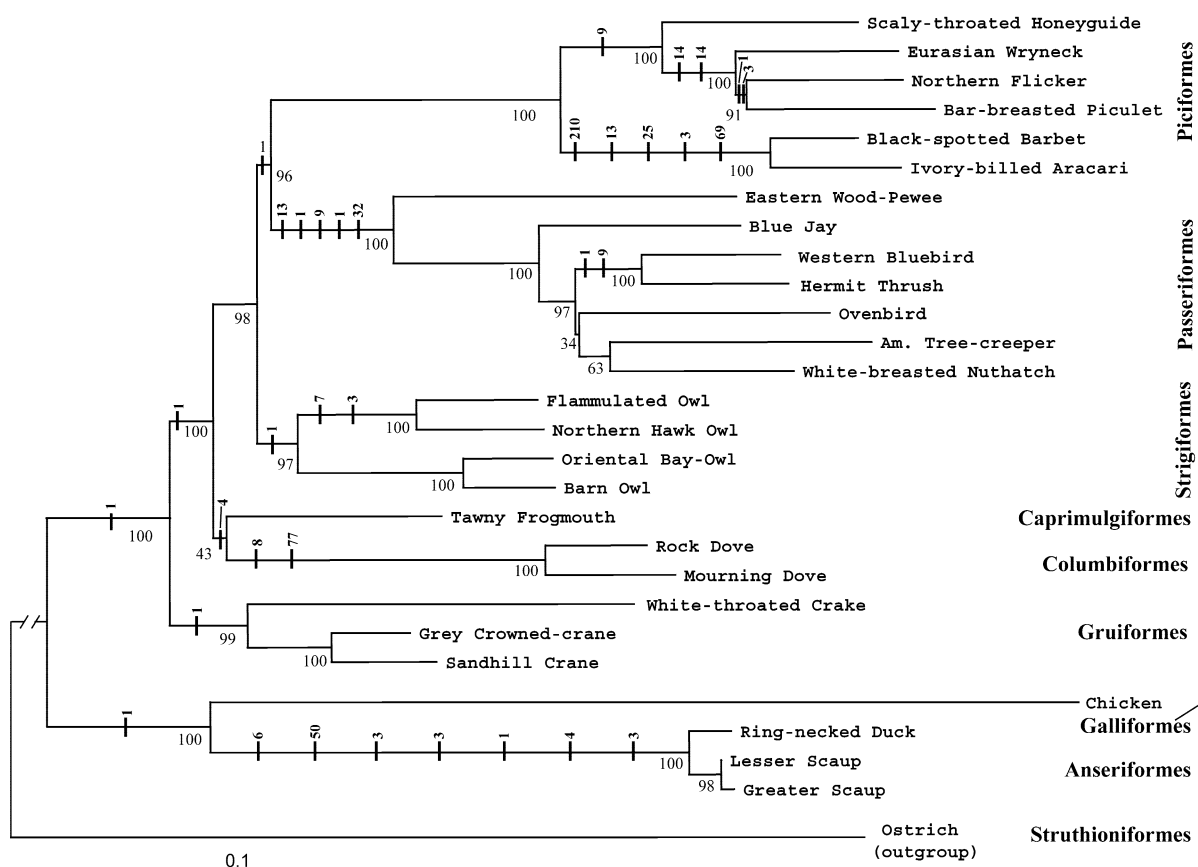


Fig. 3.—Phylogeny of avian orders based on β -fibrinogen intron 7. This topology was obtained for the maximum-likelihood tree, for the Neighbor-Joining tree, and for one maximum-parsimony tree in each of the two maximum-parsimony analyses (with and without indels). Groupings of species into orders are indicated on the right. Horizontal numbers on internodes are bootstrap proportions based on 1,000 replicates of the maximum-parsimony analysis, including indels. The vertical ticks on internodes indicate when synapomorphic indels occurred as inferred by maximum parsimony. The vertical numbers above the ticks indicate the inferred size of the indel. Branch lengths are maximum-likelihood estimates based on the GTR + G + I model. The tree is rooted with the ostrich specified as the outgroup. Species common names from Sibley and Monroe (1990).

(Prychitko and Moore 2000) and perhaps less, although mitochondrial-encoded genes appear more useful for these very recent divergences (Moore, Smith, and Prychitko 1999; Weibel and Moore 2002). The estimate of this window of resolution is only approximate because resolution of specific relationships depends on the length and depth of the internode (Lanyon 1988; Moore and DeFilippis 1997).

Nucleotide Base Composition

It was shown previously that base composition of β -*fibint* 7 varied little among woodpeckers (Prychitko and Moore 2000) or among doves (Johnson and Clayton 2000). The present analysis includes representatives from a broad range of avian orders, and base composition remains remarkably constant across lineages. Constancy of base composition is important to phylogenetic inference because variation can result in clustering of unrelated taxa with similar base composition into artificial groups (Lockhart et al. 1994). The genome structure of warm-blooded animals is characterized by isochores, homogeneous regions of DNA, 100 to 300 kb or longer, that are either AT rich or GC rich (Bernardi 1995). Whether the

homogenous base composition of isochores is maintained by selection or a biased mutational mechanism is unclear (Li 1997), but presumably β -fibrinogen is embedded in an AT-rich isochore; the uniform AT richness of β -*fibint* 7 over the diversity of modern birds suggests such a condition.

Conserved Sequences Within Introns

It is clear from figure 2 that comparison of aligned sequences from distantly related species is a powerful tool for identifying conserved, and presumably functional, regions within genomes. In our earlier analyses (Prychitko and Moore 1997, 2000), we failed to detect these regions because the woodpeckers are closely related, and too few substitutions have occurred within the family (Picidae) to reveal variation in substitution rate along the length of the intron.

Since the discovery of introns in the 1970s, their nucleotide sequences were thought not to specify function and hence not to be constrained by selection (reviewed by Rodriguez-Trelles, Tarrío, and Ayala 2000; Mattick and Gagen 2001). However, there are now reports of conserved regions within introns where it was inferred that

these regions function as alternative splice sites (Leicht et al. 1993, 1995; Croft et al. 2000), as regulatory elements (Gasch, Hinz, and Renkowitz-Pohl 1989; Schultz et al. 1991; Alder et al. 1992; Jackson and Hoffmann 1994; Metz et al. 1998), and as stem regions maintaining secondary structure of transcripts (Kirby, Muse, and Stephan 1995). (Several additional examples of introns affecting gene expression are listed on the ISIS Web site <http://isis.bit.uq.au/papers_function.html>.) Spliceosomal introns characteristically begin with the dinucleotide GT and end with AG (Keller and Noon 1985; Mount et al. 1992). As expected, these four sites are conserved across all avian orders (fig. 2). The three additional conserved sites at the 5' end of the intron (sites 5, 6, and 12) and two at the 3' end (1132 and 1147 [fig. 2]) plausibly function in splicing also. Introns characteristically have a short, conserved region (usually less than 10 nucleotides in length) towards the 3' end, the branch point, which includes the bonding site for the G at the 5' end as it loops back on the intron to form the "lariat" during excision (Mount et al. 1992; Lewin 1997). Typically, there is also a conserved pyrimidine-rich region located between the 3' splice site and the branch point, which in mammalian species has been shown to play a role in branch point recognition during splicing (Ruskin et al. 1984; Csank, Taylor, and Martindale 1990; Mount et al. 1992). Although the conserved sites near the 3' end of β -*fibint 7* (fig. 2) do not precisely satisfy these criteria, they are plausibly associated with the branch point.

At least two conspicuous clusters of conserved nucleotides remain beyond the minimal set of functionally conserved nucleotides usually associated with excision: the sequences between sites 139 and 176 and between sites 571 and 683 (fig. 2). Although these sequences are almost certainly constrained by selection, their function is unknown.

Mattick and Gagen (2001) argued that intron transcripts may play an important, but largely overlooked, role in modulating the expression of genes during development. The existence of constrained sequences within β -*fibint 7*, which seem to be in excess of those required for excision, is consistent with this hypothesis to the extent of establishing that there are conserved regions and at the same time could explain why intron-embedded regulatory elements have not been detected often in the past. The proportion of conserved sites in a large intron is small (46 of 1,155 sites in β -*fibint 7*), and hence introns as a whole evolve at rates approaching those of pseudogenes and synonymous sites of exons (Hughes and Yeager 1997; Li 1997). Moreover, these short, conserved sequences would only be detected if introns were aligned among appropriately distantly related species. This has been done rarely to date.

Avian Phylogeny

Because the mammal and reptile β -*fibint 7* sequences were too distant from the avian sequences to provide a satisfactory outgroup root, we rooted our tree by designating the ostrich as the outgroup. The basal lineage of the avian phylogeny remains somewhat controversial

(Mindell et al. 1997, 1999; Garcia-Moreno and Mindell 2000), but we think the preponderance of evidence, especially recent studies based on more slowly evolving nuclear gene sequences, favors the classical basal division of Aves into Paleognathae and Neognathae (Pycraft 1900; Cracraft 1988; Cracraft and Mindell 1989; reviewed by van Tuinen, Sibley, and Hedges 2000). Recent nuclear DNA sequence studies favoring the classical division include Groth and Barrowclough (1999), van Tuinen, Sibley, and Hedges (2000), and Garcia-Moreno and Mindell (2000) (see Braun and Kimball 2002 for a recent review). Some mtDNA studies based on additional sequences and more complex analyses also favor the classical division (Braun and Kimball 2002; Paton, Haddrath, and Baker 2002), as does a novel approach based on nuclear DNA "strings" (Edwards et al. 2002). This is our justification for outgroup rooting with the ostrich designated as the outgroup, but we acknowledge an element of uncertainty regarding the basal divergences depicted in the β -*fibint 7* phylogeny (fig. 3).

Although our study is intended as a pilot study to determine whether β -*fibint 7* has retained phylogenetic signal from the depth in antiquity of avian orders, the limited taxon sample does provide some useful insight into early avian evolution and classification. First, all of the orders represented by two or more species appear monophyletic in the ML tree (Piciformes, Passeriformes, Strigiformes, Columbiformes, Gruiformes, and Anseriformes) (fig. 3). Moreover, the relationships among the avian orders are strongly supported by bootstrap proportions with the exception of the Caprimulgiformes-Columbiformes node. Some of these relationships differ from those inferred by Sibley and Ahlquist (1990) based on DNA-DNA hybridization. For example, the β -*fibint 7* tree has the Piciformes and Passeriformes as sister orders, whereas the DNA-DNA hybridization tree infers Piciformes to be more basal. The relatively close relationship of Piciformes and Passeriformes and the derived position of the clade is consistent with more traditional classifications (e.g., Wetmore 1960; Cracraft 1981; reviewed by Sibley and Ahlquist 1990).

Within orders, relationships among subtaxa are generally strongly supported and consistent with traditional classifications. Relationships within the Piciformes corroborate current hypotheses (Short 1982; Lanyon and Hall 1994). Similarly, the relationships among passeriform taxa correspond exactly to those inferred by Sibley and Ahlquist (1990), when their tree is pruned to the limited number of species included in our study. Of particular interest here is that several studies support the traditional division of Passeriformes into suboscines (wood pewee) and oscines (other passerines) (Ames 1971 [syrinx]; Edwards, Arctander, and Wilson 1991 [mt *cyt b*]; Mindell et al. 1997 [mt *rRNA*]; Garcia-Moreno and Mindell 2000 [CHD]; Lovette and Bermingham 2000 [nuclear *c mos*]; Irestedt et al. 2001 [*c-myc* and *RAG-1*]), but some mtDNA-based phylogenies suggest that these traditional groups are not sister taxa (Mindell et al. 1999; Johnson 2001). The β -*fibint 7* data strongly support this traditional perspective of a sister-group relationship of suboscines and oscines (fig. 3). The subsequent split of the oscines into

Corvida (blue jay) and Passerida (other oscines) is strongly supported (fig. 3), consistent with several other studies (Bock 1962 [osteology]; Sheldon and Gill 1996 [DNA hybridization]; Mindell et al. 1997 [mt *rRNA*]; Pasquet et al. 1999 [mt *rRNA*]; Ericson, Johansson, and Parsons 2000 [*c-myc* indels]; Garcia-Moreno and Mindell 2000 [*CHD*]). Two recent studies (Barker, Barrowclough, and Groth 2002; Ericson et al. 2002) indicate that the suborder Tyranni (suboscines) is paraphyletic, as is the parvorder Corvida, but, again, when the trees in those studies are pruned to the taxa in the β -*fibint* 7 tree, the topologies are congruent (assuming the wood pewee represents the Tyrannida and the blue jay represents the “core Corvida” of Barker, Barrowclough, and Groth 2002 and the suboscines and the “Corvida,” respectively, of Ericson et al. 2002). The four species of owls divide into two strongly supported clades, corresponding to the widely accepted bifurcation of the owls into two families, Tytonidae (bay owl, barn owl) and Strigidae (flamulated owl, hawk owl). Our sample is deficient in species that would have allowed us to assess the uncertain relationships between owls and frogmouths and their allies (Caprimulgiformes [Sibley and Ahlquist 1990; Bleiweiss, Kirsh, and Lapointe 1994]). In our tree, the frogmouth is united with the Columbiformes as opposed to the Strigiformes (Sibley and Ahlquist 1990), but the bootstrap support is weak (43%), and the MP analysis with indels found six equally most-parsimonious trees distinguished by three placements of the frogmouth in combination with two placements of the ovenbird. Thus, the relationship of the frogmouth and the Caprimulgiformes, to which it is perhaps incorrectly assigned, remains uncertain other than to say this lineage diverged early in the radiation of the modern birds. The relationship depicted in figure 2 is supported, however, by an inferred 4-bp deletion (positions 1096 to 1099) shared by the two doves and the frogmouth. The β -*fibint* 7 data strongly support the existence of a sister-group relationship between gallinaeous birds and waterfowl, the Galloanserae (Sibley and Ahlquist 1990; Caspers et al. 1997; Groth and Barrowclough 1999; Mindell et al. 1999; Garcia-Moreno and Mindell 2000; van Tuinen et al. 2000).

Finally, the level of support for ordinal and familial relationships among birds provided by the β -*fibint* 7 data set is as strong or stronger than that in other studies reported to date. The number of taxa included in all these studies is small and a limited number of taxa are common to all of the studies; thus, conclusions based on comparisons among the studies are tentative. It is important to note, however, that the substitution rate among diverging β -*fibint* 7 lineages is greater than that reported for other nuclear genes. Relative to β -*fibint* 7 set at a rate of 1, *c-mos*, *RAG-1*, and *c-myc* evolve at 0.36, 0.30, and 0.25, respectively (based on comparison of genetic distances among taxa held in common among the studies: *c-mos*, [Lovette and Bermingham 2000], *RAG-1* [Groth and Barrowclough 1999], *c-myc* [Irestedt et al. 2001]). The rate of β -*fibint* 7 substitution appears well suited for resolving relationships among lineages that diverged 55 to 90 Myr B.P. and perhaps longer, the timeframe of the Cretaceous-Tertiary boundary, which is so impor-

tant in vertebrate evolution. Moreover, the indels, themselves, harbor significant phylogenetic information. This is apparent in the indel characters displayed on the internodes in figure 3, the increased bootstrap support when the indel matrix is included in the MP analysis and the fact that an analysis based on the indels alone yields a reasonable tree with several significantly supported nodes. The phylogenetic distribution of indels raises a number of intriguing questions. For example, there is a preponderance of deletions over insertions, which suggests the intron is, on average, becoming shorter through evolution, and the indels appear to have been frequent along some lineages and infrequent or absent along others.

Supplementary Materials

Specimens, along with voucher information, are tabulated as supplementary materials. Newly derived sequences are added to GenBank (accession numbers AY082398 to AY082425). The aligned sequences are available as both a Nexus and text file as supplementary materials. Numerical references to nucleotide sites are to that alignment. Supplementary materials are available online at the journal's Web site.

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