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Evolution

Rates of nuclear DNA evolution in pheasant-like birds: Evidence from restriction maps

(lysozyme gene/globin genes/evolutionary tree/fossil record/molecular clock)

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ABSTRACT **To examine the tempo of genomic evolution in birds, we mapped 161 restriction sites in the nuclear DNA of seven species of birds belonging to the pheasant superfamily Phasianoidea. The three regions mapped lie on different chromosomes and bear eight genes, coding for lysozyme *c*, three "α-like" globins, and four "β-like" globins. Together, the three regions span about 56 kilobases, most of which is presumably noncoding. The maps differed from one another at a minimum of 77 sites and by 9 length mutations. The extent of sequence divergence due to base substitutions was inferred to be similar for all three regions, even though the three coding regions differ by 5-fold from one another in mean rate of evolution at the amino acid level. A tree relating the maps differs in branching order from that implied by the traditional classification of phasianoid birds and is supported by published protein comparisons. Five of the nodes in the tree were associated with fossil evidence and historical biogeographic information, allowing us to estimate the mean rate of DNA divergence to be 0.34–0.40% per million years. This rate is similar to that estimated for the globin gene regions of higher primates, which validates the concept of an evolutionary clock at the DNA level. Our fossil-based calibration of DNA evolution differs by a factor of almost 2 from that proposed by others on the basis of biogeography. In consequence, published estimates of divergence times for birds and primates that are based on a biogeographically calibrated DNA clock may be too long.**

One of the most significant and controversial concepts to emerge from the comparative study of macromolecules is that mutations accumulate on surviving molecular lineages at rather steady rates (reviewed in ref. 1). This paper describes restriction mapping of three regions in the nuclear genome to examine the uniformity of the rate of DNA evolution in birds and to compare this rate with that in primates.

Members of the pheasant superfamily (Phasianoidea) offered several advantages for our work. First, this group of species includes the domestic chicken, whose DNA has been investigated more thoroughly than that of any other bird. Three regions of the chicken's nuclear genome have been especially well defined: those containing the structural genes for lysozyme *c* (2), for three "α-like" globins (3), and for four "β-like" globins (4). Because each is on a different chromosome (5), we can investigate the uniformity of the rate of DNA evolution across the genome by comparing the three chicken regions to their counterparts in other phasianoid species. Another advantage of working with these birds is that they have been compared before at the protein level (6), and this has given information about their phylogenetic relationships. The DNA comparisons now reported contribute further to a knowledge of phylogenetic relationships among phasianoid birds. Finally, the phasianoid birds whose DNAs we have compared possess a fossil record and are incapable of sustained flight. These two facts make these birds a good group with which to begin calibrating DNA evolution and erecting a tentative molecular time scale for bird evolution, with the aid of both fossil and biogeographic evidence. We can then ask how similar the mean rate of evolutionary divergence estimated for these birds is to that in primates, because the α- and β-globin regions have already been compared among species in the latter group (7, 8).

The primate globin studies have made possible the calibration of comparative restriction mapping as a method for estimating DNA divergence. Sequencing of >16 kilobases (kb) of DNA throughout the α- and β-globin regions has yielded a mean value of 1.6% sequence difference (due to base substitutions) between the noncoding regions of human, chimpanzee, and gorilla DNA (9, 10, and refs. therein). Since this agrees well with the value of 1.4% estimated from comparative restriction mapping (8), we feel justified in using this method to examine genomic evolution in birds.

MATERIALS AND METHODS

DNA Sources. High molecular weight DNA was isolated from erythrocytes, heart, and soft tissues of three White Leghorn chickens (*Gallus gallus*), five ring-necked pheasants (*Phasianus colchicus*), and one specimen each of green jungle fowl (*G. varius*), peafowl (*Pavo cristatus*), chukar partridge (*Alectoris graeca*), turkey (*Meleagris gallopavo*), and guinea fowl (*Numida meleagris*). The methods used were modifications (11) of those described by Kan and Dozy (12) and Robbins *et al.* (13).

cDNA Probes for Analysis of Restriction Endonuclease Digests. The enzyme *Pvu* II released a 3.0-kb fragment bearing chicken lysozyme cDNA from the plasmid pLys6 (14); this fragment was used as a probe for most of the lysozyme experiments. Plasmid pCGα-3 (15), containing a cDNA copy of the chicken α^A-globin mRNA, was digested with *Hin*dIII to excise the cDNA insert specifically; this fragment was used as probe in most of the α-globin experiments. Probes specific for the 5′ and 3′ ends of the lysozyme and α-globin genes were made for double-digest experiments by cutting the cDNA fragments at unique *Sac* I and *Hin*cII sites, respectively. Plasmid pCGβ-3 (16) is the β-globin analog of pCGα-3. The *Hin*dIII fragment containing the adult β-globin-gene cDNA was used as probe in all the β-globin experiments.

Southern Hybridizations. DNAs were digested with 10 restriction endonucleases (Fig. 1) under conditions specified by the supplier (New England Biolabs); electrophoresed in 0.8%, 1.0%, 1.2%, or 2.0% agarose gels; and transferred to nitrocellulose paper (7). The size standards included in the gels were bacteriophage λ and PM2 DNAs cut with *Hin*dIII, and λ DNA cut with *Eco*RI or *Sma* I. Generally, the

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Abbreviations: kb, kilobase(s); bp, base pair(s); Myr, million year(s).

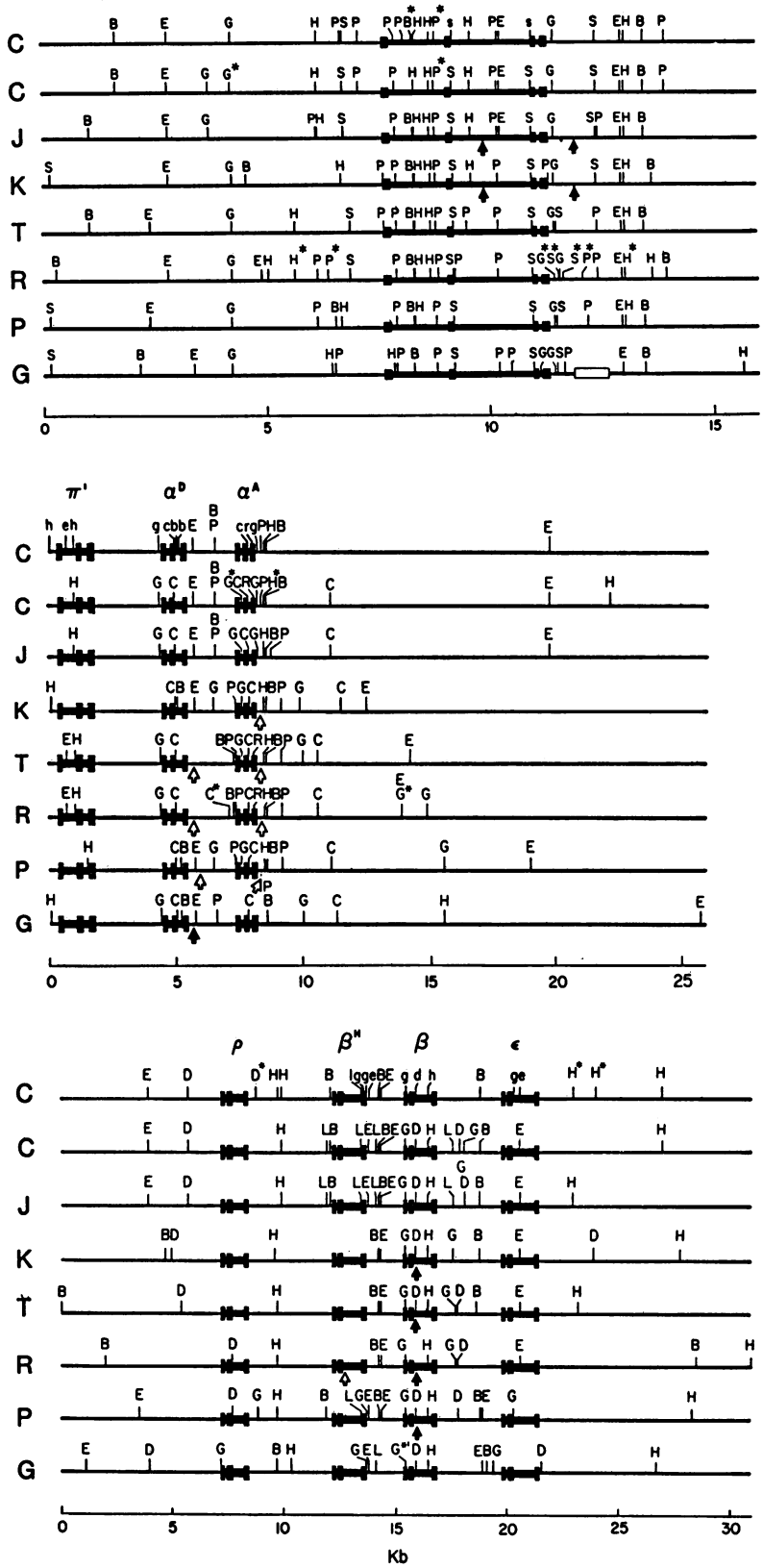

FIG. 1. Restriction maps for the lysozyme (*Top*), α-globin (*Middle*) and β-globin (*Bottom*) gene regions of phasianoid birds. The species are chicken (C), green jungle fowl (J), chukar partridge (K), turkey (T), ring-necked pheasant (R), peafowl (P), and guinea fowl (G). The thickest black bars represent gene sequences complementary to those found in the mature mRNAs; the bars of medium width are introns; the scales are in kb. The code for restriction enzyme sites is: B, *Bam*HI; C, *Hin*cII; D, *Bgl* II; E, *Eco*RI; G, *Bgl* I; H, *Hin*dIII; L, *Bcl* I; P, *Pst* I; R, *Rsa* I; S, *Sac* I. The top chicken maps in each set were compiled from the literature (11); sites labeled with lowercase letters were established by DNA sequencing and those marked with uppercase letters were placed by restriction mapping. Asterisks indicate sites that are polymorphic within the species. The *Bgl* I and *Eco*RI sites around the β^H gene could not be mapped in all of the birds, because they could not be localized by double digests. *Rsa* I (α-globin maps) and *Bcl* I (β-globin maps) were used with only some DNAs, to localize certain length mutations and restriction fragments. Solid and open arrows indicate locations of small [≤100-base-pair (bp)] DNA additions and deletions, respectively. The open bar 3′ of the guinea fowl lysozyme gene represents a 750-bp deletion. In most cases, the presence and location of length mutations were indicated by the results of digestions with two or more enzymes or enzyme pairs. Each length mutation allows alignment of a minimum of two additional sites with at least one other species.

procedure used for hybridizing the bird DNAs to our cDNA probes was that of Wahl *et al.* (17). The β-globin hybridizations were done more stringently, to eliminate reactions between the embryonic ρ- and ε-globin genes (4) and the probe (adult β-globin cDNA): filters were hybridized at 56°C, and the low-ionic-strength washes were done at 64°C. Occasionally, the β-globin filters were rehybridized at 49°C or 42°C to check on questionable bands.

Restriction Mapping. Species comparisons were first made by cutting DNAs with single enzymes and electrophoresing the digests side-by-side in the same gel. With the published chicken maps as guides, selected double digests were then done. Each species had its own gels, with the lanes containing DNA digested with two enzymes flanked by lanes containing DNA digested with the appropriate single enzymes. The initial maps were drawn independently for every species and then aligned with one another so as to maximize conservation of cleavage sites. Finally, double-digest fragments from

different species which were predicted to be the same were checked side-by-side in additional gels.

Calculations. The percentage divergence in base sequence (δ) among the DNAs was estimated from comparison of cleavage maps with equation 16 of Nei and Li (18). In making these comparisons, it was not possible to include every mapped site, because genomic restriction mapping with a cDNA probe does not allow all sites to be detected in all species. Consider, for example, the jungle fowl (J) and chukar (K) maps for the α-globin-gene region (Fig. 1). The *Eco*RI fragments containing the α^A genes of the two species share the same 5′ site (5.6*E*) but have different 3′ sites. One knows that the jungle fowl DNA lacks site 12.4*E* found in the chukar, because the fragment detected was 14.0 rather than 6.8 kb long. However, one cannot determine whether site 19.6*E* is present in the chukar DNA. The DNA fragment bounded by sites 12.4*E* and 19.6*E* would not contain any regions homologous to the cDNA copy of the α^A gene used as probe (see Fig. 1) and so would be undetectable. Therefore, site 19.6*E* is excluded when comparing the maps from these two species, though it is included when, for example, comparing the maps for chicken and jungle fowl.

RESULTS

Cleavage Maps. The cleavage maps (Fig. 1) for the three gene regions of seven phasianoid species represent mainly noncoding DNA and contain a total of 161 different sites and an average of 52 sites per species. Eleven of these sites occur in every species; included among them are all 4 of the exon sites examined in every species. Evidence for the general accuracy of the maps is provided by comparing them with the chicken reference maps (the top maps in each set in Fig. 1). Whenever a species has a site that maps to a region for which a chicken sequence is available, an appropriate site or semisite, differing by only 1 base, occurs in the chicken sequence. For example, the turkey and pheasant have a B site (GGATCC) at 7.3 kb in the map of the α-globin region (Fig. 1), which corresponds to the sequence GGGTCC 80 bp upstream from exon 1 of the chicken α^A gene.

Nineteen sites (indicated by asterisks in Fig. 1), were inferred to be polymorphic; i.e., present in some individuals and absent in others of the same species. This was most evident from comparisons of five pheasants and up to eight chickens (only three of which were tested in our laboratory for every site) as described in detail in ref. 11.

Comparison of Maps. Although the maps in Fig. 1 differ mainly in ways explainable by point mutations causing loss or gain of restriction sites, at least 9 DNA rearrangements are evident (4 additions and 5 deletions). The DNA segments involved range from 50 to 750 bp in length. For each pair of species, we calculated *s*, the fraction of sites shared between the maps, as shown in Fig. 2. When the three DNA regions were combined, the mean value of *s* varied from 0.90 for the chicken/jungle fowl comparison to 0.35 for the pheasant/guinea fowl comparison. When we treated the three regions separately, it was found in most of the species comparisons that *s* is nearly the same for each region (Fig. 2). The mean *s* value ranges from 10/16 (0.62) for the lysozyme region, to 6/10 (0.60) for the α-globin region, to 5/9 (0.56) for the β-globin region. Because *s* is a function of the sequence homology between two DNAs, this result indicates that the rates of sequence evolution do not differ much among the three regions.

The slightly lower β-globin value may be attributable to a higher average rate of evolutionary change in this region, or to the large uncertainty associated with β-globin comparisons involving the guinea fowl. Since these *s* values are based on only 1–3 shared sites and 7–8 compared sites, there is a large stochastic error associated with these comparisons of β-globin regions.* Further, several potentially conserved sites around the β^H gene (13.7*G* and 13.8*E*, Fig. 1) could not be mapped in every species and were thus excluded from the calculations. Including these sites in the comparisons of the guinea fowl with peafowl, chicken, and jungle fowl raises the *s* values for the β-globin region (see open bars in Fig. 2), bringing them into closer agreement with those for the lysozyme and α-globin regions (Fig. 2).

FIG. 2. Fraction of sites shared (*s*) for three regions of the genome and 21 pairs of species. Each set of solid bars indicates the fraction of sites shared by the lysozyme (left), α-globin (middle) and β-globin (right) maps of a given species pair. The three open bars (for P/G, C/G, and J/G comparisons) are based on additional sites mapped in the β-globin region (see text). The single-letter code for the species names is in the legend for Fig. 1.

Evolutionary Tree. From the fraction of shared sites, one can estimate approximate extents of sequence divergence, as shown in Table 1 for all possible pairs of the seven species. Such a matrix of sequence divergence values allows trees to be constructed (20) that reflect the extent of DNA relatedness among the seven species. The tree of best fit (tree I, Fig. 3) associates the chicken and jungle fowl most closely, then the chukar, then the turkey/pheasant group, followed by the peafowl and guinea fowl.

Tree I is also the most parsimonious; it accounts for the evolution of the seven groups of maps from a common ancestor with fewer mutations—32—than do trees with other branching orders. This parsimony analysis (23) is based on 23 phylogenetically informative positions. These are sites whose presence or absence can be examined in all seven species, and that are shared by two to five of the species. The informative positions in the lysozyme, α-globin and β-globin maps, respectively, are 6.7*H*, 6.9*S*, 7.7*P*, 8.8*H*, 9.7*H*, 10.4*E*, 11.5*S*, and 13.6*B*; 5.6*E*, 6.4*G*, 7.3*B*, 7.4*P*, 7.6*G*, 8.2*G*, 10.5*C*, 5.5*R*, and 8.3*R*; and 7.7*D*, 10.0*H*, 12.1*B*, 17.7*D*, 18.9*E*, and 23.1*H*. Included among these are two positions of DNA rearrangement (*R*). Tree II (Fig. 3), which has the branching order implied by the traditional taxonomic classification (21, 22), requires nine more mutations and thus is much less parsimonious than tree I.

DISCUSSION

Significance of Branching Order. The evolutionary tree inferred from the maps (Fig. 3) receives support from protein comparisons. Chukar lysozyme, for instance, is much more similar in amino acid sequence to that of chicken than is pheasant or turkey lysozyme (24). Protein evidence also suggests that the turkey is no more different from chicken than is ring-necked pheasant (ref. 6 and refs. therein), and quantitative morphological comparisons have yielded a result consistent with the molecular view (25).

*The guinea fowl results indicate that estimates of sequence divergence based on map comparisons become unreliable when the fraction of shared sites falls below 0.5.

Table 1. Extent of sequence divergence (δ) for all possible pairs of seven species*

	C	J	K	T	R	P	G
C	0.8	1.3	6.4	7.9	10.0	8.8	13.4
J	37/41	—	6.2	6.5	9.6	7.8	13.4
K	23/33	27/38	—	6.7	9.4	6.0	13.0
T	21/33	27/38	26/38	—	4.2	6.4	12.1
R	16/29	19/32	19/32	28/36	1.6	8.8	22.1
P	19/32	24/36	27/38	26/37	19/31	—	10.5
G	15/31	18/35	18/34	18/35	10/28	20/34	0.6

In the lower-left half of the matrix, the number before the slash is the number of sites shared for the lysozyme, α-globin, and β-globin gene regions, combined; the number after the slash is the total average number of sites compared for each pair of species. These numbers have been rounded off to whole numbers, and the values of δ calculated from them will in some cases differ from those listed here. In comparing any two maps, all sites whose presence or absence could be scored in both maps were usually included, even if a given site was unscorable by the cDNA method in another map. The exceptions were the α-globin *Rsa* I sites, the β-globin *Bcl* I sites, and the *Bgl* I and *Eco*RI sites around the β^H gene in the peafowl and guinea fowl maps; these sites were excluded from the calculations because the corresponding fragments and/or sites were not mapped in the other species. Only those sites in the chicken reference maps which could be mapped by Southern analysis with a cDNA probe were used in the comparisons. In the upper-right half of the matrix are the values of δ (substitutions per 100 bp) based on the maps. Where possible, these values of interspecific divergence have been corrected for intraspecific divergence (19). The numbers along the diagonal are mean amounts of intraspecific divergence. For the three individual DNA regions, the mean pairwise divergences within species were as follows. Chickens: lysozyme, 0.8%; α-globin, 0.9%; β-globin, 0.6%. Pheasants: lysozyme, 2.1%; α-globin, 1.0%. Guinea fowl: β-globin alleles, 0.6%.

*Abbreviations for species: C, chicken; J, green jungle fowl; K, chukar partridge; T, turkey; R, ring-necked pheasant; P, peafowl; G, guinea fowl.

Constancy of Sequence Change in Noncoding DNA. Since the fractions of sites shared in each of three gene regions for each species pair are similar (Fig. 2), we infer that much of the phasianoid genome is evolving at a uniform rate. This is in spite of the fact that the genes in these regions differ greatly from one another in the number and lengths of introns, as well as in total length, conservation of exons, regulation, and chromosomal location in chicken. Further evidence that the three regions have similar patterns of evolution is that tree I was suggested by every region. Because the cleavage sites of any given enzyme are usually distributed nonrandomly and restriction sites differ from one another in the probability of undergoing evolutionary change, concern has been raised about the validity of trees based on restriction maps (26). Our results showing the similarity in rates of evolutionary change in three diverse gene regions and in the trees produced from them inspire confidence in the use of nuclear restriction maps for evolutionary analysis.

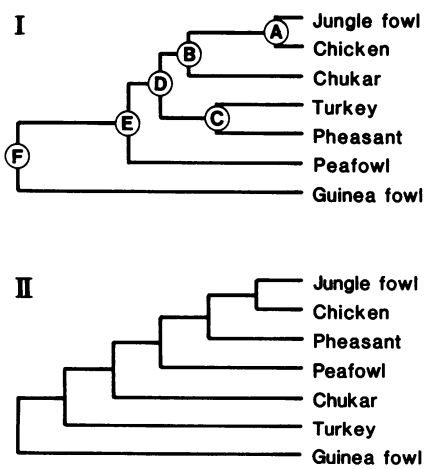

FIG. 3. Alternative trees for seven species of phasianoid birds. Tree I depicts the most parsimonious branching order from the nuclear gene maps (Fig. 1). Tree II depicts the branching order implied by the traditional classification (21, 22), according to which the superfamily Phasianoidea is divided into several families, including Numididae (guinea fowl), Meleagrididae (turkey), and Phasianidae. The family Phasianidae is further divided into two subfamilies, one of which, the Phasianinae, is further divided into the tribes Perdicini (chukar) and Phasianini. Within the latter tribe, Delacour (22) puts the chicken and jungle fowl taxonomically closer to the ring-necked pheasant than to the peafowl.

Calibrating the Rate of DNA Evolution. In theory, the absolute rate of molecular evolution can be estimated from a knowledge of the degree of molecular divergence between species and independent evidence about the time that has elapsed since the species compared had a common ancestor. In practice, one is limited by the quantity and quality of the fossil or biogeographic evidence relating to divergence times. To make the estimate as accurate as possible, it is important to use as many independent bits of information as are available (27). Table 2 and its first footnote show how the fragmentary nonmolecular data concerning divergence times can be tentatively associated with our estimates of molecular divergence and thus with five of the six nodes (A–F) in tree I (Fig. 3).

Table 2 lists the unbounded estimates for the rate of DNA evolution that are obtained by associating the divergence times with the percent sequence divergence for each node, as described by Beverley and Wilson (27). These estimates are all consistent with the mean rate of DNA divergence being >0.34%/Myr and <0.40%/Myr. Taking the midpoint of this range, 0.37%/Myr, as the mean rate of noncoding DNA evolution, we make the following provisional estimates in Myr for the nodes in Fig. 2: F, 38; E, 20; D, 19 (based only on turkey comparisons); C, 11; B, 17; A, 4.

Our estimate of the phasianoid rate is similar to the value of 0.4%/Myr estimated from restriction mapping comparisons of primate globin gene regions (8), which gives support to the concept that noncoding regions evolve as molecular clocks. It is almost twice the rate (0.22%/Myr) that has been assumed by Sibley and Ahlquist (34, 35) to apply to birds in general and to primates. Three independent bits of information imply that the phasianoid rate is >0.24%/Myr (Table 2).

A possible reason for the discrepancy is the assumptions made by Sibley and Ahlquist about times of divergence among birds (34). Their calibration method ignores fossil evidence and relies instead on historical biogeographic information, which is hazardous for birds capable of sustained flight. Consider, for example, two groups of birds, one confined today to Africa and the other to South America. The common ancestor may have lived 80 Myr ago when Africa and South America began to drift apart. Alternatively, the common ancestor may have been more recent and flown across the water gap. Even mammals, such as monkeys, appear to have bridged this gap within the last 40 Myr (36). Thus, it is not surprising that the biogeographic time scale for birds (34) is almost twice as long as those time scales that depend mainly on fossil evidence (e.g., ref. 37). We raise this issue because Sibley and Ahlquist (35) have used their biogeographic calibration for DNA evolution in birds to date divergence events within higher primates. If our fossil-based calibration were to be used, the divergence time estimates for higher primates would have to be shortened.

Table 2. Association of time estimates (T, in millions of years) with DNA sequence divergence (D)

Origin or divergence event*	T, Myr	Tree node*	$D^{\dagger}$	$D/T^{\ddagger}$
Divergence within *Gallus*	<3–4	A	1.3	≥0.32
Origin of *Gallus*	>8	B	6.2	≤0.78
Origin of turkeys	>6–8	C	4.2	≤0.70
Splitting between turkeys and pheasants	<16–20	C	4.2	≥0.21
Splitting within Phasianidae	<31	E	7.6	≥0.24
Origin of Phasianoidea	<33–41	F	14.1	≥0.34
Origin of Guinea fowl	>35–40	F	14.1	≤0.40

*Nonmolecular evidence relating to divergence times within the superfamily Phasianoidea are associated with nodes on tree I (Fig. 3) as follows. *Node A*. The green jungle fowl is native to Java and the Lesser Sunda Islands (22), which are of volcanic origin and generally conceded to have undergone uplift in the Plio-Pleistocene (<3–4 Myr ago) (28). Since the green jungle fowl line could not have split from the main jungle fowl line before the origin of Java, the age of Java gives an upper bound for the time of that divergence within *Gallus*. *Node B*. Multiple fossil bones attesting to the existence of *Gallus* have been found as far back as the Upper Miocene (8 Myr ago) of Greece (ref. 29; C. Mourer-Chauviré, personal communication). Hence, the split between *Gallus* and *Alectoris* occurred at least 8 Myr ago. [It has been suggested that a fossil bone from the Lower Miocene (19–20 Myr) of Bavaria represents the recent genus *Alectoris* (30). However, the diagnostic feature of the bone (a ridge present in *Alectoris* but not in other partridges, quails, or jungle fowls) may instead be indicative of a common ancestral form, as it is also present in pheasants, peafowl, and turkeys (31). Therefore, it is uncertain whether this fossil provides an upper or lower bound for the split between *Alectoris* and *Gallus*.] *Node C*. The oldest definite turkey fossil, *Proagriocharis*, is represented by several bones (32), the earliest of which is of Upper Miocene age (6–8 Myr) in Nebraska (38, 39). Of possible relevance is an unnamed species represented by a single bone from the Upper Miocene (9–12 Myr) deposits in Virginia. *Rhegminornis* is known by several poor fossils from the Lower Miocene (16–20 Myr) of Florida. It has characters of both the Phasianidae and Meleagrididae (32), suggesting that the families had not split before that time. *Node E*. The first phasianid fossil represented by multiple body parts is *Schaubortyx*, a skeleton impression found in the Lower Oligocene (31 Myr) of France (ref. 29; C. Mourer-Chauviré, personal communication). Since this fossil cannot be ascribed to a specific lineage within the family Phasianidae, it is unlikely that the splitting within this group occurred before this time. *Node F*. The Eocene and Lower Oligocene fossils from Quercy, France (33–40 Myr) are galliform but do not belong in the phasianoid superfamily (C. Mourer-Chauviré, personal communication). In addition, Olson (33) proposes that a single fossil bone representing an extinct genus (*Telecrex*) belongs to the family Numididae. This fossil from the Upper Eocene (35–40 Myr) of Inner Mongolia might imply that the split between Numididae and other families of phasianoid birds occurred at least 35–40 Myr ago.

†Sequence divergences were arrived at by averaging the δ values from Table 1 for all of the pertinent lineages on tree I in Fig. 3.

‡Percent sequence divergence per Myr. Rates were estimated using the values of T that gave the largest range of possible rates.

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