Pathways of Lysozyme Evolution Inferred from the Sequences of Cytochrome b in Birds

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Abstract. A reliable phylogeny relating the major groups of Galliformes was sought in order to shed light on an unusual case of coupled amino acid replacements in the lysozymes c of these birds. The New World quail and the African guinea fowl share a unique trio of amino acids at three internal positions but have been separated phylogenetically by the majority of trees based on morphological characters. Alternative hypotheses based on molecular data have suggested an arrangement that would be more parsimonious with regard to the lysozyme data. The entire mitochondrial cytochrome b gene (1,143 bp) was amplified via the polymerase chain reaction (PCR) and sequenced for nine galliforms and a representative anseriform to provide DNA sequence data for a phylogenetic reconstruction. The mode and tempo of change in these sequences were analyzed to determine the characters most appropriate for phylogenetic reconstruction. Our results place the New World quail outside all other representative game birds except the cracids. Although in conflict with various morphological analyses, this finding is consistent with the results of DNA-DNA hybridization studies. A model to account for the coupled replacements in the lysozymes is presented. Our results also suggest a rapid but ancient radiation among the Galliformes such that the majority of cytochrome b sequence differences among taxa have accumulated on the terminal branches of the reconstructed phylogenetic trees.

Key words: Mitochondrial DNA — Evolutionary trees — Coupled changes — Structure-function relationships — New World quail — Guinea fowl — Base composition — PCR and direct sequencing

Introduction

The egg-white lysozyme c sequences of galliform birds possess a unique pattern of amino acid replacements at three internally clustered residues. As shown in Fig. 1, these positions are occupied in all characterized game-bird lysozymes by Thr 40, Ile 55, and Ser 91 (TIS), with the exception of the guinea fowl and New World quail lysozymes, which have Ser 40, Val 55, and Thr 91 (SVT) at these positions (Jollès and Jollès 1984; Malcolm et al. 1990). A phylogenetic analysis of the amino acid sequences for these lysozymes placed the African guinea fowl and the New World quail together

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1 Among 20 galliform species whose lysozyme c sequences are known are seven species of New World quail, all with SVT; 12 species (i.e., all others except the guinea fowl) have TIS (E.M. Prager, personal communication summarizing published and unpublished data)
within the family Phasianidae (see Fig. 1C; Jollès et al. 1979; Jollès and Jollès 1984), an arrangement that is not suggested by morphological or other molecular evidence. Ibrahim (1979) viewed this arrangement as a phylogenetic anomaly likely attributable to convergence or parallelism in amino acid sequence.

The trio of replacements appears to have occurred in unison, as none of the six possible intermediate states (i.e., TIT, SIT, SVS, etc.) occur in any of the characterized egg-white lysozymes of galliform birds. In addition, thermostability studies of engineered lysozymes (Malcolm et al. 1990) revealed that these evolutionary intermediates lie outside a proposed neutral corridor for protein evolution, which suggests that these intermediates would be suboptimal states and therefore unlikely to persist. The traditional morphological arrangement for the game birds requires this trio of replacements to occur independently on two separate lineages (Fig. 1A). This would suggest an instance of convergent evolution in game-bird lysozymes. However, an alternative placement of the New World quails could necessitate fewer concerted replacement events.

The placement of the New World quails within this order is one of the most intriguing problems in systematic studies of the Galliformes. Traditional morphological classifications (e.g., Wetmore 1960; Johnsgard 1986) placed New World quails (Odontophorinae) as a subfamily within the family Phasianidae (Fig. IA) and the guinea fowl (family Numididae) outside the other phasianoids. Alternatively, the DNA-DNA hybridization studies of Sibley and Ahlquist (1985, 1990) place New World quails outside the guinea fowl. Several other molecular methods have been used to develop phylogenetic hypotheses or to determine phylectic relationships within Galliformes, including restriction mapping of mtDNA (Glaus et al. 1987) and nuclear DNA (Helm-Bychowski and Wilson 1986), allozyme electrophoresis (Gutiérrez et al. 1983), protein sequencing (Jollès and Jollès 1984), and chromosome banding-pattern analysis (Stock and Bunch 1982). However, neither the molecular studies nor ongoing morphological studies have been successful at resolving several issues regarding alternative taxonomic organizations within the order Galliformes.

With the development of the polymerase chain reaction (PCR) (White et al. 1989), it has become possible to generate DNA sequence data for a large number of taxa rapidly and efficiently for the purpose of understanding phylogenetic relationships. Mitochondrial DNA (mtDNA) is often employed for such studies at or near the species level, primarily due to its fast rate of evolution (Brown 1985); for a review, see Harrison 1989). The increasing availability of mtDNA sequences from many groups of organisms made it possible to design oligonucleotide primers with a wide taxonomic utility (Kocher et al. 1988; Kocher and White 1989) for PCR amplification of a small region (307 bp) of cytochrome b. This short segment served to successfully resolve phylogenetic questions in birds at or near the genus level (e.g., Edwards and Wilson 1990; Smith et al. 1991). Deeper evolutionary relationships could not
be resolved with this limited data set (Kocher et al. 1989); however, longer cytochrome b sequences made it possible to answer phylogenetic questions for higher taxonomic categories: Edwards et al. (1991) used a 924-bp region of cytochrome b to resolve a deep branching pattern among perching birds, and the DNA sequences for the entire cytochrome b coding region for 20 mammals were used to investigate the phylogenetic relationships of hoofed mammals (Irwin et al. 1991).

In this paper, we present DNA sequences for the entire gene encoding cytochrome b of nine species in the order Galliformes and the Muscovy duck. We have established guidelines for use of this data set in phylogenetic reconstruction based in part on previously established tests for analysis of cytochrome b evolution (Edwards et al. 1991; Irwin et al. 1991). Additionally, we present data for the nucleic acid codon of amino acid residue 91 of lysozyme c from the chachalaca, a representative of the family Cracidae. It was previously assumed that the cracid would have an AGY at codon position 91 encoding the serine residue, as is the case for chicken lysozyme. Since the codon TCN also encodes serine, we sought to shed light on the pathway of lysozyme evolution by determining which serine codon is used in the chachalaca lysozyme at position 91.

Materials and Methods

**DNA Sources.** Total genomic DNA samples were prepared from fresh or frozen tissues for chicken (Gallus gallus), Japanese quail (Coturnix coturnix), chukar partridge (Alectoris chukar), turkey (Meleagris gallopavo), pheasant (Pavo cristatus), silver pheasant (Lophura nycthemera), Gambel quail (Lophortyx gambellii), guinea fowl (Numida meleagris), and chachalaca (Ortalis vetula) by extraction with a solution containing sodium dodecyl sulfate and proteinase K followed by phenol/chloroform extraction as described previously (Kocher et al. 1989). Muscovy duck (Cairina moschata) DNA was provided by K.M. Helm-Brychowski.

**Cytochrome b Sequences.** Mitochondrial fragments containing the cytochrome b gene were amplified from the total genomic DNA preparations via PCR and directly sequenced. Primers used for amplification and sequencing are shown in Table 1 and Fig. 2. Single-stranded DNA for sequencing was generated in one of three ways. Generally, double-stranded DNA fragments were amplified first using PCR conditions as described previously (Kocher et al. 1989), with each cycle consisting of denaturation for 1 min at 93°C, hybridization for 1 min at 45-60°C, and extension for 1-3 min at 72°C for 30-35 cycles. The double-stranded DNA products purified by electrophoresis in low-melting-temperature agarose (Sambrook et al. 1989) then served as template DNA for asymmetric PCR followed by direct sequencing (Gyllenstein and Eritsch 1988). Alternatively, asymmetric PCR was performed directly from whole DNA preparations for some primer pairs and the products were utilized for direct sequencing. Occasionally, single-stranded DNA template suitable for sequencing could not be obtained by these methods. For those cases, one primer in a pair was phosphorylated and a double-stranded PCR amplification was performed. Lambda exonuclease was then used to digest the kinase strand, leaving a
Fig. 3. Sequences of cytochrome b genes for nine galliform birds and the Muscovy duck. Dots indicate identity to the chicken sequence. Numbering of nucleotides is according to the chicken mtDNA sequence. The predicted amino acid sequence of the chicken protein appears in the single-letter code above the DNA sequences. Continued.
which employs a six-parameter stochastic model of DNA sequence evolution (Felsenstein 1981; Hasegawa et al. 1985; Kishino and Hasegawa 1989). The rate of change at second positions of codons relative to first positions was assumed to be 0.25 and a transition-to-transversion ratio of 2.0 was assumed for all maximum likelihood calculations. These distances were used in the NEIGHBOR program (version 3.4-6) of the PHYLIP package to reconstruct trees based on the neighbor-joining distance algorithm (Saitou and Nei 1987). For distance analysis based on amino acid replacement data, neighbor-joining reconstruction was performed with the NJTREE computer program of Saitou and Nei (1987) using a matrix of calculated amino acid substitutions inferred from the predicted cytochrome b protein sequences. A comparison of the maximum likelihood values for different phylogenetic hypotheses was done with user-defined trees in the DNAML (DNA maximum likelihood) program of PHYLIP, version 3.4, according to the aforementioned six-parameter model with the same specifications. In all phylogenetic analyses of the nucleic acid data, third positions of codons were omitted and the first positions of all leucine codons were converted to generic pyrimidine characters because of saturation effects (see Results, Tempo and Mode of Substitution).

**Lysosome Sequences**. The region of the chachalaca egg-white lysozyme c gene encoding amino acid position 91 was amplified from genomic DNA via PCR using two flanking primers: chl91nt (5'-CTTAAGCCCTGCTGGGCGGACACAT-3') and chl207nt (5'-GTCTCCTACCCAGGCATTGAT-3'). These primers were designed based on DNA sequence data for the chicken gene (Jung et al. 1980) and on the amino acid sequence of the chachalaca protein. The double-stranded 94-bp PCR product was purified by electrophoresis on a 6% polyacrylamide gel and used as a template for asymmetric PCR and direct DNA sequence determination.

**Results and Discussion**

**Cytochrome b Sequences**

The sequences for the entire cytochrome b gene for nine galliform species plus the Muscovy duck are presented in Fig. 3. During the course of this work, the entire chicken mtDNA sequence was published (Desjardins and Morais 1990). Comparison of our chicken cytochrome b sequence with that of Desjardins and Morais (1990) reveals a single silent difference at the third position of codon 124, confirming that this is the mitochondrial cytochrome b gene and ruling out any possibility that we have obtained a nuclear copy or a pseudogene. For all the species examined in this report, flanking sequences adjacent to the cytochrome b gene implicate a gene order identical to that reported for the chicken. All 10 gene sequences are 1,143 nucleotides in length and appear to encode a 380-amino-acid protein as expected, with no apparent deletions or insertions.

We did come across a putative duplicate copy of cytochrome b for one of our samples, the chukar partridge. With one of the primer pairs, L14851 and H15298 (No. 2 and 5 in Table 1 and Fig. 2), we obtained a clean sequence from both strands that did not correspond to the sequence obtained for the chukar partridge using L14464 (primer 1) or L14990 (primer 3) with H15298. This different sequence was most related to the normal chukar partridge cytochrome b in a parsimony analysis, but it had four additional replacement changes and two codon deletions over 381 bases (data not shown). This sequence is most likely to be part of a duplicated mtDNA segment that has been translocated to the nuclear genome, although an intramitochondrial duplication cannot be ruled out. A nuclear homologue sequence that contains base deletions and substitutions not observed in the corresponding mitochondrial sequences has been reported in snow geese (Quinn 1992). In addition, the potential for amplification of nuclear copies of cytochrome b with certain primers has been reported and discussed thoroughly by Smith et al. (1992).

**Base Composition**

Table 2 summarizes the base compositions for the nine galliform and the Muscovy duck cytochrome b sequences for each codon position. The values of the nucleotide compositional bias index are very similar to the values reported for mammalian cytochrome b sequences (Irwin et al. 1991), and they reflect previous observations for mtDNA base composition (Brown 1985; Kocher et al. 1989; Edwards et al. 1991).

Third positions of codons are strongly biased against guanine residues, a trait characteristic of metazoan mtDNA in general (W.K. Thomas, personal communication). A lesser but pronounced bias against thymines in third positions is also evident. From sequences for a 307-bp segment of cytochrome b (Kocher et al. 1989), it appeared that birds had an unusual bias against third-position thymines in this gene. However, longer or complete sequences (Edwards et al. 1991; Irwin et al. 1991; this report) revealed that in mammalian cytochrome b genes there is a pronounced anti-thymine bias that is frequently as strong as in birds. The second positions of the bird cytochrome b genes we studied are thymine-rich and the base composition of first positions is relatively unbiased (Table 2)—observations made also for other birds (Edwards et al. 1991) and for mammals (Irwin et al. 1991).

Interspecific variation in base composition is reflected in the standard deviation values in Table 2 and is highest for third positions (mean standard deviation = 2.22), a finding that can be accounted for by the fact that third positions of codons are often degenerate and therefore allow high numbers
Table 2. Base composition at first, second, and third positions of codons

<table>
<thead>
<tr>
<th>Species</th>
<th>First</th>
<th>Second</th>
<th>Third</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>Chicken</td>
<td>20.7</td>
<td>26.8</td>
<td>22.8</td>
</tr>
<tr>
<td>Japanese quail</td>
<td>20.2</td>
<td>27.3</td>
<td>22.6</td>
</tr>
<tr>
<td>Chukar partridge</td>
<td>20.7</td>
<td>27.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Gambel quail</td>
<td>20.7</td>
<td>27.8</td>
<td>22.6</td>
</tr>
<tr>
<td>Guinea fowl</td>
<td>20.5</td>
<td>27.0</td>
<td>21.8</td>
</tr>
<tr>
<td>Peafowl</td>
<td>21.0</td>
<td>26.8</td>
<td>23.9</td>
</tr>
<tr>
<td>Silver pheasant</td>
<td>21.3</td>
<td>25.7</td>
<td>21.4</td>
</tr>
<tr>
<td>Turkey</td>
<td>21.3</td>
<td>26.8</td>
<td>23.9</td>
</tr>
<tr>
<td>Choasalaca</td>
<td>21.3</td>
<td>25.5</td>
<td>22.6</td>
</tr>
<tr>
<td>Muscovy duck</td>
<td>23.6</td>
<td>24.7</td>
<td>22.9</td>
</tr>
<tr>
<td>Mean</td>
<td>21.1</td>
<td>26.6</td>
<td>23.0</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.95</td>
<td>0.74</td>
<td>0.68</td>
</tr>
<tr>
<td>Bias*</td>
<td>0.079</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bias in base composition is calculated as

\[
C = \frac{2}{n} \sum_{i=1}^{4} (c_i - 0.25)
\]

where \(C\) is the compositional bias and \(c_i\) is the frequency of the \(i\)th base.

of silent substitutions. Conversely, second positions of codons, which are most constrained in the genetic code, have the lowest level of interspecific variation (average standard deviation = 0.36). The guanine content at third positions of the Muscovy duck gene is significantly higher than that of the nine galliform sequences \((P < 0.001\) in a G-test for independence using William’s correction).

 Tempo and Mode of Substitution

Variation as a Function of Position

Cytochrome b is the only protein (of nine or 10) in complex III of the mitochondrial oxidative phosphorylation system that is encoded by the mitochondrial genome (Hasef 1985), and it is the best characterized in terms of structure/function relationships using evolutionary comparisons and studies of mutants (Howell and Gilbert 1988; Howell 1989; di Rago et al. 1990a,b). Both redox centers, \(Q_1\) and \(Q_2\), involved in electron transfer from ubiquinol to cytochrome c are contained in cytochrome b, which also has several putative membrane-spanning regions.

Because of its functional importance and structural limitations, we might expect variability in cytochrome b to be relatively low. In comparing the nine galliform sequences, we find 398 variant nucleic acid positions, with 73 of these occurring at the first positions of codons, 26 at the second positions, and 299 at the third positions. Over half of these variable positions are phylogenetically informative, with 29 informative sites at the first positions of codons, 9 at second positions, and 188 at third positions. Thus, most of the potential data for making phylogenetic and distance calculations reside in the third positions of codons.

The majority of variant amino acid positions are in membrane-spanning domains of the protein, as is the case for mammalian cytochromes b (Irwin et al. 1991). These are often conservative replacement changes from one hydrophobic residue to another (e.g., leucine-isoleucine, leucine-valine, etc.). A theoretical estimate of the variable sites (codon positions) by the capture-recapture method of Sidow et al. (1992) results in a value 69.3 of the 380 total codons. The apparent low level of variability in these sequences places an upper limit on the amount of phylogenetic information available for tree construction.

Rates of Change and the Fossil Record

Examination of rates of change in cytochrome b are important for determining the utility of transition and transversion changes in each set of codon positions. In previous studies using cytochrome b as a phylogenetic probe for deep relationships, it was shown that changes at third positions of codons require special consideration due to base-compositional constraints and the saturation of transition changes at silent positions (Irwin et al. 1991; Ed-
Table 3. Estimated divergence dates for some major branches within the Galliformes

<table>
<thead>
<tr>
<th>Event</th>
<th>Time (Myr)</th>
<th>Node</th>
<th>Pairwise comparisons*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin of Gallus</td>
<td>&gt;8</td>
<td>B</td>
<td>Chicken vs. peafowl, pheasant</td>
</tr>
<tr>
<td>Splitting of Phasianidae</td>
<td>&lt;31</td>
<td>C</td>
<td>Japanese quail vs chicken, peafowl, pheasant, Partridge vs chicken, peafowl, pheasant</td>
</tr>
<tr>
<td>Origin of Numididae</td>
<td>&gt;40</td>
<td>D</td>
<td>Guinea fowl vs Phasianidae, turkey</td>
</tr>
<tr>
<td>Origin of Cracidae</td>
<td>&gt;50</td>
<td>E</td>
<td>Chachalaca vs all</td>
</tr>
</tbody>
</table>

* For simplicity, these dates have been generalized. See text for details about the fossil record
b Nodes correspond to the tree inferred in Fig. 5A
c These pairwise comparisons are used for the data presented in Fig. 4

wards et al. 1991). Transition changes for first and second positions should be examined, as well as transversions for all codon positions, to consider the possibility that changes of all kinds may saturate within the time frame of deep divergences, particularly for coding sequences of highly conserved proteins.

The fossil record for the order Galliformes offers little in the way of definitive dates for divergence among its member taxa. However, a reasonable attempt to calibrate temporally molecular genetic distance data from nuclear DNA restriction maps was made for a group of galliforms by Helm-Bychowski and Wilson (1986). In this study, we include several of the divergence points defined in that analysis, as well as additional paleontological information for other deep divergence dates.

Table 3 summarizes available time estimates for several nodes in the phylogenetic tree in Fig. 5A inferred from cytochrome b data. The oldest galliform fossil is Gallinuloides wyomingensis Eastman from the Lower Eocene (Wasatchian) deposits of the Green River formation of Wyoming. Gallinuloides has been generally considered a member of the family Cracidae (Olson 1985), although it has been suggested to be closer to the phasianoids (Craik 1973). Thus, it seems likely that the cracids diverged from other Galliformes at least 50 Myr ago. Telecere girgenti Wetmore, a fossil from the Upper Eocene of Inner Mongolia in China, has been assigned to the family Numididae based on its similarity to modern guinea fowl (Olson 1974), suggesting that the guinea fowl lineage could be at least 35–40 Myr old. The earliest fossil assigned to Meleagrididae (turkeys) is Rheginornis calabotes Wetmore, from the Lower Miocene (Hemphfordian, 16–20 Myr) of Florida. The fragmented nature of these specimens makes Rheginornis difficult to place decisively and it may represent a more phasianid intermediate (Steadman 1980). A later fossil species, Proagriocaris kimballensis Martin and Tate from the Upper Miocene (6–8 Myr) of Nebraska, has been definitively assigned to Meleagrididae (Steadman 1980). Therefore, turkeys split from other phasianids at least 6–8 Myr ago and possibly more than 20 Myr ago. This level of uncertainty limits the value of using the turkey in a rate comparison. A more useful comparison point might be obtained from the fossil species Schaubornyx, a skeletal impression from the Lower Oligocene (31 Myr) of France (Brodskorb 1964). Because this fossil cannot be assigned to a particular lineage within the Phasianidae, it has been hypothesized that the splitting within this group did not occur before this time. (See Helm-Bychowski and Wilson 1986.) The existence of Gallus as a separate genus has been documented by several fossils dating as far back as the Upper Miocene of Greece (Brodskorb 1964), making that divergence at least 8 Myr old.

The results of plotting the observed pairwise differences (both transitions and transversions) at each codon position for the species pairs listed in Table 3 are shown in Fig. 4. It is clear that third-position transition changes have been saturated in the divergence of these game birds. It appears that transversions in the third positions of codons slowly continue to accumulate following a short period of rapid change early in the phylogenetic history. However, it is virtually impossible to determine which sites may have saturated and would thus confound phylogenetic analyses. Therefore, all third positions have been omitted for those analyses using nucleic acid data. Transition changes in the first position of codons also exhibit some level of saturation, due to silent changes in the disproportionate number of leucine codons. Thirty of the 73 variant sites in first positions are due to silent leucine substitutions, and this type of silent change accounts for nearly one-half of the 29 phylogenetically informative sites in the first positions. In light of the potentially erroneous phylogenetic information represented by these changes, all leucine codon first positions were converted to generic pyrimidines for phylogenetic analyses. Transversions at first positions as well as transitions and transversions at second positions of codons show little evidence of saturation and require no special treatment or modifications.
Fig. 4. Nucleotide differences among bird cytochrome b genes relative to time. Pairwise sequence differences for the comparisons listed in Table 3 were averaged for both transitions (dashed, open arrow) and transversions (solid arrows). The averages are plotted against time for (A) first, (B) second, and (C) third positions of codons. Each arrow has its base at the minimum or maximum time tabulated in Table 3 and points in the direction of uncertainty.

Phylogenetic Analyses

Tree Construction and Statistical Testing

Parsimony analysis of nucleic acid changes at first and second positions of codons yielded four trees of equal length (Fig. 5A). It is notable that in three of these four trees, the New World quail representative, the Gambel quail, is placed outside all other members of Galliformes except the chachalaca. In the fourth equally parsimonious tree, guinea fowl replaces the Gambel quail in the outside position. We attempted to resolve this question by using predicted amino acid sequences in a parsimony analysis. By using the PROTPARS substitution matrix, we gain additional phylogenetic information from third positions of codons, where transversion changes sometimes result in amino acid replacements. This resulted in a single tree with a topology identical to one of the four nucleic acid trees (Fig. 5B), in which the Gambel quail is placed in the outside position.

The results of distance analyses using calculated maximum likelihood distances for first and second positions of codons are shown in Fig. 6A. A transition to transversion ratio of 2.0 was used in this six-parameter model. Again, the Gambel quail is placed outside the rest of the noncracid game birds, a feature also present in Fig. 6B, which depicts the phylogenetic tree inferred from the predicted pairwise amino acid replacement distance data. It is also of interest that the peafowl and pheasant are sister taxa in all the phylogenetic analyses (Figs. 5 and 6).

Statistical tests for these analyses have included bootstrap resampling methods for both parsimony and maximum likelihood algorithms as well as evaluation of various proposed phylogenetic trees from the current analyses and from previous hypotheses by the maximum likelihood method. Bootstrap resampling did show the highest levels of support for the placement of Gambel quail outside the other phasianoids (63–65%), followed by the guinea fowl, and for the grouping of peafowl and silver pheasant; however, statistical significance was not demonstrated for any branch (data not shown).

A comparison of the relative likelihoods of various phylogenetic hypotheses using the paired-sites test (Kishino and Hasegawa 1989) in the DNAML program of Felsenstein's PHYLIP package, version 3.4, is summarized in Table 4. The trees presented in Figs. 5 and 6 for the parsimony and distance analyses of cytochrome b data do not differ significantly from the best-fit tree constructed by the DNAML program. Since the placement of Gambel quail...
the grouping of pheasant with peafowl are identical for all of these trees, this result is not surprising. The difference in likelihood is also relatively small for the DNA-DNA hybridization tree (Sibley and Ahlquist 1985, 1990), while the differences are greatest for the morphological hypotheses (Table 4). A given tree is significantly worse than the best-fit tree if the ln L (likelihood) value for that tree is two standard deviations away from the ln L value for the best-fit tree. Although the morphological arrangements appear least likely of those evaluated in Table 4, none of the phylogenetic hypotheses tested could be absolutely ruled out by this criterion.

Our phylogenetic analyses of cytochrome b sequence data support the hypothesis that the New World quails (represented in the current study by the Gambel quail[a]) fall outside all other galliforms

Table 4. Evaluation of several phylogenetic hypotheses by a maximum likelihood method

<table>
<thead>
<tr>
<th>Tree</th>
<th>lnL</th>
<th>ΔlnL</th>
<th>SD in lnL</th>
<th>ΔlnL/SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-1,567.41</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>-1,568.63</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>-1,574.95</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>-1,578.08</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
<tr>
<td>5</td>
<td>-1,590.27</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
<tr>
<td>6</td>
<td>-1,604.96</td>
<td>-1.22</td>
<td>4.15</td>
<td>0.29</td>
</tr>
</tbody>
</table>

[a] User-defined trees are given in unrooted form as in the following example: (Tree: Source (taxon1,taxon2,(taxon3,taxon4))). Abbreviations for six of the species correspond to the first four letters in Fig. 5: the rest are jppu, Japanese quail; gamaq, Gambel quail; gulf, guinea fowl.

Tree 1: DNA-DNA best-fit tree
(chac.gamq,jppu,(turk,part),(chic,(peaf,peha)))))

Tree 2: Fig. 5B (this study)
(chac.gamq,jppu,(turk,part),(chic,(peaf,peha)))))

Tree 3: Fig. 6A (this study)
(chac.gamq,jppu,(turk,part),(chic,(peaf,peha)))))

Tree 4: DNA-DNA hybridization (Sibley and Ahlquist 1985, 1990)
(chac.gamq,(jppu.part),(chic,(peaf,peha)))

Tree 5: Morphological example 1 (Johnsgard 1966)
(turk.igmq,(jppu.part),(chic,(peaf,peha)))

Tree 6: Morphological example 2 (Cawte 1988)
(chac.gamq,(jppu.part),(peaf,(peha,turk),(chic,(peaf,peha))))

lnL is the natural log of the maximum likelihood value calculated for the tree using Felsenstein’s DNAML computer program as described in Materials and Methods

with the exception of the cracids (represented by the chachalaca[a]). This is in close agreement with the DNA-DNA hybridization tree of Sibley and Ahlquist (Fig. 1B) and contrasts with the majority of traditional morphological trees. (See Fig. 1A, Table 4.)

Rapid, Ancient Cladogenesis?
It is possible that the ongoing debate regarding the phyletic organization of the major groups in Galliformes into the various families and subfamilies has not been resolved satisfactorily by any method to date because of an inherent problem in the timing of separation of these groups. Specifically, we suspect that a relatively rapid radiation occurred among the Galliformes at least 30 Myr ago and that

[a] Strong molecular support for the monophyletic and close relationship of New World quails to each other relative to other phasianoids comes from allozyme electrophoresis (Gutiérrez et al. 1983), DNA hybridization (Sibley and Ahlquist 1990), and transferrin immunological comparisons (E.M. Prager, personal communication). In this last study, an antisemur to bobwhite quail (Colinus virginianus) transferrin yielded immunological distances of 0-15 to members of four other genera of New World quails, compared to distances of >38 to a wide variety of phasianoid species outside the Coliopophoridae

[b] Strong molecular support for a close, monophyletic relationship of the cracids comes from DNA hybridization (Sibley and Ahlquist 1990) and transferrin immunological comparisons (Prager and Wilson 1976)
the majority of sequence differences between taxa have accumulated along the terminal branches. This hypothesis is difficult to test in the absence of a definitive fossil record, but our analyses are consistent with inferences made from mtDNA sequence analysis for a group of mammals for which a rapid radiation has been suggested (Kraus and Miyamoto 1991). In that case, it was suggested that the long terminal branch lengths relative to the internal branches were indicative of a rapid cladogenesis. It is evident that the phylogenetic trees inferred from cyt b for these birds exhibit the expected short internal branch lengths that would indicate that most of the evolutionary change has occurred since these taxa separated from one another. (See especially Fig. 6.) For the mammalian example above, rapid radiation was a possibility that had been previously suggested. In our case, there have been no strong arguments for a rapid radiation in the Galliformes, although it has been suggested that the earliest interordinal divergences (i.e., the separation of lineages leading to the present-day cracids and megapodes from the lineage leading to the remaining galliforms) was due to the breakup of Gondwanaland and that these members of Galliformes were distributed in the Southern Hemisphere (Cracraft 1973). Subsequently, dispersals to North America, Africa, and Eurasia would have produced the New World quails, the African guinea fowls, and the Old World phasianids, respectively. This scenario is most consistent with a rapid radiation of the Galliformes as long ago as 50–60 Myr, prior to the separation of the Americas and when the North Atlantic land connection to Eurasia was intact, and is supported by the cyt b data presented here.

Lysozyme Evolution

With respect to the original question of concerted change in the lysozyme amino acid sequences of galliform birds, our data support the idea that, while the New World quails and the guinea fowl do not form a clade, they are not separated in the phylogenetic tree. This indicates that the Ser 40, Val 55, Thr 91 (SVT) type of lysozyme c did not arise on two separate occasions. However, as Fig. 1B illustrates, this arrangement still implies two occurrences of the coupled amino acid replacements because the chachalaca has the Thr 40, Ile 55, Ser 91 (TIS) type at these three internal residues. To address this aspect of the question, we obtained the nucleic acid sequence for the chachalaca serine codon at position 91 to determine whether it is the same codon used for chicken Ser 91 and inferred from considerations of parsimony to be used also in the other phasianids and turkey (Fig. 7).

Chachalaca has the serine codon TCN at position 91 while the phasianids and turkey have the serine codon AGY at the same position in the amino acid trio. Figure 8 shows a possible order of replacement events that accounts for this difference in codon usage within the phylogenetic framework presented in this work. Because the next-closest avian group to the Galliformes is the order Anseriformes (Prager and Wilson 1980; Sibley and Ahlquist 1985, 1990), we can use the duck as an outgroup for this comparison. Duck (Anas platyrhynchos) lysozymes c possess an alanine at position 91, giving it a Thr 40, Ile 55, Ala 91 (TIA) designation. A possible intermediate state giving rise to the two lysozyme trios observed within Galliformes would be TTT (Fig. 8) because it requires only a single nucleotide change for the replacement of alanine by threonine. As previously stated, the putative intermediate states, TTT and SVS, shown in Fig. 8 would not be expected to persist evolutionarily because they do not fall within the predicted neutral corridor of protein stability (Malcolm et al. 1990). The model in Fig. 8 suggests that the TTT type may have been a shared ancestral intermediate for the cracid TIS type and for the subsequent SVT type. It is possible that TIA...
References


Kraus F, Miyamoto MM (1991) Rapid cladogenesis among the