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Analyses of mitochondrial DNA nest ratite birds within the Neognathae: supporting a neotenous origin of ratite morphological characters

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It is commonly acknowledged that the basal divergence among extant birds is between Palaeognathae and Neognathae. However, recent analyses of mitochondrial sequences have challenged that notion. In order to investigate this fundamental phylogenetic question, the complete mitochondrial DNA (mtDNA) molecule of the rook *Corvus frugilegus* (Passeriformes) was sequenced and included in phylogenetic analyses with the previously reported complete mtDNAs of the chicken *Gallus gallus* (Galliformes) and two ratite species, the ostrich *Struthio camelus* and the rhea *Rhea americana* (Struthioniformes). The analyses reconstructed a split between Passeriformes and a branch including Galliformes and Struthioniformes. Thus, the result is inconsistent with the traditional understanding of a basal avian divergence between Palaeognathae and Neognathae. The findings suggest that the morphological characteristics of the ratites are secondarily acquired, probably through neoteny and that the ratites are descendants of flying, neognathous ancestors.

Keywords: mitochondrial DNA; molecular phylogeny; *Corvus frugilegus*; Passeriformes; ratites; neoteny

1. INTRODUCTION

Living palaeognathous birds include the South American tinamous and the ratites, represented by the kiwis (New Zealand), the ostrich (Africa) and the rheas (South America) plus the emu and the cassowaries (Australia). Analyses of both nuclear and mitochondrial data sets have demonstrated that the ostrich, rhea and emu constitute a monophyletic group (Sibley & Ahlquist 1990; Härlid *et al*. 1998), hereafter referred to as Struthioniformes. All other living birds, more than 9000 species, are neognathous. The traditional view is that of a basal divergence between Palaeognathae and Neognathae and is primarily based on the belief that the palate of paleognaths is primitive compared to that of other birds (Huxley 1867), but a Palaeognathae–Neognathae dichotomy has also been advocated on the basis of some molecular studies (Stapel *et al*. 1984; Sibley & Ahlquist 1990; Caspers *et al*. 1997).

The basal divergence between Palaeognathae and Neognathae has recently been challenged in character state analyses of protein-coding mitochondrial DNA (mtDNA) genes (Härlid *et al*. 1997, 1998; Mindell *et al*. 1997). In contrast to DNA–DNA hybridization studies (Sibley & Ahlquist 1990), character state analyses make it possible to establish the polarity of the avian tree by applying unequivocal rooting. One of these studies (Härlid *et al*. 1998), which was based on the complete sequence of the mitochondrial cytochrome *b* gene, placed the Passeriformes basal to an assembly of six other avian orders (Procellariiformes, Gruiformes, Caprimulgiformes, Anseriformes, Galliformes and Struthioniformes), joining the Galliformes and the Struthioniformes on a common branch.

It has previously been shown that phylogenetic analyses of short sequences may yield inconsistent topologies (Arnason & Johnsson 1992) and that these shortcomings are progressively ameliorated as longer alignments are used (Cao *et al*. 1994). For this reason and because recent analyses of short nuclear genes (305 and 607 nucleotides (nt), respectively; Caspers *et al*. 1997) contradict our findings, we have sequenced the complete mtDNA molecule of a passeriform bird, the rook (*Corvus frugilegus*). This was then included in phylogenetic analyses along with all other available complete mtDNA bird sequences: the chicken (Desjardins & Morais 1990), the ostrich (Härlid *et al*. 1997) and the rhea (Härlid *et al*. 1998). Rooting of the avian tree was performed using either the complete mtDNA of the alligator (Janke & Arnason 1997) or of various mammals (Bibb *et al*. 1981; Anderson *et al*. 1982; Janke *et al*. 1997). These four avian mtDNA sequences, unequivocally rooted, make it possible to establish the relationship between Galliformes, Struthioniformes and Passeriformes, i.e. to test the hypothesis of a basal avian divergence between the Palaeognathae and the Neognathae.

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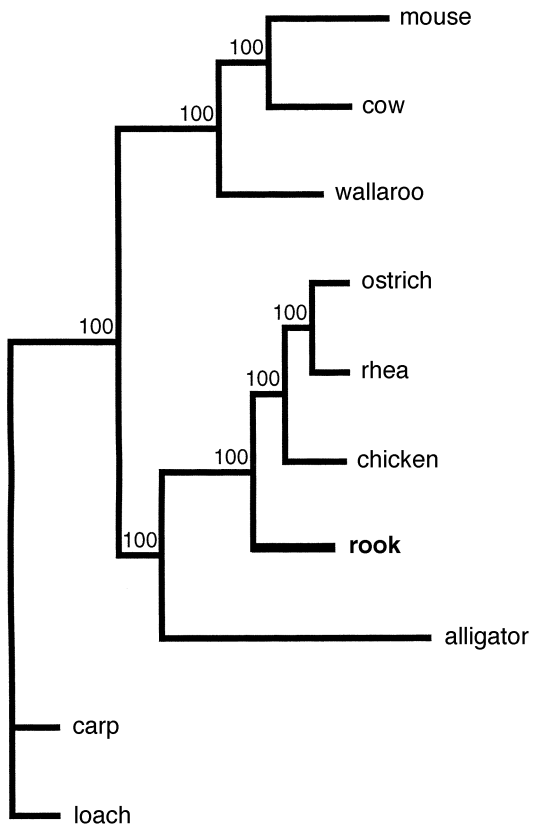

Figure 1. Amniote relationships, reconstructed by rooting the tree with the carp and the loach. The avian relationship with the rook (Passeriformes) basal to the chicken (Galliformes) and the ostrich and rhea (Struthioniformes) was identified using either the alligator or mammals as outgroup to the avian taxa. The tree is from ML–QP (maximum likelihood–quartet puzzling) analysis of the concatenated aa sequences of 11 protein-coding mtDNA genes. Support values obtained from ML analysis using 100 replicates are given above the branches. The reconstructed tree gave no support to the hypothesis that the basal avian divergence is that between Palaeognathae and Neognathae.

2. METHODS

Mitochondrial DNA was isolated from fresh heart tissue of a single rook specimen following the procedure described by Arnason *et al*. (1991). The mtDNA was cleaved separately with *Hin*dIII and *Spe*I and run on a preparative agarose gel. The restriction fragments were excised and ligated into M13. Positive clones were identified by plaque hybridization. The clones were sequenced (LICOR DNA sequencer model 4000L) using a Thermo Sequenase kit (Amersham). All of the clones sequenced were natural (not PCR) clones. The complete mitochondrial sequence of the rook *C. frugilegus* has been deposited at EMBL with accession number Y18522.

Phylogenetic analyses were performed on the concatenated sequences of 11 mitochondrial protein-coding genes from the following ten species: loach (Tzeng *et al*. 1992), carp (Chang *et al*. 1994), alligator (Janke & Arnason 1997), rook (present study), chicken (Desjardins & Morais 1990), rhea (Härlid *et al*. 1998), ostrich (Härlid *et al*. 1997), wallaroo (Janke *et al*. 1997), cow (Anderson *et al*. 1982) and mouse (Bibb *et al*. 1981). The phylogenetic methods used were maximum likelihood (ML; Adachi & Hasegawa 1996; Strimmer & von Haeseler 1996), neighbour-joining (NJ; Felsenstein 1991) and maximum parsimony (MP; Felsenstein 1991) and both amino acid (aa) and nucleotide (nt) data were analysed. The nt analysis was performed on first plus second codon positions, excluding synonymous leucine transitions at first codon positions. The mtREV-24 model (Adachi & Hasegawa 1996*b*) was applied to the aa analysis and the Tamura–Nei (TN; Tamura & Nei 1993) model to the nt analysis.

3. RESULTS

The examination of the alleged basal avian divergence between Palaeognathae and Neognathae was performed using complete mtDNA sequences. Two genes (*NADH3* and *NADH6*) were not included in the analysis. The *NADH3* gene was omitted due to the different length of this gene (one additional nt) in Struthioniformes relative to the passeriform rook and other vertebrates (Härlid *et al*. 1997, 1998). The presence of the additional cytosine at position 174 disrupts the reading frame unless this is remedied by RNA editing. Since the nature of this particular feature has not been thoroughly examined we have chosen to exclude the gene from the phylogenetic analyses. The same additional nt as in the *NADH3* gene of the Struthioniformes has been identified in the *NADH3* gene of the chicken after resequencing (A. Härlid and U. Arnason, unpublished data). The H strand-encoded *NADH6* gene was excluded because of the deviating nt composition of the gene compared to the L strand-encoded genes. After the removal of gaps and ambiguous sites adjacent to gaps, the length of the alignment was 9255 nt, corresponding to 3085 aa.

All methods of phylogenetic analysis reconstructed the same topology, irrespective of the data set used (aa or nt), with a basal split between Passeriformes and a branch consisting of Galliformes and Struthioniformes (figure 1). The separation between Passeriformes and Galliformes–Struthioniformes was supported by bootstrap and reliability values of 97.9% or higher. Alternative topologies to that in figure 1 were tested using both ML analysis (Kishino & Hasegawa 1989) and Templeton (1983) tests (table 1). According to both tests, the best tree was consistent with that shown in figure 1. The natural log-likelihood values (ln values) for the two alternative trees, Galliformes basal to Passeriformes–Struthioniformes and Struthioniformes basal to Passeriformes–Galliformes (traditional topology), were $\geqslant 2$ s.e. smaller than the ln value for the best tree, making the alternative topologies highly unlikely. In the Templeton (1983) test, each of the alternative topologies required a considerably larger number of steps than the best tree. Thus, both the ML and Templeton (1983) tests supported Passeriformes as basal to the Galliformes–Struthioniformes split, giving no support to a basal avian split between Palaeognathae and Neognathae. Indeed, the traditional tree with Struthioniformes basal received the least support in both tests. Analyses of complete cytochrome *b* sequences using a broader taxon sampling (Härlid *et al*. 1998) also

Table 1. *Tests of three alternative topologies of avian relationships* (*ML analysis and Templeton test*)

(Abbreviations: Pas, Passeriformes; Gal, Galliformes; Pal, Palaeognathae; OG, outgroup includes: mammalian, reptilian and piscine sequences; Δln L, ln-likelihood value; s.e., standard error; steps, additional steps required to reconstruct alternative tree; s.d., standard deviation.)

	ML test for aa		Templeton test for aa	
topology	Δln L	s.e.	steps	s.d.
OG, (Pas, (Gal, Pal))	0.0	—	(4613)	—
OG, (Gal, (Pas, Pal))	−60.2	±22.6	+20	±10.6
OG, (Pal, (Gal, Pas))	−69.9	±21.6	+36	±10.1

supported the position of Passeriformes basal to Galliformes–Struthioniformes. This study has now been extended to include four additional orders (Falconiformes, Columbiformes, Piciformes and Psittaciformes). Again, this extended analysis supported the basal position of the Passeriformes and the sister-group relationship between Galliformes and Struthioniformes (A. Härlid and U. Arnason, unpublished data).

The cytochrome *b* gene study of Härlid *et al*. (1998) indicated that this gene evolves at a similar rate among the different avian orders included. Both the branch lengths of the tree presented here (figure 1) and relative rate tests (Sarich & Wilson 1973) on the current data set, comprising 11 protein-coding mitochondrial genes, support a similar rate of evolution among the avian taxa, irrespective of which non-avian taxa are used as the outgroup.

In addition to the phylogenetic analyses, it is of interest to note that the length of the *NADH3* gene of the passeriform rook corresponds to that of vertebrates in general, whereas the inclusion of an additional cytosine at position 174 of the *NADH3* gene is synapomorph for the chicken and the two ratite representatives. This finding is consistent with the reconstructed relationship of Passeriformes basal to Galliformes and Struthioniformes.

Most mammalian orders appear to have arisen during the Cretaceous period (Janke *et al*. 1997). The same has been suggested for most ordinal, avian divergences (Cooper & Penny 1997), although the topology of that study was inconsistent with the present findings. Using the split between mammals (Synapsida) and birds plus crocodiles (Diapsida) at 310 million years before present (Ma BP) (Benton 1990) as a molecular calibration point, the divergence between Crocodylia and Aves has been dated at 254 Ma BP (Janke & Arnason 1997). The divergence time between the rook and the group including the ostrich, the rhea and the chicken was estimated as a proportion of the time since the separation of crocodylians and birds 254 Ma BP. Assuming a constant evolutionary rate on the avian branches, use of this reference gives an estimate of 114 Ma for the divergence between the Passeriformes and Galliformes–Struthioniformes. A similar figure is obtained when the Synapsida–Diapsida reference (310 Ma BP) is used, with the passeriform divergence being estimated at 110 Ma BP.

4. DISCUSSION

The deep position of Passeriformes in the phylogenetic tree is highly intriguing for the interpretation of passerine evolution. Passerine birds (*ca*. 5700 species) are divided into two main groups: the suboscines, which are largely restricted to South America, and the nearly worldwide oscines. Due to the primitive morphology of both the syringeal muscles and the stapes in the middle ear, the New Zealand wrens (Acanthisittidae) are definable neither as oscines or suboscines (Feduccia & Olson 1982). They are thus recognized as primitive within the order Passeriformes. This relationship has also been suggested on the basis of analysis of DNA–DNA hybridization (Sibley & Ahlquist 1990). As the New Zealand wrens are endemic to New Zealand, it has been proposed that Passeriformes originated in the Australian region (Feduccia & Olson 1982; Sibley & Ahlquist 1990). The oldest passeriform fossils are Australian, from the early Eocene period (54 Ma BP) (Boles 1995). While small non-passerine birds are highly abundant in the fossil records of the Northern Hemisphere, passeriform fossils did not appear in this region until the late Oligocene period (28 Ma BP). During the mid-Tertiary period, the northern radiation of passeriforms would appear to have been extremely fast, with passeriform fossils in sediments from this time exceeding the total number of all other avian fossils (Ballmann 1969). A passeriform origin in the early Cretaceous period would support a southern origin of Passeriformes, an isolation of suboscine passerines in South America and a mid-Tertiary period radiation into the Northern Hemisphere. All Cretaceous avian fossils investigated so far appear to belong either to the Enanthiornithines or to the Ornithurines. The absence of Neornithic fossils from the Cretaceous period, with the exception of a probable loon fossil, might cause some concern. However, if we hypothesize a southern origin of Passeriformes and possibly other bird orders, it may well be the case that the less well-investigated fossil records of the Southern hemisphere have merely not yet provided evidence of the existence of modern bird orders in the Cretaceous period.

The phylogeny presented here implies that the palaeognathous characteristics are not primitive, but have instead been secondarily acquired from the corresponding morphological features characterizing flying birds. Several features of the ratites such as the palaeognathous palate, the late closure of the cranial sutures and the downy plumage, have been described as juvenile characteristics representing stages which all neognathous birds pass through during ontogeny. For this reason it has been argued that ratites are neotenous descendants of flying neognathous ancestors (De Beer 1956). Neoteny, i.e. the maintenance of juvenile characteristics in adulthood, is a common phenomenon proposed to be responsible for major morphological changes (Gould 1977) and it is known to be the result of a lower level of thyroid hormones during the development of many amphibians (Hanken 1992). Juvenile characteristics similar to those occurring in the ratites have been induced in neognathous birds, with thyroidectomy of young starlings leading to unproportionally long legs, short and wide beaks, and large and protuberant eyes (Dawson *et al*. 1994). In

addition, the fusion of the skull sutures was delayed and the plumage of thyroidectomized starlings remained downy with underdeveloped barbules. Furthermore and most significantly, the palatine bones remained juvenile at a size corresponding to that of three-week-old 'normal' starlings. In spite of these severe deviations, the thyroidectomized starlings became sexually mature. This experiment shows that a low level of thyroid hormones induces ratite-like features in developing neognathous birds, suggesting that a hormonal shift of this kind may have led to the establishment of neotenous characteristics in the ratites. This is consistent with observations that plasma levels of the thyroxine hormone are low in developing ostriches compared to other birds (Dawson *et al*. 1996).

In the light of the present findings, it is of interest that examination of the extensive DNA–DNA hybridization studies performed by Sibley & Ahlquist (1990) actually did suggest a clustering between Struthioniformes and Galliformes. Sibley & Ahlquist (1990) dismissed these affinities, however, after the introduction of extensive calibrations, allegedly related to the age at sexual maturity of different lineages. Unlike Sibley & Ahlquist's (1990) studies, the present and previous (Härlid *et al*. 1997, 1998) analyses included outgroups against which evolutionary rates could be tested. Quite inconsistent with Sibley & Ahlquist's (1990) studies, these analyses have not shown slower evolutionary rates in the Struthioniformes than in the other avian lineages examined. Our findings are also inconsistent with previous morphological interpretations and analyses of both aa and nt sequences (Stapel *et al*. 1984; Cracraft 1988; Caspers *et al*. 1997; Cooper & Penny 1997) which have supported a basal position for Struthioniformes in the avian phylogenetic tree.

The present findings, in conjunction with endocrinological studies (Dawson *et al*. 1994, 1996), suggest that the morphological characteristics of the ratites are not primitive but might be the result of thyroid-dependent neoteny. If this is correct, then it provides a unique example of thyroid-dependent neoteny being put into an avian phylogenetic context. The non-basal phylogenetic position of the Struthioniformes calls for further investigation of early avian evolution, with the particular aim of identifying additional avian divergences that might be basal to the Galliform–Struthioniform split.

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