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α-Crystallin Sequences Support a Galliform/Anseriform Clade

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An unresolved issue in higher avian systematics is the position of the fowl-like and the duck-like birds, Galliformes and Anseriformes, respectively. Most studies place these orders at the base of the neognath radiation. While DNA hybridization data support a sister-group relationship of Galliformes and Anseriformes, macromolecular sequence analyses have not yet been able to provide a clear-cut answer. In this study, we present nucleotide sequences coding for the eye lens proteins αA- and αB-crystallin of a palaeognath, a galliform, an anseriform, and two other neognathous birds. Phylogenetic analyses of this data set clearly support a galliform/anseriform clade, to the exclusion of other neognaths.

INTRODUCTION

Over the years, different phylogenetic hypotheses for the early lineages of birds have been proposed. Most studies placed the ratite/tinamou clade (Palaeognathae) as the sister-group of all other extant birds (Neognathae), as reviewed by Sheldon and Bledsoe (1993): morphology (Cracraft and Mindell, 1989), transferrin and albumin immunological distances (Prager and Wilson, 1976), reassessment of DNA hybridization data (Sibley and Ahlquist, 1990, p. 256), and amino acid sequences of the eye lens protein αA-crystallin (Stapel *et al.*, 1984; Caspers *et al.*, 1994). The aforementioned molecular studies, except for the albumin immunological distances (Prager and Wilson, 1976), also recognized a clade consisting of Neognathae without Galliformes (fowl-like birds, cracids, and megapodes) and Anseriformes (duck-like birds and screamers).

Whether Galliformes and Anseriformes diverged independently from the early neognathous lineage or rather are sister clades is less clear. Cracraft and Mindell (1989) recognized 11 morphological synapomorphies for a galliform/anseriform clade, but none for the remaining neognaths. Olson and Feduccia (1980), however, considered Galliformes and Anseriformes not to be sister taxa, and they linked Anseriformes to Charadriiformes (shorebirds), based on fossil evidence. Molecular studies have not been conclusive either. DNA hybridization data placed Galliformes and Anseriformes as sister-groups (Sibley and Ahlquist, 1990, p. 256), as did transferrin immunological distances (Prager and Wilson, 1976). However, upon reanalysis of the transferrin immunological distances, Cracraft and Mindell (1989) found two shorter trees, one with anseriforms outside a galliform/other neognath clade and one with galliforms outside an anseriform/other neognath clade. Ribosomal DNA restriction fragment length differences appeared to unite anseriforms and tinamous (Mindell and Honeycutt, 1989). Maximum parsimony analysis of ovomucoid amino acid sequences rendered Anseriformes paraphyletic and excluded them from a galliform/other neognath clade (Laskowski and Fitch, 1989). Maximum parsimony analysis of tandemly combined α- and β-hemoglobin sequences placed a clade consisting of galliforms, songbirds, and parrots as a sister-group to Anseriformes; in this study, however, the neognathous storks were the first offshoot of the avian lineage (Czelusniak *et al.*, 1990). A tree based on mitochondrial 12S and 16S rRNA gene sequences indicated a sister-group position of chicken and duck, albeit with a very low confidence probability value (Hedges *et al.*, 1995).

αA-Crystallin amino acid sequences had been unable to resolve the galliform/anseriform/other neognath trichotomy (Stapel *et al.*, 1984), but a 146-bp fragment of the αA-crystallin gene placed the duck as a sister-group of a galliform clade represented by chicken and silver pheasant (*Lophura nycthemera*), although with a low confidence probability value (Hedges *et al.*, 1995). This prompted us to investigate whether longer αA-crystallin nucleotide sequences would give more conclusive evidence as to the position of Galliformes and Anseriformes in avian phylogeny. To broaden our analysis, we also included sequences coding for another lens protein, αB-crystallin, in this study.

MATERIALS AND METHODS

αA-Crystallin cDNA sequences, coding for amino acid positions 12–160 of the 173-amino-acid-residue pro-

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tein, were amplified from domestic pigeon (*Columba livia*) total lens RNA and from a domestic duck (*Anas platyrhynchos*) lens cDNA library in phage lambda gt11 as described earlier (Caspers *et al.*, 1994; Hedges *et al.*, 1995). αB-Crystallin sequences, coding for amino acid positions 9–61, were amplified from total heart RNA of a thrush, the Eurasian blackbird (*Turdus merula*), and from elegant crested-tinamou (*Eudromia elegans*), as well as from pigeon genomic DNA. Primers and hybridization conditions for αA-crystallin sequences were as described in Caspers *et al.* (1994); primers and hybridization conditions for αB-crystallin sequences were as in Caspers *et al.* (1996), except that in the case of pigeon genomic DNA a hybridization temperature of 45°C instead of 55°C was used. Amplification products were cloned and sequences were determined as in Caspers *et al.* (1996). The sequences have been deposited in the EMBL database (Accession Nos. X96592–X96596).

Corresponding αA-crystallin sequences of chicken (*Gallus gallus;* Accession No. M17657), thrush (U31942), tinamou (L25850), red-eared slider turtle (*Trachemys scripta elegans;* U31938), human (*Homo sapiens;* U05569), and mouse (*Mus musculus;* J00375/J00376), as well as αB-crystallin sequences of chicken (S53164), duck (L08078), turtle (U31939), human (M28638), and mouse (M63170), were extracted from the aforementioned publications and from the databases.

Sequences were aligned with the program PILEUP from the GCG package (Devereux *et al.*, 1984). The position of a 3-bp gap in the avian and turtle αB-crystallin sequences, compared to the mammalian sequences, was manually adjusted to match the amino acid alignment (Caspers *et al.*, 1996). Phylogenetic analyses were performed with neighbor-joining methods (Saitou and Nei, 1987) (DNADIST and NEIGHBOR, or TREECON), using Kimura two-parameter distances (Kimura, 1980) or transversions only (Tajima and Nei, 1984). In addition, maximum parsimony and maximum likelihood methods (DNAPARS and DNAML, respectively) were employed. TREECON has been written by Van de Peer and De Wachter (1994); the other programs are from the PHYLIP package (Felsenstein, 1993). Confidence in the neighbor-joining and maximum parsimony analyses was assessed by bootstrapping (Felsenstein, 1985).

RESULTS AND DISCUSSION

α-Crystallins belong to the small heat-shock protein family (Caspers *et al.*, 1995). They occur abundantly in the vertebrate eye lens as multimeric complexes, composed of two types of homologous subunits, αA- and αB-crystallins (Groenen *et al.*, 1994). αB-Crystallin is also present in other tissues, most notably in the heart (Bhat and Nagineni, 1989). Both subunits are encoded by single copy genes (King and Piatigorsky, 1983; Quax-Jeuken *et al.*, 1985), which avoids the problem of paralogy in comparative studies. While αA-crystallin sequences have already contributed to resolving some important problems in avian phylogeny (Stapel *et al.*, 1984; Caspers *et al.*, 1994; Hedges *et al.*, 1995), avian αB-crystallin sequences have only been reported for chicken (Sawada *et al.*, 1992) and duck (Lee *et al.*, 1993).

We now determined nucleotide sequences coding for amino acid residues 12–160 of αA-crystallin for an anseriform (duck) and another neognath bird (pigeon), and sequences coding for residues 9–61 of αB-crystallin for a palaeognath (tinamou) and a pigeon. The alignments of these sequences with other available avian sequences and relevant outgroups have been deposited in the EMBL database under Accession No. DS27786.

A Kimura-distance neighbor-joining tree based on the tandemly aligned αA- and αB-crystallin sequences (607 bp) is shown in Fig. 1A. This tree groups chicken and duck together, with a bootstrap value of 97%, to the exclusion of pigeon. The palaeognathous tinamous constitutes the outgroup to this neognathous clade. Transversions-only neighbor-joining analysis, as well as maximum parsimony and maximum likelihood analyses, produces the same topology (not shown).

To include another taxon from the nongalliform, nonanseriform neognaths in our analyses, we deter-

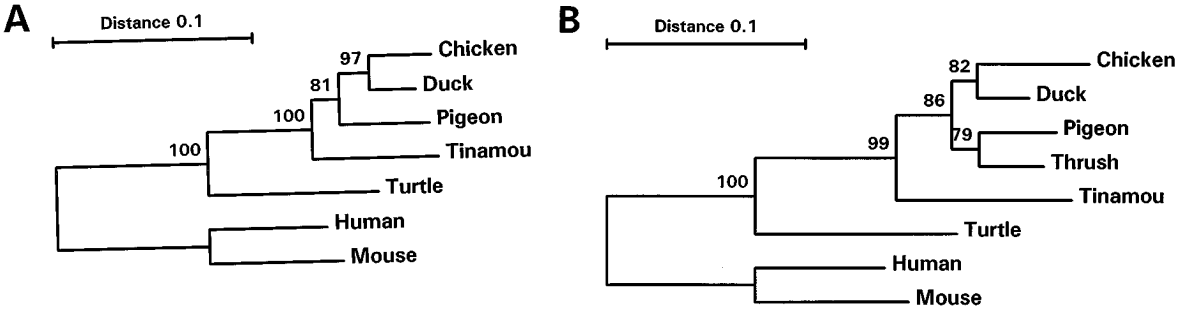

FIG. 1. Avian relationships inferred from (A) 607 and (B) 305 bp of combined αA- and αB-crystallin coding sequences, respectively. Neighbor-joining trees using Kimura distances are shown, constructed with TREECON (Van de Peer and De Wachter, 1994). Distances are proportional to the minimum number of mutations per residue. Bootstrap percentages from 1000 replications are indicated.

mined the corresponding αB-crystallin sequence of a thrush, the Eurasian blackbird. As we did not succeed in amplifying a larger part of the thrush αA-crystallin sequence than the 146 bp already reported in Hedges *et al.* (1995), this shortened our alignment from 607 to 305 bp. Nevertheless, in the Kimura-distance neighbor-joining analysis (Fig. 1B) chicken still clusters with duck, with a bootstrap value of 82%, and thrush clusters with pigeon in another neognath clade, with a bootstrap value of 79%. The transversions-only, maximum parsimony, and maximum likelihood analyses produce the same topology (not shown).

To see whether additional sequences that might shed light on the galliform/anseriform relationship are hidden in the databases, we searched the SwissProt (version 32.0), PIR (version 45.0), EMBL (version 44.0), and GenBank (version 89.0) databases for amino acid and protein-coding nucleic acid orthologous sequences of galliforms, anseriforms, and other neognaths, for which outgroup sequences were also available. We tried to construct phylogenetic trees from the few retrieved data sets, but this did not render promising results. Cytochrome b sequences appear unable to resolve higher order avian relationships (Edwards *et al.*, 1991). Cytochrome c and pancreatic polypeptide sequences contain insufficient phylogenetic signal (Eernisse and Kluge, 1993), and orthology of available keratin sequences could not be established. Sequences coding for another eye lens protein, δ-crystallin, presented another problem: Wistow and Piatigorsky (1990) noted that, while δ1-crystallin is a structural lens protein and δ2-crystallin is an active enzyme (argininosuccinate lyase), δ1- and δ2-crystallin of duck share the highest percentage identity with each other, as do chicken δ1- and δ2-crystallin. Indeed both chicken and both duck sequences are sister-groups in phylogenetic analyses using mammalian argininosuccinate lyase sequences as outgroups (not shown), indicating that they may have undergone gene conversion. This makes the position of the single known pigeon δ-crystallin sequence (Lin and Chiou, 1992) in the trees rather ambiguous.

This report presents the first macromolecular sequence analysis, based on nuclear genes, in which a sister-group relationship of galliforms and anseriforms is well supported. However, in this study only one galliform and one anseriform are included. Further research should evaluate more taxa, since monophyly of both the orders Galliformes and Anseriformes is not entirely warranted. Monophyly of the traditional Galliformes (i.e., including cracids and megapodes) is supported by DNA hybridization (Sibley and Ahlquist, 1990), ovomucoid amino acid sequences (Laskowski and Fitch, 1989), and transferrin and ovalbumin immunological distances (Prager and Wilson, 1976). Albumin and lysozyme c immunological distances, however, placed a cracid outside a clade consisting of other galliforms and anseriforms (Prager and Wilson, 1976). Ovomucoid amino acid sequences placed anhimids (screamers) outside a galliform/other neognaths clade. This anhimid/galliform/other neognath clade was in turn a sister-group of the other anseriforms (Laskowski and Fitch, 1989). Thus, subsequent studies on the phylogenetic position of Galliformes and Anseriformes should at least include a cracid, a megapode, and an anhimid as well. The present results demonstrate that even relatively short nuclear gene sequences may be informative in untangling such relationships.

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