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Phylogenetic relationships of the hoatzin, an enigmatic South American bird

(DNA sequences/phylogeny/systematics)

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Contributed by Charles G. Sibley, September 5, 1995

ABSTRACT **The hoatzin (*Opisthocomus hoazin*) lives in the humid lowlands of northern and central South America, often in riparian habitats. It is a slender bird ≈65 cm in length, brownish with lighter streaks and buffy tips to the long tail feathers. The small head has a ragged, bristly crest of reddish-brown feathers, and the bare skin of the face is bright blue. It resembles a chachalaca (*Ortalis*, Cracidae) in size and shape, but its plumage and markings are similar to those of the smaller guira cuckoo (*Guira guira*). The hoatzin (pronounced Watson) has been a taxonomic puzzle since it was described in 1776. It usually has been viewed as related to the gallinaceous birds, but alliances to other groups have been suggested, including the cuckoos. We present DNA sequence evidence from the 12S and 16S rRNA mitochondrial genes, and from the nuclear gene that codes for the eye lens protein, αA-crystallin. The results indicate that the hoatzin is most closely related to the typical cuckoos and that the divergence occurred at or near the base of the cuculiform phylogenetic tree.**

Most species of birds have obvious living relatives and are members of well-characterized groups. The hoatzin is one of the few that differ in so many ways from other birds that its nearest surviving kin have been uncertain. It was first described in 1776 as *Phasianus hoazin*, thereby suggesting a relationship to the galliform birds (pheasants, grouse, quail, etc.). Subsequent authors assigned the hoatzin to various groups, including the turacos (Musophagidae), hornbills (Bucerotidae), pigeons, cuckoos, and gruiforms (cranes, bustards, etc.). Some considered it a "link" between galliforms and cuckoos or placed it in a monotypic order, Opisthocomiformes, but an affinity to the Galliformes has been the most frequent opinion (for review, see ref. 1). Sibley and Ahlquist (2) compared the starch gel electrophoretic patterns of the egg-white proteins of the hoatzin with those of all pertinent groups and concluded "that it is most closely allied to the neotropical Crotophaginae"—the anis, a group of cuckoos. Sibley and Ahlquist (3) added comparisons of electrophoretic patterns of egg-white proteins produced by isoelectric focusing in acrylamide gel and came to the same conclusion. DNA–DNA hybridization comparisons were also interpreted as supporting this alliance (1). Bock (4) argued that the anisodactyl feet (three toes forward, one back) of the hoatzin precluded its derivation from a common ancestry with the cuckoos, which have zygodactyl feet (two toes forward, two back). Avise *et al.* (5) found that >900 bases of the mitochondrial gene for cytochrome *b* from 18 species of birds did not provide a clear answer to the question. These workers suggested that much longer sequences, including nuclear genes, might be required to resolve the older nodes in the phylogeny.

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The hoatzin feeds primarily on the tender young leaves, twigs, and shoots of trees and marsh plants, which are ingested into a huge, muscular crop with a deeply ridged interior lining where active foregut fermentation occurs. The hoatzin is the only bird that uses microbial foregut fermentation to convert cellulose into simple sugars, as do some groups of mammals, such as the ruminant ungulates, colobine monkeys, sloths, and macropodid marsupials. The bony sternum and pectoral girdle are modified to accommodate the filled crop, and there is a callosity on the bare skin of the breast where the heavy crop is rested on a branch. The proventriculus and gizzard are small, and the lower esophagus is sacculated, which delays the passage of particles into the lower gut, where additional fermentation occurs in the paired caeca. The contents of the crop and esophagus can account for up to 10% of total body weight. The rate of food passage through the digestive system is rapid in many birds, but hoatzins retain liquids for ≈18 hr and solids for 24–48 hr, similar to retention times in sheep, which also must digest leafy food (6–8).

A bacteriolytic lysozyme is expressed at high levels in the stomach and serves to aid in the digestion of the fermentative foregut bacteria as they pass through the stomach, thus preventing the loss of nutrients assimilated by the microorganisms. The amino acid sequence of this lysozyme is more similar to that of the rock pigeon (*Columba livia*) than to that of the gallinaceous domestic fowl (*Gallus gallus*) (9).

Hoatzins form large nonbreeding flocks, but with the first rains they break up into smaller groups of two to eight birds and defend small territories. They are communal (or cooperative) breeders with each breeding group consisting of an adult pair alone or with up to five helpers, usually previous offspring of the adults. Nests are usually built in bushes or trees over water. All group members participate in building the nest, incubating the eggs, and caring for the young. The clutch is usually two eggs; one or three eggs occur in ≈20–25% of nests. This communal pattern of breeding behavior is similar to that of the anis (*Crotophaga*) and the guira cuckoo (*Guira guira*). Nestling hoatzins have two claws on the wing, which they use to clamber about near the nest before fledging. If alarmed they may drop into the water and hide or swim under water using their wings and feet. They can climb back into the nest when the danger is past (7, 10).

In this paper we present additional DNA sequence data from two mitochondrial genes (12S and 16S rRNA) and a nuclear gene (αA-crystallin) relating to the phylogenetic position of the hoatzin.‖ These sequences provide strong statistical support for the conclusion that the hoatzin shares

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‖The sequences discussed in this paper have been deposited in the GenBank data base [accession nos. are X87853–X87868 for 12S and 16S rRNA (DS21861 for alignment) and U31938, U31940, U31942–U31947 for the αA-crystallin sequences].

a most recent common ancestry with the cuckoos (Cuculiformes).

MATERIALS AND METHODS

Mitochondrial 12S and 16S rRNA. DNA sequences of portions of these two mitochondrial genes totalling 1.7 kb of aligned sequence were obtained by two of us (S.B.H. and M.D.S.) from each of the following eight species of birds: hoatzin (*Opisthocomus hoazin*), greater ani (*Crotophaga ani*), yellow-billed cuckoo (*Coccyzus americanus*, a New World cuckoo), pallid cuckoo (*Cuculus pallidus*, an Old World cuckoo), rock pigeon (*C. livia*), budgerigar (*Melopsittacus undulatus*, an Australian parrot), mallard duck (*Anas platyrhynchos*), and greater rhea (*Rhea americana*). Corresponding sequences from an American alligator (*Alligator mississippiensis*; accession no. L28074) and a domestic fowl (*G. gallus*; accession no. X52392) were obtained from the data bases for comparison. Latin and English names are from Monroe and Sibley (11).

Methods of DNA amplification, dideoxynucleotide sequencing, and sequence analysis were the same as those described (12), except that four additional primers were used: 12L1 (refs. 13 and 14; in combination with 12H4), 16H1 (ref. 14; with 16L1], 16L10 (ref. 14; with 16H10), and 16H14 (International Union of Pure and Applied Chemistry code: AYY CTT GTT ACT CAT WTT ARC A; with 16L2a). The same two portions of the 12S and 16S rRNA genes were amplified and sequenced. As before, sequence was obtained from both complementary strands, except for the initial 105 bp of the 16S fragment (light strand only) and the 50-bp region spanning primer 16L1 (heavy strand only).

Sequences were aligned (15), and both sequenced regions were combined for analysis with MEGA (16). The neighbor-joining method (17) was used with Jukes–Cantor (18) distances, excluding sites containing gaps and ambiguities, and statistical confidence was inferred by a *t* test for the significance of the difference between branch length and zero (interior-branch test), expressed as the complement of the probability (P_c; refs. 16 and 19). Parsimony analysis was performed with MEGA. Recently, it was shown that the interior-branch test may give overestimates of statistical confidence in some cases, although there is little or no difference for P_c values >90% (20).

αA-Crystallin. DNA sequences of the amplified third exon of the αA-crystallin nuclear gene were obtained by three of us (M.A.M.v.D., G.-J.C., and W.W.d.J.) from each of the following species: hoatzin, groove-billed ani (*Crotophaga sulcirostris*), silver pheasant (*Lophura nycthemera*), sooty tern (*Sterna fuscata*), and a thrush, the Eurasian blackbird (*Turdus merula*). Corresponding fragments of αA-crystallin cDNA sequences were obtained from rock pigeon (*C. livia*) total lens RNA according to the method described by Caspers *et al.* (21), and from a mallard duck (*A. platyrhynchos*) lens cDNA library in phage λgt11 (22). Corresponding sequences from the elegant crested-tinamou (*Eudromia elegans*; accession no. L25850) and red-eared slider turtle (*Trachemys scripta elegans*) were obtained from previous studies (ref. 22; G.-J.C., G.-J. Reinders, J. A. M. Leunissen, J. Wattel, and W.W.deJ., unpublished data). Sequences from domestic fowl (*G. gallus*; accession no. M17627), house mouse (*Mus musculus*; accession no. J00376), and human (*Homo sapiens*; accession no. U05569) were obtained from the data bases for comparison.

Most of the third exon of αA-crystallin sequences (146 bp) was amplified by the PCR method using the Biometra (Tampa, FL) TRIO-thermoblock. Degenerated oligonucleotides (Eurogentec, Seraing, Belgium) were designed (OLIGO 4.0) to amplify sequences coding for amino acids 112–159 of the complete αA-crystallin chain. The sequences of these primers are: 5′-GAY GAC CAY GGC TAC ATN TC-3′ and 5′-TTY TCC TCC YGN GAC ACN G-3′. A total reaction volume of 50 μl was used. The dNTPs were from Boehringer Mannheim; the *Taq* polymerase was from Wiljan Hendriks (University of Nijmegen, Nijmegen, The Netherlands); and the buffer contained 2.0 mM $MgCl_2$, 20 mM Tris·HCl, 50 mM KCl, 0.27% Tween 20, 0.27% Nonidet P-40, and bovine serum albumin at 0.2 mg/ml. As template we used genomic DNA of the Eurasian blackbird isolated from liver, nuclear DNA of the hoatzin, groove-billed ani, and silver pheasant, and nuclear DNA of the sooty tern. To each reaction, 200 ng of template were added. Optimization of the PCR led to the following program: 10 min at 95°C, 30 cycles of 2 min at 95°C denaturation; 1 min at 54°C annealing and 30 sec at 72°C extension, and finally 7 min at 95°C denaturation. To obtain a specific reaction, 5 μl of PCR product of the hoatzin, groove-billed ani, and silver pheasant were reamplified using the same program. The PCR products were directly ligated into a T-vector using a TA cloning kit (Invitrogen or Promega). All sequences were amplified in triplicate and sequenced in both directions, using the Sequenase version 2.0 sequencing kit (United States Biochemical).

Sequences were aligned with PILEUP from the Genetics Computer Group package (24). Phylogenetic analyses were done with neighbor-joining (17), MEGA (16), maximum parsimony [DNAPARS in PHYLIP (25)], and maximum-likelihood [PHYLIP (25)]. The neighbor-joining method used a Kimura (16, 26) distance using only transversions. Statistical confidence in the neighbor-joining analyses was assessed by the interior branch test (19) in PHYLTEST (27).

RESULTS

Mitochondrial 12S and 16S rRNA. Sequences from these two genes totaled 1725 aligned sites (12S rRNA = 396 bp; 16S rRNA = 1329 bp), including 677 variable (excluding sites containing gaps and ambiguities) and 388 parsimony sites. A phylogenetic tree of nine avian taxa (Fig. 1*A*), rooted with alligator, joins the Old World and New World cuckoos (P_c = 99%), all three cuckoos (P_c = 98%), and places the hoatzin as the closest relative of these cuculiforms (P_c = 96%). The relationships of the remaining orders are well supported, and most aspects of the branching pattern agree with the results of

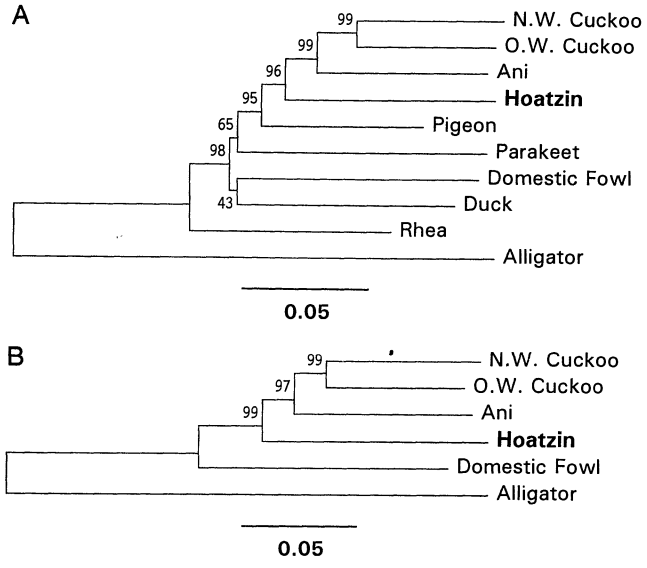

FIG. 1. Phylogenetic relationships of the hoatzin and eight other species of birds inferred from DNA sequences of portions of the mitochondrial 12S rRNA and 16S rRNA genes (1.7 kb). Trees were constructed by the neighbor-joining method with Jukes–Cantor distance; confidence probability values (P_c) are indicated on the nodes. (*A*) All sequences. (*B*) Subset of taxa including the hoatzin, two cuckoos and a galliform, with a reptile as the outgroup.

DNA–DNA hybridization (1) and with traditional classifications based on morphology. The use of other distance measures [Kimura, Kimura (transversions), Tajima–Nei, Tamura, Tamura–Nei, Kimura-γ (γ parameter = 0.80) and Kimura-γ (transversions)] with neighbor-joining all yielded strong support for a hoatzin–cuculiform relationship; only the position of the duck differed from Fig. 1*A* in some of the trees, branching after the domestic fowl rather than together with it. When sequences of 15 additional species of birds of the order Ciconiiformes (12) were added to the alignment and analyses, the hoatzin also clustered with the cuculiforms (P_c = 93%).

Because the position of the hoatzin in the avian tree has centered on its relationship to either gallinaceous birds (Galliformes) or cuckoos (Cuculiformes), a separate analysis was done using only the DNA sequences for those groups. The same result was obtained (Fig. 1*B*), but with greater statistical confidence for a hoatzin–cuckoo relationship (P_c = 99%). Parsimony analysis of all taxa, and of the galliform–cuculiform subset, each resulted in a single tree showing a hoatzin–cuculiform relationship (data not shown).

αA-Crystallin. The nucleotide sequence of most of the third exon of the αA-crystallin gene of the hoatzin was determined and corresponds to residues 112–159 of the protein. This sequence contains the phylogenetically most informative amino acid replacements in avian αA-crystallins (28). The hoatzin has residues Ala-127 and Ala-152, which are thought to have originated in the neognathous lineage after the divergence of the galliforms and anseriforms (21, 28). At position 135, however, it shares serine with galliforms and paleognaths. The hoatzin shares a unique replacement with the groove-billed ani, Ser-155 → Val, which requires two base substitutions. These observations favor a closer relationship between the hoatzin and the cuckoos than between hoatzin and the galliforms. However, because of the small number of amino acid replacements, tree constructions were done on the nucleotide sequences.

The neighbor-joining tree (Fig. 2*A*) places the hoatzin as the closest relative of the cuculiforms (P_c = 79%). When only hoatzin, cuckoo, galliform, and an outgroup (turtle) are compared (Fig. 2*B*), the support for the hoatzin–cuckoo grouping increases (P_c = 91%). Branch lengths in both trees show rate variation, but this is expected because the small number of total sites was reduced even further by considering only transversions and thereby increasing the variance. The maximum-parsimony consensus tree of the total sequences yielded the same topology as in Fig. 2*A*, and the maximum-likelihood tree also grouped hoatzin with the cuckoo and joined that clade with the pigeon and tern clade. Thus, the αA-crystallin data support a closer relationship between the hoatzin and the cuckoo than between the hoatzin and any other taxon in this analysis.

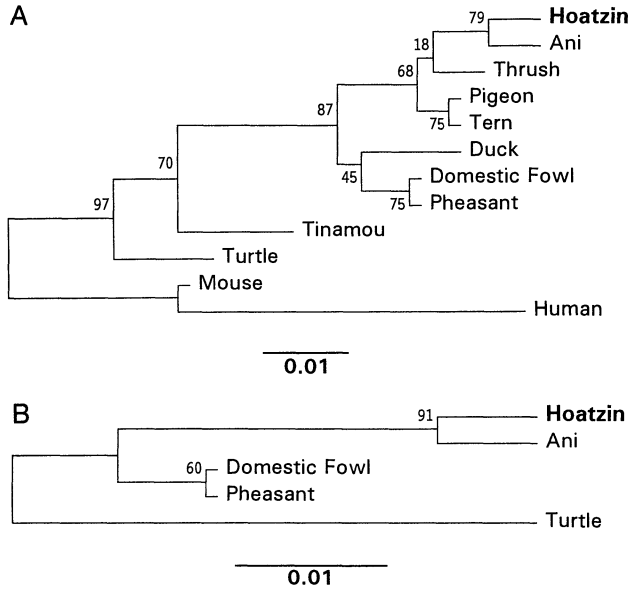

FIG. 2. Phylogenetic relationships of the hoatzin and other birds inferred from DNA sequences of a portion of the nuclear gene encoding αA-crystallin (146 bp). Trees were constructed by the neighbor-joining method with a transversion distance. Confidence probability values (P_c) are indicated on the nodes. (*A*) All available sequences. (*B*) Subset of taxa including the hoatzin, a cuckoo, and two galliforms, with a turtle as the outgroup.

Table 1. Molecular evidence for the closest relative of the hoatzin

	Sites, no.		Closest relative,* %	
	Total	Variable	Galliform	Cuculiform
12S and 16S rRNA	1725	651	0 (0)	99 (100)
Cytochrome *b*	961	435	2 (0)	97 (99)
αA-crystallin	146	42	2 (0)	96 (91)
Combined	2832	1128	0 (0)	100 (100)

*Bootstrap *P* values (ref. 23; 2000 replications) and P_c values (in parentheses) for neighbor-joining analyses using a transversion distance (16, 26) and four taxa: hoatzin, a cuculiform (ani), a galliform (domestic fowl), and an outgroup (alligator for 12S and 16S rRNA, human for cytochrome *b*, and turtle for αA-crystallin).

DISCUSSION

Analyses of both data sets provide support for a closer relationship between the hoatzin and the cuckoos than between the hoatzin and any other group of living birds examined. This result agrees with DNA–DNA hybridization evidence (1) but not with most morphological analyses. The principal alternative hypothesis is that the hoatzin is a galliform or more closely related to that group than to the cuckoos. Analyses of the present data are in Figs. 1*B* and 2*B* for comparisons among the hoatzin, cuckoos, and galliforms. Support for a hoatzin–cuckoo relationship is stronger in these comparisons (P_c = 99%, 12S and 16S rRNA; 91% αA-crystallin). The problem can be further reduced by treating this as a four-taxon question and using taxa in common among the three genes examined herein and the cytochrome *b* data (5). When this is done, the available DNA sequences provide statistically significant support for the hoatzin–cuckoo relationship (Table 1; Fig. 3).

Three major lineages of cuckoos may be recognized (1): the Old World cuckoos and coucals (Cuculidae, Centropodidae), the American cuckoos (Coccyzidae), and the mainly Neotropical roadrunners, anis and guira cuckoo (Neomorphidae, Crotophagidae). Representatives of each lineage are included in the 12S and 16S rRNA data, and they form a statistically significant monophyletic group (P_c = 97–98%) with the hoatzin the next closest relative of this clade. This result differs from the interpretation of the DNA–DNA hybridization results (1), in which the hoatzin was placed in a basal cuculiform

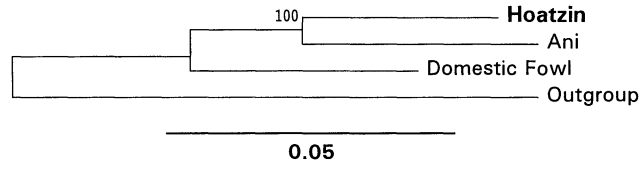

FIG. 3. Phylogenetic tree of the hoatzin, a cuckoo, a galliform, and an outgroup species inferred from DNA sequences of portions of the mitochondrial 12S and 16S rRNA genes, cytochrome *b* gene (5), and the nuclear αA-crystallin gene (total = 2832 bp). The tree was constructed by the neighbor-joining method with a transversion distance. The confidence probability value (P_c) and bootstrap *P* value for the hoatzin–cuckoo relationship are 100%.

clade, but closer to the crotophagids than to the other lineages. The DNA sequence data suggest that the hoatzin is the sister group to the other cuculiforms, and we propose to place it in the suborder Opisthocomi with the other cuckoos in the suborder Cuculi.

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