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# Identification of the Extinct Hawaiian Eagle (*Haliaeetus*) by mtDNA Sequence Analysis

ROBERT C. FLEISCHER,[1,2,5] STORRS L. OLSON,[3] HELEN F. JAMES,[3] AND ALAN C. COOPER[2,4]
[1]*Department of Biological Sciences, University of Durham, Durham DH1 3LE, United Kingdom;*
[2]*Molecular Genetics Laboratory, National Zoological Park, Smithsonian Institution, Washington, D.C. 20008, USA;*
[3]*Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560, USA; and*
[4]*Departments of Zoology and Biological Anthropology, University of Oxford, Oxford, OX2 6QS, United Kingdom*

Among the many bones of extinct birds discovered in the first extensive deposits studied in the Hawaiian Islands were a few from Molokai and Oahu that clearly belonged to a large sea-eagle of the genus *Haliaeetus*, as shown by their large size and the fusion of the phalanges of the inner toe (Olson 1982, Olson and James 1982). Even with the discovery of a nearly complete skeleton of this eagle on Maui, its specific identification could not be entirely resolved. Size and other characters eliminated most species of the genus, but no qualitative osteological characters could be discerned between the Hawaiian fossils and available skeletons of the Old World White-tailed Eagle (*H. albicilla*) and the New World Bald Eagle (*H. leucocephalus*; Olson and James 1991). Unable to make a morphological resolution, Olson and James (1991:62) were constrained to list this interesting specimen as "*Haliaeetus* sp., aff. *H. leucocephalus*/*H. albicilla*." To identify the Hawaiian eagle more satisfactorily, we turned to "ancient" DNA methods to obtain mitochondrial DNA (mtDNA) sequences from its bones.

The bones used in ancient DNA analysis were obtained from a nearly complete skeleton found on 4 April 1988 in a good state of preservation in Puu Makua Cave, a lava tube at 1,463 m elevation on the south slope of Mt. Haleakala, Maui (Olson and James 1991). The eagle had died in a cave that served as a natural trap for flightless species such as moa-nalos, rails, and ibises. The only entrance to the cave is a skylight of approximately 5 m diameter above a vertical drop of approximately 22 m. The eagle may have flown into the cave to scavenge flightless birds that had fallen in beforehand. The eagle presumably became trapped because the entrance was too small for such a large bird to be able to fly back out.

Previous analyses showed that the skeleton contained 4.1% nitrogen and amino acids in proportions only slightly different from those of modern cow bone (T. Stafford, Jr. pers. comm.). An AMS radiocarbon date on the skeleton gave an age of 3,300 ± 60 years (two-sigma calibrated range 3,389 to 3,689 years; see James 1995). Because the nitrogen and amino acid content suggested that organic compounds

[5] Address correspondence to Molecular Genetics Laboratory, National Zoological Park, Smithsonian Institution, Washington, D.C. 20008, USA. E-mail: rfleischer@nzp.si.edu

were well preserved in the skeleton, and despite its antiquity, we chose this specimen for ancient DNA analysis.

We isolated DNA from two bones from the skeleton in two separate laboratories dedicated to ancient DNA analysis. DNA was amplified from the Hawaiian eagle bones for three different gene regions of mtDNA. We compared sequence from part of the cytochrome-*b* (cyt *b*) gene with cyt-*b* sequences obtained from GenBank for seven of the eight species of *Haliaeetus* known worldwide (Seibold and Helbig 1996). We also obtained sequences of the ATP subunit 8 gene (ATPase8) and part of the 12S ribosomal RNA gene (12S rRNA) and compared them with sequences we obtained from individuals of *H. leucocephalus, H. albicilla,* and *H. leucogaster.*

*Methods.*—Two bones were selected for analysis from the nearly complete skeleton found in Puu Makua Cave (USNM 431238): a thoracic rib fragment and a pedal phalanx. We obtained two tissue samples of *H. albicilla* from the Swedish Museum of Natural History (nos. 501 and 502) and feathers of *H. albicilla* and *H. leucogaster* (White-bellied Fish-Eagle) from captive birds in the United States. DNA samples of *H. leucocephalus* were obtained from W. Piper.

All pre-PCR protocols were conducted in isolated ancient DNA laboratories located in buildings different from the ones containing the primary DNA laboratories. DNA isolations and PCRs for ATPase8 and 12S rRNA segments were conducted at the National Zoological Park (NZP) by A. C. Cooper, and DNA isolations and PCRs for cyt *b* and 12S rRNA were conducted at the University of Durham by R. Fleischer. Methods to reduce the probability of contamination followed Handt et al. (1994) and included the use of barrier pipet tips; sterile solutions; DNA destruction with UV illumination; liberal washing of equipment and bench surfaces with bleach solutions; and the use of extract, PCR, and "large DNA" controls. In addition, equipment and supplies were dedicated to the ancient DNA laboratories, and work was not conducted in an ancient DNA laboratory after working in the main laboratory on the same day.

Isolation protocols in the two laboratories were very similar. A small piece of each *Haliaeetus* bone was cut away and cleaned in sterile water. This was followed by a wash with rotation at room temperature in sterile 0.5 M EDTA, after which the EDTA was discarded. At Durham, the bone was wrapped in heavy aluminum foil (which had been decontaminated of DNA by UV illumination for 20 min) and crushed in a mortar and pestle (decontaminated by soaking in 50% bleach solution). At the NZP, a bleach-sterilized coffee grinder was used to pulverize the bone sample. The crushed bone was placed in 5 mL of sterile UV-treated lysis buffer (0.01 M Tris, ph 8.0; 0.002 M EDTA; 0.01 M NaCl; 10 mg/mL DTT; 1% SDS; and 1 mg/mL Proteinase K) and rotated overnight at 60°C. The sample was then extracted twice with 5 mL of phenol and once with 5 mL of chloroform. The supernatant was placed in 1 mL of sterile water in a Centricon 30 microconcentrator and centrifuged until about 50 to 100 μL covered the membrane. A second 1 mL of sterile water was added and the centrifugation repeated. The remaining sample was collected from the membrane by a short centrifugation and diluted to 200 μL total volume. From 2 to 4 μL of this were used in subsequent PCR experiments.

We attempted PCR amplifications with primers that amplify different-sized segments of the three mtDNA genes: 12S rRNA, ATPase8, and cyt *b*. We designed a set of primers (AncEag. 2: forward, 5′-AGGAATCTGCCTACTAACACA-3′, 3′ is base 15,026 of *Gallus gallus* sequence; Desjardins and Morais 1990; reverse, 5′-TTCGACATGTATGGGCTACG-3′, 3′ is base 15,090) from *Haliaeetus* eagle cyt-*b* sequence (Seibold and Helbig 1996) such that they flanked a small (63 base pairs [bp]) region that contained four transitional substitutions between *H. albicilla* and *H. leucocephalus* and also differed by at least this much from the other five *Haliaeetus* species. The AncEag. 2 product size was 104 bp.

Primers cyt-*b* 2-rc (5′-TGAGGACAAATATCC TTCTGAGG-3′, 15,320 of *Gallus,* reverse complement of cyt *b* 2; Kocher et al. 1989) and cyt-*b* ack (5′-CCTCCTCAGGCTCATTCTAC-3′, 15,376 of *Gallus*) amplified a 98-bp segment of cyt *b* disjunct from AncEag. 2. The 12S rRNA segments were amplified and sequenced using primers 12SA, 12SH, 12SJ, and 12SB2 (Cooper 1994), producing 392 bp of sequence. The ATPase8 segment included 35 bp of tRNA-lysine and was amplified and sequenced with "Birds-R-Us" (5′-TGGTCGAAGAAGCTTAGGTTCA-3′, 9,241 of *Gallus*) and t-lys (5′-CACCAGCACTAGCCTTTT AAG-3′, 9,051 of *Gallus*).

Final PCR components in a 50-μL reaction volume were 1X *taq* buffer (Perkin Elmer), 1.25 units of *Taq*-gold polymerase, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 3 mg/mL non-acetylated BSA, and 1 μM of each primer. PCR was conducted for 45 cycles beginning with a "hot-start" for 10 min at 94°C and ending with a 6-min soak at 72°C. Conditions per cycle were 92°C denaturation for 40 s, 50°C annealing for 1 min, and 72°C extension for 1 min.

Successful amplification was determined by visualizing PCR products on a 2% agarose gel. Single-banded products were cleaned by centrifugation using a Qiagen kit following the manufacturer's instructions. About 100 ng of product were used for cycle sequencing of both strands using dye-labeled dideoxynucleotide terminators; resulting sequence reactions were run on an ABI 377 sequencer. We assessed chromatograms for accuracy and aligned sequences using Sequencher 3.0.

We compared sequences from the Hawaiian eagle bones directly with those of other *Haliaeetus* species. We also conducted a total-evidence phylogenetic

analysis of the sequences of the Hawaiian bone and composite sequences from three outgroup *Haliaeetus* taxa. Replicate sequences for *H. leucocephalus* and *H. albicilla* for ATPase8, and for *H. leucocephalus* for 12srRNA, were identical; thus, we used only one sequence from these to represent each taxon in the analysis. The cyt-*b* sequences of *H. albicilla, H. leucocephalus,* and *H. leucogaster* were from a different study and therefore arise from different individuals than the sequences obtained for these same species in this study. Nonetheless, we combined the gene regions into the full 704 bp of sequence (shown abridged in Table 1 as variable sites only) and conducted an exhaustive search, with no character weighting, in PAUP* (Swofford 1999). We also ran a bootstrap (1,000 replications) using a branch-and-bound search algorithm to determine the relative support for different clades. In addition, we constrained the Hawaiian bone to be a sister taxon to *H. albicilla* in one tree and *H. leucocephalus* in another and then used a Kishino-Hasegawa test (Kishino and Hasegawa 1989) to determine whether the alternative trees differed significantly in length.

*Results.*—We obtained a total of 704 nucleotide sites of sequence from the Hawaiian eagle bones: 392 nucleotide sites of 12S rRNA, 118 bp of cyt *b*, and 194 bp of ATPase8 (Table 1). We compared the cyt-*b* sequences with those obtained by Seibold and Helbig (1996) for seven species of *Haliaeetus,* and the 12S and ATPase8 sequences with ones we obtained from *H. albicilla, H. leucocephalus,* and *H. leucogaster* (Table 1). The cyt-*b* sequence of the Hawaiian *Haliaeetus* was identical to that of *H. albicilla* and differed by 5 to 17 bp from all other *Haliaeetus* taxa, including *H. leucocephalus.* The more-conserved 12S sequence was identical to that of *H. albicilla* but differed by two transitions from the sequence for *H. leucocephalus* and by 24 substitutions from *H. leucogaster.* The Hawaiian eagle bone ATPase8 sequence differed at only 3 positions from *H. albicilla,* but differed at 6 positions from *H. leucocephalus* and at 24 positions from *H. leucogaster.*

In summary, the Hawaiian *Haliaeetus* bone sequences differed at only 3 of 704 bp (0.4 %) from the White-tailed Eagle, but by 13 of 704 (1.8 %) from the Bald Eagle. Divergence of the Hawaiian eagle bone sequence from the Bald Eagle sequence was significantly greater than its divergence from the White-tailed Eagle sequence (Fisher exact test, $P = 0.021$). Divergence of the Hawaiian bone sequence from the White-tailed Eagle sequence was not significantly different from zero (Fisher exact test, $P = 0.250$).

The three ATPase8 sequence differences between the Hawaiian eagle skeleton and White-tailed Eagle sequences included two A-G transitions and one A-T transversion (Table 1). Because of the rarity of A-T transversions in mtDNA at this level of divergence, and the fact that the T site of the latter was unique to the Hawaiian eagle bone, it is possible that it repre-

TABLE 1. Sixty-eight variable nucleotide sites from 704 sites of mtDNA sequence obtained from two bones of an eagle skeleton found in Puu Makua Cave, Maui, Hawaii (USNM 431238) aligned against sequences of three species of *Haliaeetus.* Outgroup sequences for cyt *b* are from Seibold and Helbig (1996). GenBank accession numbers are X86753 for *H. albicilla,* Z73467 for *H. leucocephalus,* and Z73468 for *H. leucogaster;* outgroup sequences for the other two genes are from this study. Nucleotide sites 1 to 15 are from 118 bp of cyt-*b* sequence, sites 16 to 41 are from 392 nucleotide sites of 12S rRNA sequence, and sites 42 to 68 are from 194 nucleotide sites of ATPase8 sequence. Dots indicate identity of the base with the Hawaiian eagle sequence.

| Sample | 1 2 3 4 5 6<br>123456789012345678901234567890123456789012345678 |
|---|---|
| Hawaiian bone | TCCGTTTTTTTCAACACACACTTCAATTCTCGGCTCTGAGAAAGTCCAACTATTTACAAACTTAAACGC |
| *H. albicilla* | ..... A..... G.A. |
| *H. leucocephalus* | C..AC.C.C..... G..GG.A..G...T..C..... A. |
| *H. leucogaster* | CTTACCCCCCTCGTGTCACCTGGCCTCTAATCTCA.AG..AGTT.GACTCC.GTGGCTCCCGGTCG |

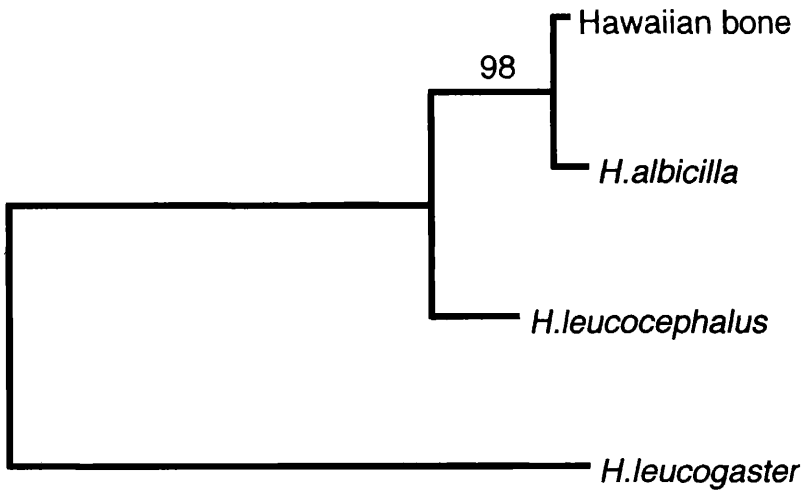


FIG. 1. Total-evidence parsimony phylogram showing relationships among mtDNA sequences from Hawaiian bone and from three species of *Haliaeetus* (see Table 1). Note that the outgroup sequences are chimeric for individuals: ATPase8 and 12S rRNA were obtained from individuals sequenced as part of this study, and cyt-*b* sequences are from different individuals described in Seibold and Helbig (1996). The 98% bootstrap value for the Hawaiian bone and *H. albicilla* clade is on the clade's node.

sents a postmortem artifact rather than a real substitution. Other than this single change, which does not modify our conclusions, no other differences are suggestive of artifacts owing to environmental modification.

The reconstructed phylogeny (Fig. 1) shows the close relationship of the Hawaiian bone sequences to those of *H. albicilla* and their more-distant relationship to *H. leucocephalus* and the outgroup taxon *H. leucogaster.* Bootstrap support for a Hawaiian bone/*H. albicilla* clade was very high (98%). Constraining the Hawaiian bone sequences to be sister to *H. leucocephalus* increased the tree length by five steps, but the Kishino-Hasegawa test was not quite significant in supporting the Hawaiian bone/*H. albicilla* clade over the Hawaiian bone/*H. leucocephalus* clade ($t$ = 1.9, $P$ = 0.058).

*Discussion.*—We obtained mtDNA sequences from two subfossil Hawaiian eagle bones that are nearly identical to ones from *Haliaeetus albicilla.* Our analyses were conducted in two separate laboratories using independently purchased supplies, solutions, and synthesized primers. No outgroup samples of *Haliaeetus* were present in the laboratories used at Durham University (where the cyt-*b* and some 12SrRNA sequences were obtained). In the NZP laboratory (where the ATPase8 and 12SrRNA sequences were obtained), all outgroups were analyzed after DNA isolation from the subfossil. Thus, it is extremely unlikely that these sequences are the result of contamination by external sources of DNA.

It is remarkable that DNA and amino acids are well preserved in this skeleton that has lain in a cave in a subtropical climate for perhaps 3,500 years, especially when considering that the eagle skeleton was exposed to dripping water in the cave and was moist when collected. Factors that may have favored preservation of organic compounds include the relatively constant, cool temperatures of this high-elevation cave, and the fact that the eagle corpse was always protected from UV radiation, being preserved in darkness.

Our results show that DNA sequences of the Hawaiian *Haliaeetus* are more similar to sequences of the White-tailed Eagle than they are to those of the Bald Eagle. They do not support a hypothesis that the Hawaiian eagle skeleton is conspecific with the Bald Eagle. The protein-coding sequence divergence (i.e. cyt *b* and ATPase8, combined) between the two (3.5%) is higher than might be expected for a within-species level divergence in birds and more typical of divergence between congeneric species (Klicka and Zink 1997, Avise and Walker 1998). On the other hand, the divergence between the White-tailed Eagle and Hawaiian bone protein-coding sequences (1.0%) is much lower and is within the typical range of within-species variation. Our results do not allow us to reject the hypothesis that the Hawaiian skeleton is that of a White-tailed Eagle, or its very close relative. Therefore, we suggest that the Hawaiian eagle is conspecific with and represents a disjunct Hawaiian population of the White-tailed Eagle.

The similarity of the sampled sequence from the Hawaiian eagle to that of the White-tailed Eagle also suggests that this species colonized the archipelago at a relatively late date, although one that apparently

preceded the arrival of humans (James 1995). The relative recency of colonization is also supported by the absence of eagle fossils in the sediments of a Pleistocene lake at Ulupau Head, Oahu (James 1987) and the lack of skeletal differences between the Hawaiian eagle and *H. albicilla* (Olson and James 1991). The Hawaiian eagle and the Black-crowned Night-Heron (*Nycticorax nycticorax*) are the only lineages of Hawaiian birds that successfully colonized the archipelago without the aid of humans, but that have not differentiated at least to the level of subspecies. This, and other evidence (e.g. Olson and James 1991, Fleischer and McIntosh 2000) suggest that colonization of the Hawaiian Islands is an ongoing process and that "equilibrium" diversities predicted by theories of island biogeography have not yet been reached.

Although the eagle appears to have been the only indigenous predator capable of depredating the large flightless waterfowl of the islands (see Olson and James 1991), genetic and other data indicate that these waterfowl existed in the islands well before the arrival of *H. albicilla* (Paxinos 1998, Sorenson et al. 1999).

Our study demonstrates the utility of analyzing fossil or ancient DNA for identifying species when other approaches will not work. The identification of the Hawaiian eagle adds another Palearctic element to the sources of colonization of the Hawaiian avifauna. Most avian lineages in Hawaii appear to have colonized from the New World, particularly North America, and very few from the Palearctic (Mayr 1943, Fleischer and McIntosh 2000). Other lineages of Palearctic origin include the Laysan Rail (*Porzana palmeri*; Olson 1973a,b; Fleischer and McIntosh 2000) and possibly the *Acrocephalus* warblers and the drepanidines (Groth 1994, Fleischer et al. 1998). Historical records of eagles in the Hawaiian chain include a Golden Eagle (*Aquila chrysaetos*) resident on Kauai for 17 years (Berger 1981, Pyle 1997) that may have originated in either North America or Asia, and the Asian Steller's Sea-Eagle (*H. pelagicus*) that was recorded from the Northwestern Hawaiian Islands of Kure and Midway (Pyle 1997). *Haliaeetus albicilla* is a latitudinal migrant in Eurasia, especially in the eastern part of its range (del Hoyo et al. 1994), and sometimes is a vagrant and thus could be expected to have reached the islands.

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# Are Nest Predation and Brood Parasitism Correlated in Yellow Warblers? A Test of the Cowbird Predation Hypothesis

CELIA M. MCLAREN AND SPENCER G. SEALY[1]
*Department of Zoology, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada*

Brown-headed Cowbirds (*Molothrus ater*) are generalist brood parasites that reduce the productivity of many of their passerine hosts through egg removal and competition between cowbird and host nestlings (Lowther 1993, Payne 1998, Lorenzana and Sealy 1999). Recently, it has been suggested that cowbirds exert a previously unappreciated influence on the demography of their hosts through predation (Arcese et al. 1992, 1996; Smith and Arcese 1994; Arcese and Smith 1999). The cowbird predation hypothesis (Arcese et al. 1992) was suggested to explain the frequently observed link between nest predation and interspecific brood parasitism in many passerines (e.g. Wolf 1987, Payne and Payne 1998). Specifically, the hypothesized link is a direct result of cowbird predation of host nests that are discovered too late in the host's nesting cycle for successful parasitism. This creates future opportunities for parasitism by causing hosts to renest (Arcese et al. 1996). Alternatively, a link between predation and parasitism may result if cowbird parasitism facilitates predation, or if both factors are correlated with a third variable such as nest vulnerability (Arcese and Smith 1999). Determining the independent effects of brood parasitism and nest predation on host populations is difficult because of this potential functional dependence (Pease and Grzybowski 1995, Arcese and Smith 1999).

In a resident population of Song Sparrows (*Melospiza melodia*) on Mandarte Island, British Columbia, the frequency of nest failure was positively related to the frequency of cowbird parasitism (Arcese et al. 1996). In 5 of 19 years when cowbirds were rare, absent, or removed on the island, nest failure was lower

[1] Address correspondence to this author. E-mail: sgsealy@cc.umanitoba.ca