Distinct and extinct: Genetic differentiation of the Hawaiian eagle

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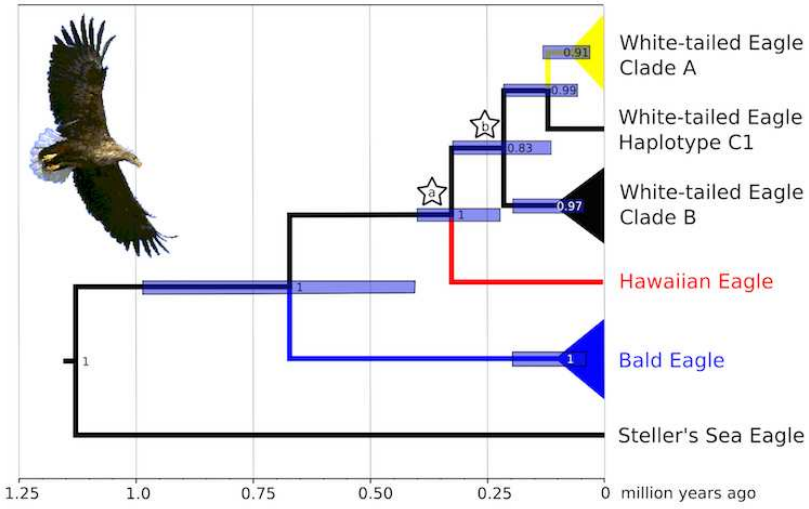
One-sentence summary:
Ancient DNA sequences show that eagle fossils from the Hawaiian Islands represent an extinct, long-term endemic lineage of *Haliaeetus*.

Keywords
Ancient DNA, bald eagle, island endemic, *Haliaeetus albicilla*, *Haliaeetus leucocephalus*, mtDNA



Eagles currently occur in the Hawaiian Islands only as vagrants, but Quaternary bones of *Haliaeetus* eagles have been found on three of the major islands. A previous study of a ~3,500 year-old skeleton from Maui found its mtDNA more similar to White-tailed (*H. albicilla*) than to Bald (*H. leucocephalus*) Eagles, but low intraspecific resolution of the markers and lack of comparative data from mainland populations precluded assessment of whether the individual was part of the diversity found in Eurasia, or whether it represented an endemic Hawaiian lineage. Using ancient DNA techniques, we sequenced part of the rapidly evolving mtDNA control region from the same specimen, and compared it to published range-wide control region data from White-tailed Eagles and newly generated sequences from Bald Eagles. Phylogenetic analyses indicated that the Hawaiian eagle represents a distinct (>3% divergent) mtDNA lineage most closely related to those of extant White-tailed Eagles. Based on fossil calibration, we estimate that the Hawaiian mtDNA lineage diverged from mainland sequences around the Middle Pleistocene. Although not clearly differentiated morphologically from mainland forms, the Hawaiian eagle thus likely constituted an isolated, resident population in the Hawaiian archipelago for more than 100,000 years, where it was the largest terrestrial predator.

1. Introduction

Although many distinctive species of birds have been found in the recent fossil avifauna of the Hawaiian Islands (Olson and James 1982, 1991; James and Olson 1991), some fossil species appear relatively undifferentiated at skeletal traits from extant mainland taxa, suggesting evolutionarily recent (i.e., Holocene) colonization or non-resident status. One such taxon was an eagle placed in the genus *Haliaeetus* on the basis of comparative osteology (Olson 1982, Olson and James 1982), represented by remains of a few individuals that were found on Oahu and Molokai (see Appendix A). No resident population of eagles has existed in Hawaii in modern times, although a White-tailed Eagle (*Haliaeetus albicilla*) and two Steller's Sea Eagles (*H. pelagicus*) have arrived in the islands as vagrants since the late 1970s (Zaun 2009, Pyle and Pyle 2009).

An earlier study (Fleischer et al., 2000), based on a ~3,500-year-old bone from the skeleton collected on Maui, showed from analysis of 704 basepairs (bp) of DNA sequences from three mtDNA genes (cytochrome *b*, ATPase8, and 12S rRNA), that the extinct Hawaiian eagle was more closely related to the Eurasian White-tailed Eagle than to the North American Bald Eagle (*H. leucocephalus*). However, the markers employed provided relatively little intraspecific resolution, and there was insufficient comparative data from populations of White-tailed Eagles to establish whether (i) Hawaiian eagle bones represent non-resident visitors to the islands from eastern Asia (Pyle and Pyle 2009, Zaun 2009), or (ii) the Hawaiian eagle was an endemic Hawaiian lineage of *Haliaeetus* that had colonized so recently as not to have become differentiated morphologically (Olson and James 1991, James 1995). Under the first hypothesis, Hawaiian eagle sequences would be highly similar or identical to sequences from the mainland. Under the second hypothesis, Hawaiian eagle sequences would be divergent from mainland sequences, indicative of long-term independent evolution in the archipelago.

Since the publication of Fleischer et al. (2000), extensive studies have been conducted on the phylogeography of the White-tailed Eagle, examining the highly variable mtDNA control region (Hailer et al., 2007, 2006; Honnen et al., 2010; Langguth et al., 2013; Ponnikas et al., 2013). Analysis of that locus in the Hawaiian eagle might therefore elucidate whether the lineage was evolutionarily distinct from Eurasian mainland populations. To investigate the two above hypotheses, we therefore obtained mtDNA control region sequences from the Hawaiian eagle, placing it in a range-wide phylogeographic context of White-tailed and Bald Eagles. We also generated a dated phylogeny of various taxa of *Haliaeetus*.

2. Material and methods

2.1. Lab procedures

We sequenced the same ancient skeleton of Hawaiian eagle that was studied by Fleischer et al. (2000), found in Puu Makua Cave on Maui (catalog number USNM 431238; Olson and James 1991; details in Appendix A). Amplifications utilized a

phenol-chloroform extract from 1998 (Fleischer et al., 2000). Primers were designed to amplify six overlapping fragments, each 122-143 base pairs (bp) long (see Appendix A). After assembly, this yielded the homologous 500 bp mtDNA control region fragment that has been characterized across the range of extant White-tailed Eagles. All PCR setup was done in a facility solely dedicated to ancient DNA work, following stringent protocols to avoid and detect potential contamination, using positive and negative amplification controls throughout (Fleischer et al., 2006, 2000). No *Haliaeetus* control region sequences had been amplified in the lab prior to this project. Two replicate PCRs and sequencing bouts were conducted, and two additional replicates that used an aliquot of the Hawaiian DNA extract that was treated with Uracil-N-glycosylase (UNG) to avoid potential sequencing artifacts resulting from DNA damage (Hofreiter et al., 2001). For further details of the PCRs and sequencing see Appendix A.

For comparison, data from recent phylogeographic studies of White-tailed Eagles (Hailer et al., 2007, 2006; Honnen et al., 2010; Langguth et al., 2013; Ponnikas et al., 2013) were used, plus the sequence of a Steller's Sea Eagle (GenBank accession AM156946). Because no mtDNA control region samples from Bald Eagles were available on GenBank, we also sequenced 12 Bald Eagle nestlings from northern Wisconsin, each from a different nest. All newly obtained sequences have been submitted to GenBank (accession numbers: LN623677 – LN623682, LN624632).

2.2. Statistical analyses

Phylogenetic networks were constructed in TCS 1.21 (Clement et al., 2000), and time-calibrated phylogenetic trees were obtained using BEAST 1.8.0 (Drummond et al., 2012) (see Appendix A). We applied two dating schemes, both utilizing fossil-based calibration points: In *scenario 1*, we used a wide, hence relatively uninformative lognormal prior for the divergence between White-tailed and Bald Eagles, with a mean at 800 thousand years ago (ka), truncating it at a minimum of 300 ka (Jefferson, 1985), and conservatively assumed a minimum age for the split of the Steller's Sea Eagle lineage at 800 ka (see Appendix A). We also included a maximum age for the Hawaiian lineage of 400 ka, based on the absence of *Haliaeetus* fossils in the paleontologically diverse and well-collected deposits at Ulupau Head lake that date back to 320-400 ka (James 1987; Hearty et al. 2005). At this site, fossils from other species of birds that were likely found in habitats similar to those of *Haliaeetus* are common, suggesting that the absence of *Haliaeetus* at this site indicates that the eagle colonized the archipelago more recently (see Appendix A). In a second scenario (*scenario 2*), more in line with the 2.5% divergence at *CytB* between White-tailed and Bald Eagles (Seibold and Helbig, 1996; Wink et al., 1996; see Appendix A) and published divergence rates (Lerner et al., 2011; Weir and Schluter, 2008), we utilized less conservative priors on the divergence times in BEAST, setting the divergence between Bald and White-tailed Eagles to be between 1.2-1.8 million years ago. In these analyses, we did not constrict the maximum age of the Hawaiian lineage, allowing for the possibility that it was present in the archipelago, but has not yet been recovered in older deposits. Both scenarios utilized a tip dating approach (Drummond et al., 2002), using the radiocarbon-dated age of the Hawaiian eagle bone (median probability: 3,531 calendar years). Convergence of the Bayesian analysis in BEAST was verified in Tracer (http://tree.bio.ed.ac.uk/software/tracer).

3. Results and discussion

Consistent with the findings of Fleischer et al. (2000), we infer that the Hawaiian eagle was either a conspecific of the White-tailed Eagle, or a very close relative. Further, our results indicate that the Hawaiian eagle may have persisted on the Hawaiian Islands from the mid Pleistocene until the late Holocene.

Several PCR replicates and overlapping reads from different primer pairs gave identical results for the Hawaiian eagle bone, whether the extract had been treated with UNG or not. This indicates that, despite the sample age, DNA damage was not common enough in the ancient DNA extract to significantly alter Sanger sequencing results. Consistent with this, the Hawaiian eagle sequence did not show any unusual substitution patterns when compared with congeneric sequences: along the 500 bp fragment, divergence of the Hawaiian eagle from extant White-tailed Eagles co-varied with that among published *Haliaeetus* sequences (Appendix A, Fig. S1).

The Hawaiian eagle sequence was on average 5.4% divergent from the six haplotypes we found in 12 Bald Eagle individuals, but only 3.3 and

3.2 % divergent (uncorrected p-distances) from the previously described Western (clade A) and Eastern (clade B) haplotypes of White-tailed Eagles from Eurasia. Similarly, phylogenetic analyses in BEAST confirmed with high posterior support (p>0.99: node "a" in Fig. 1B) that the Hawaiian eagle was more closely related to White-tailed than to Bald Eagles, lending further support to the results of Fleischer et al. (2000). An increased sampling of Bald Eagles is warranted to better understand the species' evolutionary and demographic history, but is unlikely to change our main conclusions. Our comparison of the Hawaiian eagle specimen's control region sequence with distribution-wide data from White-tailed Eagles revealed that the Hawaiian lineage is highly distinct from extant haplotypes. However, statistical support for intraspecific branchings in bifurcating trees was <95% (node "b" in Fig. 1B), precluding assessment of the exact placement of the Hawaiian lineage compared to extant White-tailed Eagle diversity. Nevertheless, our findings indicate that the Hawaiian eagle population was likely founded by individuals from Eurasia rather than North America, mirroring the arrival of vagrant Eurasian White-tailed and Steller's Sea Eagles in recent times.

Based on our employed calibration points for scenario 1, we estimate that the Hawaiian eagle lineage diverged from extant White-tailed Eagles during the Middle Pleistocene (95% highest posterior density (HPD) range: 223-400 ka: Fig. 1B). Based on scenario 2, all posterior divergence estimates were older, with the Hawaiian lineage diverging at 0.39 - 1.12, and Steller's Sea Eagles at 1.3-3.0 million years ago (95% HPD). Despite our use of a tip calibration, time dependency of the molecular clock could imply that we are overestimating divergence times, due to faster ticking of the molecular clock at recent times (Ho and Larson, 2006). Our dating is thus limited by a lack of precise fossil calibrations for *Haliaeetus*, and it should be verified using longer fragments of mtDNA, plus independently inherited loci. Hence, while we note that the inferred time frame should only be understood as a rough estimate, our results show that, across a broad range of conceivable calibration scenarios, the Hawaiian eagle diverged from extant *Haliaeetus* sequences long before the Holocene.

Our divergence time estimate suggests that the Hawaiian eagle was present in the Hawaiian avifauna for a relatively long period of time, during which it would have been the largest terrestrial predator on the islands of the archipelago. The eagle would have been capable of killing adults of the largest endemic flightless birds such as the moa-nalos *Thambetochen xanion* on Oahu and *T. chauliodous* and *Ptaiochen pau* on Maui Nui, and the flightless ibises of the genus *Apteribis* on Maui Nui (Olson and James 1991, James 1995). The extinction of the Hawaiian eagle could have been related to human-induced ecological changes, as was the case for the majority of Hawaii's endemic birds (James 1995). To date, however, there is no direct evidence for temporal overlap of the Hawaiian eagle with humans, so the factor(s) behind its extinction are unknown.

Other taxa in the Hawaiian avifauna have shown rapid adaptation to the island ecosystem, such as geese and rails that evolved flightlessness (Paxinos et al., 2002; Slikas et al., 2002). Among Hawaiian raptorial species, the harrier *Circus dossenus* evolved a morphology that is more *Accipiter*-like, presumably reflecting a shift in its prey base from rodents to birds (Olson and James 1991). Based on our results, the Hawaiian eagle would thus be a counter-example to the above cases, showing no apparent morphological changes in its skeleton compared with its mainland ancestors, despite being a relatively old colonizer of the archipelago. This indicates that the Hawaiian eagle may not have experienced such a clear shift in its niche after the colonization of Hawaii.

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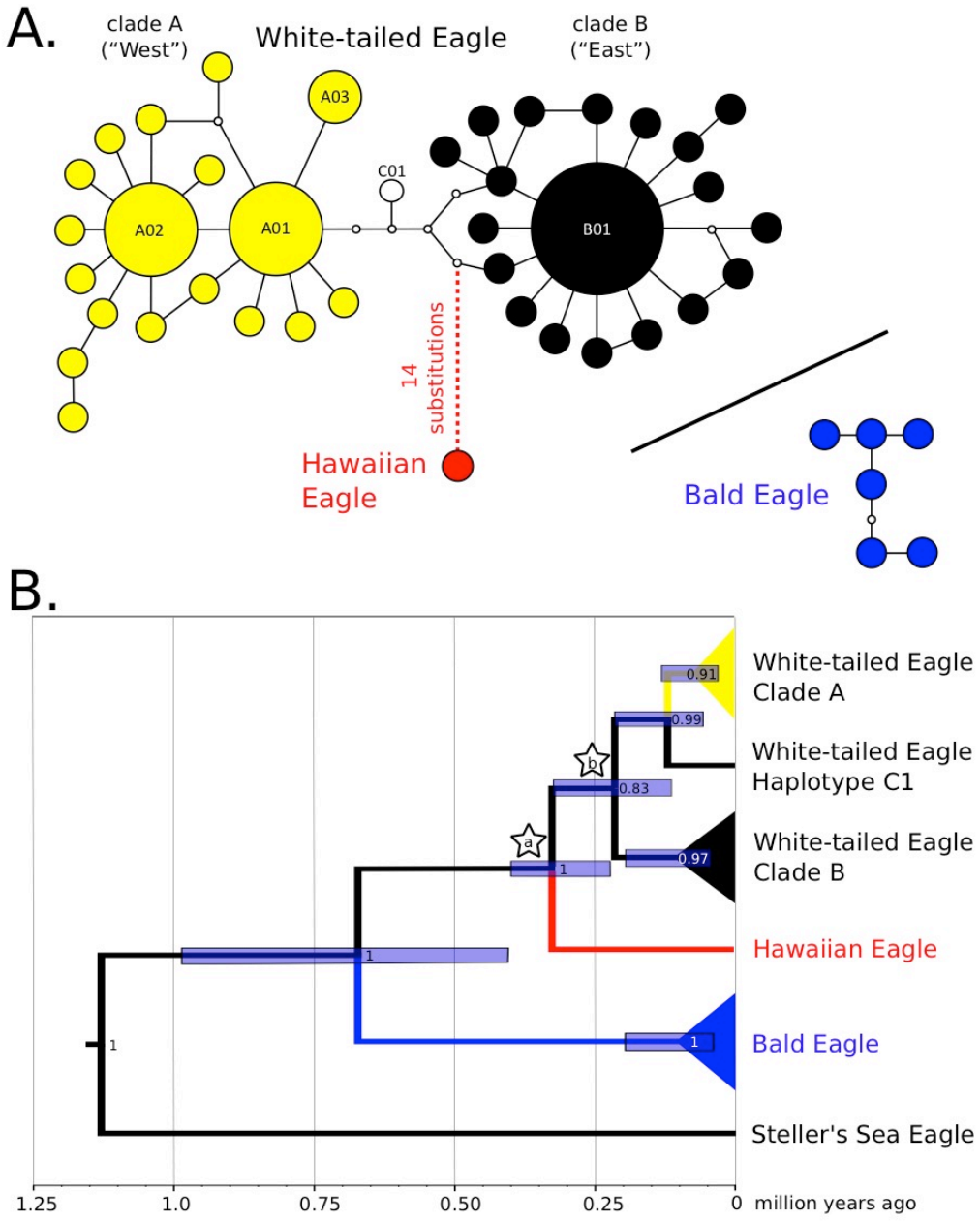

Fig. 1: Phylogenetic analyses of mtDNA control region data from the Hawaiian eagle

A. Haplotype networks: circle size reflects haplotype frequency, small white circles: inferred intermediate steps. Major haplotypes are labeled to facilitate comparison with previous studies. The Hawaiian haplotype was only connected (stippled line) to clade B haplotypes for connectivity threshold settings of $p \leq 0.91$. Bald Eagle haplotypes were separated by at least 20 mutational steps from White-tailed Eagle haplotypes (major clades shown in yellow, black and white) and could not be connected with certainty ($p < 0.9$). **B.** Maximum clade credibility tree. The Hawaiian lineage is dated to branch off from extant lineages at 223-400 ka (95% HPD). Note that the shown calibration scenario 1 yielded younger divergence time estimates for all nodes than calibration scenario 2 (see text for details). Numbers at nodes: posterior probabilities; bars: 95% HPD for node heights. Stars (a) and (b): nodes discussed in detail in the text.

Appendix A. Supplementary data file 1

Distinct and extinct: genetic differentiation of the Hawaiian eagle

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1. Details of laboratory and statistical analyses, and results from Bald Eagles

Previous phylogenetic analyses have found extant Bald and White-tailed Eagles to be sister taxa, with Steller's Sea Eagles as the next most closely related extant lineage (Seibold and Helbig, 1996; Wink et al., 1996). Our phylogenetic analysis of the Hawaiian eagle lineage therefore includes mtDNA control region data from all these taxa.

Primers used for ancient DNA analysis were (all given in 5' to 3' direction; primers with corresponding numbers in the names were used together): Hal-HVR1F (CCCCCCCTATGTATTATTGT), HalHVR-S1R (TGTATGCCCGRATTACAT), HalHVR-S2F (GACATATTAATGTATGTATTATAGTCA), HalHVR-S2R (CCTRTGGCATGGGTTTAG), HalHVR-S3F (TGCACTCTCGGACCATTT), HalHVR-S3R (GATCATGCAGA-AATCTGGTG), HalHVR-S4F (ACTACGGATCAGTGGACTGC), HalHVR-S4R (ATGATCTCTCTGGGACCGAC), HalHVR-S5F (CCAAGAAGGGCTAGGTTATCT), HalHVR-S5R (AGAGGCACAAAAGAGCAAGT), HalHVR-S6F (CGGTGCACGTATAGATCCTA), HalHVR-S6R (CAGTGAAGAGCGAGAGAACG). Except for the first primer (Hal-HVR1F; Hailer et al., 2007), all primers were newly designed for the purpose of this study.

Amplifications of bald eagle DNA were done using the Hal-HVR1F and HalHVR-S6R primers in 15 µl reactions at 58°C annealing temperature, followed by sequencing as described above. PCRs using other primer combinations (Hal-HVR1F & HalHVR-S4R, Hal-HVR1F & HalHVR- -S5R, HalHVR-S2F & HalHVR-S6R, HalHVR-S4F & HalHVR-S6R; tested for a subset of samples) yielded identical results for bald eagles. The purpose of amplifying overlapping pieces of the control region using various primer combinations was to check for any mismatching DNA sequencing results among primer combinations. The latter, had it been observed, could have indicated that at least some primer combinations amplify nuclear copies of the control region rather than the mitochondrial fragment. We did not observe any mismatches in Bald Eagles, consistent with extensive tests in previous studies of *Haliaeetus*, which all failed to detect any signal challenging the true mitochondrial origin our analyzed fragment (Hailer et al., 2007, 2006; Honnen et al., 2010; Langguth et al., 2013).

PCR amplifications were conducted in 25 µl reaction volumes in 1x of buffer II (Applied Biosystems) with3 µl of extract, 2 mM $MgCl_2$, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems), 1 mg/ml BSA, 1 µM of each primer, and 0.2 mM of each dNTP. PCR temperature profiles comprised 5 min at 95°C, 45 cycles of 30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C, completed by a final elongation step of 10 min at 72°C. PCR products were cleaned using EXOSAP (USB Scientific). Both strands of DNA were cycle-sequenced with the PCR primers using BigDye v. 3.1 (Applied Biosystems), followed by Sephadex clean-up. Sequences were run on an ABI 3130xl instrument and assembled and edited in Sequencher 4.8.

Phylogenetic analyses with BEAST 1.8.0 (Drummond et al., 2012) utilized a random starting tree, the sequence evolution model parameters suggested by jModeltest2 (Darriba et al., 2012) (HKY+G, alpha=0.021 with four categories), and a constant-size coalescent tree prior. Initial analyses using the uncorrelated relaxed clock model in BEAST yielded results that indicated non-significant deviation from a constant clock rate, so we used the strict clock model in all following analyses. No specific rate calibration for the mtDNA control region has been identified for Accipitridae (Bunce et al., 2005), but several studies have reported that the cytochrome B (CytB) gene diverges at a rate of approximately 1.4% (Lerner et al., 2011) to 2.1% (Weir and Schluter, 2008) per million years in birds. For *CytB*, White-tailed Eagles differ from Bald and Steller's Sea Eagles by ca. 2.5% and 7.7%, respectively (Seibold and Helbig, 1996; Wink et al., 1996), suggestive of divergence times from the Bald Eagle lineage of at least several 100 thousand years (ka), and clearly >1 million years from Steller's Sea

Eagles. Unfortunately, we are not aware of fossils that would be useful for calibration of the split from Steller's Sea Eagles. However, fossil data suggest the presence of Bald Eagles in North America by 300 ka (Jefferson, 1985). These considerations were taken into account when setting priors for the BEAST analyses. The main run for producing Fig. 1B sampled from a Markov Chain Monte Carlo procedure with in total 50 million steps, sampling every 10.000th step. We used TreeAnnotator from the BEAST 1.8.0 package to generate a maximum clade credibility tree based on median node heights, discarding the first 20% of the trees as burn-in.

Control region sequences from our twelve Bald Eagle samples formed six distinct haplotypes, in the following referred to as *Hle.H01* - *Hle.H0106*. Of these, one haplotype was found in five individuals (*Hle.H01*: Hle629_38209, Hle629_38242, Hle629_38248, Hle629_38249, Hle629_38252), two were found in two individuals each (*Hle.H02*: Hle629_38207, Hle629_38208; *Hle.H03*: Hle629_38219, Hle629_38227), and three were found in one individual each (*Hle.H04*: Hle629_38211, *Hle.H05*: Hle629_38213, *Hle.H06*: Hle629_38216).

2. Fossil record of *Haliaeetus* in Hawaii, and details about the analyzed specimen

Fossil evidence of a sea-eagle of the genus *Haliaeetus* on Hawaii was first discovered in the Moomomi Dunes of Molokai and in a sinkhole deposit at near Barbers Point, Oahu (Olson and James 1982a,b). At both localities the distinctive, very large fused phalanges of the inner toe (digit II) characteristic of *Haliaeetus* (Olson 1982) were found. However, even with the later discovery of a nearly complete associated skeleton at a high elevation site on Maui, it proved impossible to find any morphological distinctions to separate the Hawaiian bird from the North American Bald Eagle or the Eurasian White-tailed Eagle (Olson and James 1991).

Both the Molokai and Oahu eagle sites are essentially at sea-level, close to the modern coastline. Eagle bones from the Moomomi Dunes on Molokai are Holocene in age. The dune sands themselves were dated at about 8,300 radiocarbon years B.P. (Hearty et al. 2000), although the bones are more likely somewhat younger. The sinkhole deposits on the Ewa Plain of Oahu are likewise Holocene. At least two individual eagles are represented among the initial finds from a large sinkhole (Bishop Museum archaeological site 50-Oa-B6-22; Olson and James 1982b), and additional eagle bones have been found more recently in smaller sinkholes on Kalaeloa Unit of the Pearl Harbor National Wildlife Refuge. The Oahu eagle bones have not been directly radiocarbon dated, but 23 published radiocarbon ages determined on bones of other extinct birds from excavations in Ewa Plain sinkholes range from about 1,000 to 8,000 years old (Athens et al. 2002). The eagle skeleton from Maui was from a deep lava vent cave at 1,463 m elevation on the south slope of Mt. Haleakala. A previously reported radiocarbon date on purified collagen from the skeleton (3,300 ± 60 conventional radiocarbon years before present, Fleischer et al. 2001) has a 95% probability range of 3,400 – 3,680 calendar years before present and a median probability age of 3,531 calendar years before present, after calibration (Reimer et al. 2009).

The fossil record thus establishes that by the mid- to late Holocene, *Haliaeetus* was present on Oahu (three individuals), Molokai (at least one, possibly two individuals) and Maui (one complete skeleton), and that it ranged from sea level up to montane regions. That its remains were found in a montane natural trap site where remains of flightless birds accumulated indicates that the eagle formerly preyed on the archipelago's extinct flightless terrestrial birds. Eagle fossils have not been found on several of the major islands (Hawaii, Lanai, Kauai), perhaps because the fossil record is deficient. The islands of Hawaii and Lanai clearly have incomplete fossil records (Dove and Olson 2011, Olson 2014), which can explain the absence of eagle remains from those islands. The island of Kauai has a more complete fossil record, and the absence of eagle bones from that island might reflect a true gap in distribution (Olson and James 1982b, 1997, Burney et al. 2001).

More important for the consideration of genetic divergence times is the absence of *Haliaeetus* from the Middle Pleistocene lake deposits at Ulupau Head, Oahu (James 1987, Hearty et al. 2005). These deposits have yielded a diverse avifaunal assemblage including commonly, fossils of waterfowl (*Anas* spp.) and hawks (*Buteo sp*.). The Ulupau Head lake deposits formed in a volcanic tuff cone most likely during the Middle Pleistocene Marine Isotope Stage (MIS) 11 interglacial at roughly

400 ka, or possibly during MIS 9 at roughly 320 ka (Hearty et al. 2005). It appears unlikely that there was a resident population of *Haliaeetus* present on Oahu at the time of deposition.

Thus, the available evidence suggests that the maximum amount of time available for the colonization and differentiation of *Haliaeetus* in the Hawaiian Islands would be the equivalent of the MIS 10 glacial period, beginning about 374,000 years ago, but it could have been considerably earlier.

3. Pattern of nucleotide substitutions along the mtDNA control region fragment

The patterns of polymorphism and sequence conservation along the fragment are similar for the Hawaiian eagle sequence (compared to White-tailed Eagle haplotype B01) and the remaining data (pooled data from White-tailed, Bald and Steller's Sea Eagles), confirming that sequencing errors cannot be a major factor behind the large divergence of the Hawaiian eagle sequence.

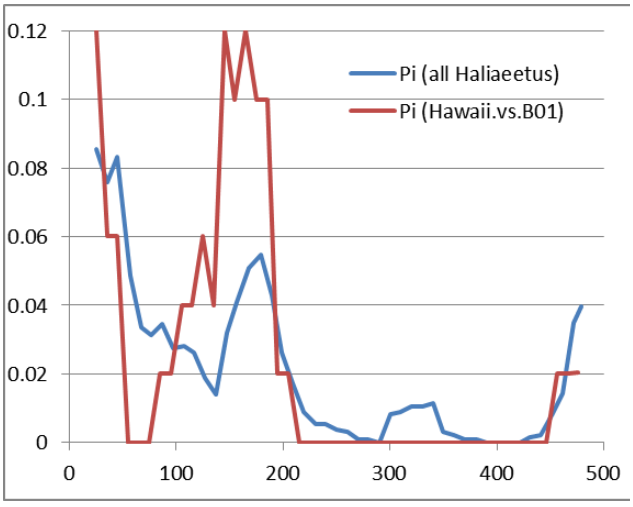

Fig. S1: Sliding window analysis of nucleotide diversity (π) across the mtDNA control region fragment.

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