Ancient DNA reveals elephant birds and kiwi are sister taxa and clarifies ratite bird evolution

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Abstract: The evolution of the ratite birds has been widely attributed to vicariant speciation, driven by the Cretaceous breakup of the supercontinent Gondwana. The early isolation of Africa and Madagascar implies that the ostrich and extinct Madagascan elephant birds (Aepyornithidae) should be the oldest ratite lineages. We sequenced the mitochondrial genomes of two elephant birds and performed phylogenetic analyses, which revealed they are the closest relatives of the New Zealand kiwi, and distant from the basal ratite lineage of ostriches. This unexpected result strongly contradicts continental vicariance and instead supports flighted dispersal in all major ratite lineages. We suggest that convergence towards gigantism and flightlessness was facilitated by early Tertiary expansion into the diurnal herbivory niche following the extinction of the dinosaurs.

One Sentence Summary: Ancient DNA reveals massive Madagascan elephant birds and tiny NZ kiwi are closest relatives, and supports flighted origin of ratites.

Main Text:

Despite extensive studies, the evolutionary history of the giant flightless ratite birds of the southern hemisphere landmasses, and the related flighted tinamous of South America, has remained a major unresolved question. The ratites and tinamous, termed palaeognaths due to their shared 'basal' palate structure, form the sister-taxon to all other living birds (neognaths). The living ratites are one of the few bird groups comprised largely of giant terrestrial herbivores and include: the emu and cassowary in Australia and New Guinea; kiwi in New Zealand; ostriches in Africa; and rhea in South America. In addition, two recently extinct groups included the largest birds known: the moa from New Zealand (*1*) [up to 2-3 m and 250 kg] and elephant birds from Madagascar [2-3 m height but up to 275 kg] (*2, 3*). Ratites have been believed to have originated through vicariant speciation driven by the continental breakup of Gondwana on the basis of congruence between the sequence of continental rifting and the presumed order of lineage divergence and distribution of ratites (*4, 5*).

New Zealand is the only landmass to have supported two major ratite lineages, the giant herbivorous moa and the chicken-sized, nocturnal, omnivorous kiwi. Morphological phylogenetic analyses initially suggested that these two groups were each other's closest relatives (*6, 7*), presumably diverging after the isolation of an ancestral form following the separation of New Zealand and Australia in the late Cretaceous ca. 80-60 million years ago (Ma) (*8*). However, subsequent studies suggest that kiwi are more closely related to the Australasian emu and cassowaries (*9, 10*), while the closest living relatives of the giant moa are the flighted South American tinamous (*11-14*). The latter relationship was

completely unexpected on morphological grounds, and suggests a more complex evolutionary history than predicted by a model of strict vicariant speciation. By rendering ratites paraphyletic, the relationship between moa and tinamous also strongly suggests that gigantism and flightlessness have evolved multiple times among palaeognaths (*12, 13*).

Perhaps the most enigmatic of the modern palaeognaths are the recently extinct giant Madagascan elephant birds. Africa and Madagascar were the first continental fragments to rift from the supercontinent Gondwana, separating from the other continents (and each other) completely during the Early Cretaceous (~130-100 Ma)(*15*). Consequently, the continental vicariance model predicts that elephant birds and ostriches should be the basal palaeognath lineages (*16*). Most molecular analyses recover the ostrich in a basal position, consistent with a vicariant model. However, the phylogenetic position of the elephant birds remains unresolved as cladistic studies of ratite morphology are sensitive to character choice and may be confounded by convergence (*17*), while aDNA studies have been hampered by the generally poor molecular preservation of elephant bird remains (*18*).

We sequenced near-complete mitochondrial genomes from both elephant bird genera, *Aepyornis* and *Mullerornis,* using hybridization enrichment with in-solution RNA arrays of palaeognath mitochondrial genome sequences and high-throughput sequencing. Phylogenetic analyses placed the two taxa, *Aepyornis hildebrandti* (15,547 bp) and *Mullerornis agilis* (15,731 bp) unequivocally as the sister-taxa to the kiwi (Figs. 1, S1). This result was consistently retrieved regardless of phylogenetic method or taxon sampling, and was strongly supported by topological tests (*19*). To our knowledge, no previous study has suggested this relationship, likely due to the disparate morphology, ecology and distribution of the two groups. Elephant birds were herbivorous, almost certainly diurnal and among the largest birds known, while kiwi are highly derived omnivores, nocturnal and about two orders of magnitude smaller. Indeed, elephant birds more closely resemble the moa, and analyses of morphology have suggested a close relationship between these taxa (*17*). However, when morphological characters were added to our molecular dataset it increased support for the relationship between elephant birds and kiwi (Figs. S2, S3), and several distinctive character states could be identified that diagnose this clade (see list in *19*).

Speciation by continental vicariance provides a poor explanation of the close relationship between elephant birds and kiwi. Madagascar and New Zealand have never been directly connected and molecular dates calculated from the genetic data suggest that kiwi and elephant birds diverged after the breakup of Gondwana (Figs. 1, S4). However, mean node age estimates among palaeognath lineages are sensitive to taxon sampling (Fig. 2), so molecular dating provides limited power for testing hypotheses about ratite biogeography. Depending on taxon sampling, estimates for the basal divergence among palaeognaths are equally consistent with the separation of Africa ~100 Ma (*15*) and the Cretaceous/Tertiary (KPg) boundary (~65 Ma) (Fig. 2). Thus, topological comparisons may be a more robust tool to test hypotheses of vicariance and connection.

The phylogenetic placement of the elephant birds as sister to kiwi creates a marked discordance between the order of continental breakup (Fig. 3A, B) and the sequence of palaeognath divergences (Fig. 3C). Instead, it appears that the common ancestor of elephant birds and kiwi was likely flighted and capable of long-distance dispersal, which is supported by a small kiwi relative from the Early Miocene of New Zealand that may have been flighted (*20*). Together, the phylogenetic position of the flighted tinamous and apparent flighted ancestor of the kiwi and elephant bird imply that every major ratite lineage independently lost flight (Fig. 1). We suggest that flighted dispersal was the primary driver of the distribution of palaeognath lineages, and that the discordance between distribution and phylogeny is more consistent with lineage turnover in a phylogenetically diverse, flighted and widespread clade. Early Tertiary palaeognaths were capable of long distance dispersal, with remains found well outside the range of modern ratites, including the flighted lithornithids in North America and Europe and flightless *Palaeotis* and *Remiornis* in Europe (Fig. 3A, *21*). Rapid diversification through flighted dispersal also provides an explanation for the short and often poorly supported internodes amongst basal extant ratite lineages (*13*, *14*).

Early ratite evolution appears to have been dominated by flighted dispersal and parallel evolution, with flightlessness evolving a minimum of six times, and gigantism a minimum of five (Fig. 1, *22*),

suggesting that adaptations for cursoriality may have confounded phylogenetic inference. Elsewhere, avian gigantism and flightlessness is almost exclusively observed in island environments in the absence of mammalian predators and competitors (e.g. the dodo). However, each of the landmasses occupied by ratites (excluding New Zealand) is now home to a diverse mammalian fauna. We suggest that the initial evolution of flightless ratites began in the ecological vacuum following the KPg mass extinction event and the extinction of the dinosaurs (*12*, *21*). Most mammals appear to have remained relatively small and unspecialized for up to 10 Ma after the KPg extinction (*23*), potentially providing a window of opportunity for the evolution of large flightless herbivores in continental bird lineages. The early Tertiary fossil record supports this interpretation, with geographically widespread flighted palaeognath fossils (Fig 3A, *22*) and the appearance of other flightless avian herbivores such as gastornithids in Europe and North America, dromornithids in Australia, and *Brontornis* from South America (*21*). After the early Tertiary, the increasing prevalence of morphologically diverse mammalian competitors is likely to have prevented flightlessness developing in other continental bird lineages.

The kiwi and tinamous are the only recent palaeognath lineages to not exhibit gigantism, and both taxa co-occur with a second palaeognath lineage (moa and rhea respectively) that is both much larger and not their closest relative. We suggest that the disparity in size between co-occurring lineages may be a result of the relative timing of arrival of ancestral flighted palaeognaths coupled with competitive exclusion: the first palaeognath to arrive on each landmass monopolized the available niche space for large flightless herbivores/omnivores, forcing subsequent arrivals to adopt an alternative role and remain much smaller. For example, the South American ancestors of the rhea lineage (*Diogenornis*) were already large and flightless at 55Ma (*21*) when the tinamou lineage originated. The absence of sympatric lineages of small palaeognaths on other landmasses in the recent past may reflect unavailability of alternative niches upon arrival (*e.g.* due to diversification of herbivorous mammals during the early Tertiary), or subsequent competition with mammals and/or neognathous birds. It is presumably the latter that has necessitated the maintenance of flight in the tinamous.

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Acknowledgements

This study was funded by the NZ Marsden Fund and Australian Research Council. Grid computing facilities were provided by eRSA (e-research South Australia) and CIPRES (Cyberinfrastructure for Phylogenetic Research). We thank the Museum of New Zealand Te Papa Tongarewa (P. Millener, S. Bartle, A. Tennyson), Natural History Museum Oslo (N. Heintz) and National Museum of Natural History Paris (C. Lefèvre) for samples. We acknowledge D. Penny, M. Phillips, D. Burney and R. Ward for valuable advice and assistance. All data associated with this study are available on GenBank and DRYAD (doi:XXXXXX).

Fig. 1: Phylogenetic position of the elephant birds from mitochondrial sequence data. Bayesian posterior probabilities and maximum likelihood bootstrap are presented in black below each branch; asterisks (*) mark branches that received maximum possible support (Bootstrap = 100%, Bayesian posterior probability = 1.0). Divergence dates (blue numbers above branches; blue bars represent 95% highest posterior density or HPD intervals) were inferred with six well-supported node age constraints (Table S5). Blue arrows mark the minimum date for the evolution of flightlessness in lineages for which fossil evidence is available (*21, 22*). Scale is given in millions of years before present. Silhouettes indicate relative size of representative taxa. Species diversity for each major clade is presented in parentheses, with extinct groups colored red.

Fig. 2: Sensitivity of palaeognath age estimation to taxon sampling and genetic loci used. Mean and 95% HPD intervals are displayed for the age (basal divergence) of crown palaeognaths as inferred under several dataset permutations. The y-axis represents time before present in millions of years, while age estimates for individual datasets are arrayed on the x-axis for both mitochondrial (light grey circles) and

nuclear loci (dark grey triangles). Results are presented for: A) a full taxon-set including ratites, tinamous, neognaths and crocodilians; B) ratites, tinamous and neognaths only C) ratites, neognaths and crocodilians only; and D) ratites and neognaths only. Taxon sets A and C are each calibrated with six fossil node constraints, while taxon sets B and D are calibrated with a subset of four relevant constraints (Table S5). Taxon sets are represented visually with silhouettes of an ostrich (ratites), a flying tinamou (tinamous), a duck (neognaths) and an alligator (crocodilians). Analyses excluding the rate-anomalous tinamous (C, D) retrieve a young age near the KPg boundary.

Fig. 3: Conflict between inferred palaeognath phylogeny and the topology predicted by continental vicariance. A) Relative position of continents during the Late Cretaceous and Tertiary. Continental landmasses are colored according to order of severance from the remaining Gondwanan landmass: Africa and Madagascar first (dark grey; 100 – 130 Ma), followed by New Zealand (red; 60 - 80 Ma), and finally Australia, Antarctica and South America (green; 30 - 50 Ma). Palaeognath-bearing fossil localities from the late Palaeocene and Eocene *(21, 22)* are represented by black circles (flighted taxa) and triangles (flightless taxa). B) Predicted phylogeny of ratites under a model of speciation governed solely by continental vicariance. C) Palaeognath phylogeny as inferred in the present study (see Fig. 1).

Supplementary Materials:
Materials and Methods
Supplementary Text
Figures S1-S9
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Supporting Online Material

Materials and Methods

Extraction

The two elephant bird specimens were *Mullerornis agilis* (MNZ S38300, tibiotarsus from Beloha, Madagascar) and *Aepyornis hildebrandti* (MNZ S38301, femur from central Madagascar). The extraction procedure was performed as outlined in a previous study, which also analyzed MNZ S38300 (*18*). A Dremel tool and disposable carborundum disks were used to remove approximately 2 mm of the exterior surface of each bone, and a 0.1 g fragment of cortical bone was collected. Each bone fragment was powdered using an 8 mm tungsten ball bearing in a Braun Mikrodismembrator U (B. Braun Biotech International, Germany) for 30 s at 2000 rpm in a sterilized stainless steel container. The powder was decalcified overnight in 10-30 vol of 0.5 M EDTA (pH 8.0) at room temperature, along with a negative control (no bone powder). The resulting sediment was collected by centrifugation and digested with proteinase-K/DTT overnight at 50-55 °C, then extracted twice with Tris-saturated phenol and once with chloroform. The DNA was desalted using Centricon-30 filter units (Millipore) and concentrated to approximately 100-150 μL.

Library Preparation

DNA templates were enzymatically repaired and blunt-ended, and had custom adapters (Table S1) ligated following the protocol of Meyer and Kircher (*24*). The 5' adapter featured a diagnostic index sequence to allow identification and exclusion of any contamination resulting from the downstream hybridization and sequencing reactions. In order to produce the quantity of DNA required for hybridization-enrichment (~200 ng) each library was subjected to a series of short PCR amplifications using primers complementary to the adapter sequences (Table S2). Cycle number was kept low and each round of amplification was split into 8 separate PCRs in order to minimize PCR bias. Each individual PCR (25 μL) contained 1 × PCR buffer, 2.5 mM MgCl2, 1 mM dNTPs, 0.5 mM each primer (Table S2), 1.25 U AmpliTaq Gold and 2 μL DNA library. Cycling conditions were as follows: 94 °C for 12 min; 12 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 + (2 x cycle number) s; and 72 °C for 10 min. PCR products were pooled and purified using AMPure magnetic beads (Agencourt), eluting in 30 μL TLE buffer.

Hybridization enrichment

Biotinylated 80-mer RNA baits, synthesized commercially by MYcroarray (MI, USA) were used to enrich the target library for avian mitochondrial DNA (mtDNA). Baits were designed using published whole mitochondrial genome sequences (excluding D-loop) and included the palaeognaths: *Anomalopteryx didiformis* (little bush moa; NC_002779), *Dinornis robustus* (giant moa; NC_002672), *Emeus crassus* (eastern moa; NC_002673), *Apteryx owenii* (little spotted kiwi; NC_013806), *Apteryx haastii* (great spotted kiwi; NC_002782), *Rhea americana* (greater rhea; NC_000846), *Struthio camelus* (ostrich; NC_002785) and *Tinamus major* (great tinamou; NC_002781); see Table S3 for a complete list of probe taxa. One hybridization reaction was performed for each of the two elephant bird libraries. Each reaction was performed according to manufacturer's instructions in a final volume of 26 μL: 5.2 × SSPE, 5.2 × Denhardt's, 5 mM EDTA, 0.1% SDS. This solution was incubated for 44 hr (3 hr at 60 □C, 12 hr at 55 □C, 12 hr at 50 □C, 17 hr at 55 □C) with a total of 200 ng of library DNA. After incubation the RNA baits were immobilized on magnetic streptavidin beads and washed once with 1 × SSC + 0.1% SDS for 15 min at room temperature, and then twice with 0.1 × SSC + 0.1% SDS for 10 min at 50 □C. The beads were subsequently resuspended in 0.1M NaOH pH 13.0, which destroyed the RNA baits and released the captured DNA into solution. The captured DNA molecules were purified using a Minelute spin-column (Qiagen), and eluted in 20 μL EB buffer.

Sequencing

The overall quantity of DNA remaining after enrichment was extremely small. To increase the DNA yield, we performed a post-hybridization round of amplification for each library: 8 × 25 μL: 1 × PCR buffer, 2.5 mM MgCl2, 1 mM dNTPs, 0.5 mM each primer (Table S2), 1.25 U AmpliTaq Gold and 2 μL DNA library (Cycling: 94 □C for 12 min; 12 cycles of 94 □C for 30 s, 60 □C for 30 s, 72 □C for 40 + [2 x cycle number] s; and 72 □C for 10 min). To add IonTorrent recognition sequences to the sequencing libraries, we performed a short round of PCR with fusion primers: 5 × 25 μL reaction: 1 × PCR buffer, 2.5 mM MgCl2, 1 mM dNTPs, 0.5 mM each primer (Table S2), 1.25 U AmpliTaq Gold and 1 μL DNA library (Cycling: 94 □C for 12 min; 7 cycles of 94 □C for 30 s, 60 □C for 30 s, 72 □C for 40 + [2 x cycle number] s; and 72 □C for 10 min). PCR products were purified using AMPure magnetic beads (Agencourt), eluting in 30 μL TLE buffer. The two libraries were then pooled in preparation for sequencing.

The pooled library was diluted to 11.6 pM, linked to proprietary micron-scale beads (Ion Sphere Particles; Life Technologies), and clonally amplified according to manufacturer's protocols via emulsion PCR on an Ion OneTouch system (Life Technologies) using the Ion OneTouch 200 Template Kit v2 DL (Life Technologies). The template beads were then loaded onto a 316 chip and sequenced on an IonTorrent PGM (Life Technologies) using the 200 bp sequencing chemistry. In order to increase read coverage and ensure reproducibility, this template preparation and sequencing protocol was performed twice. The resulting reads were pooled for downstream analysis.

Sequence processing and quality filtering

Following sequencing, base calling was performed using Torrent Suite v3.2.1 (Life Technologies). Sequencing reads were immediately demultiplexed according to 5' Index II (Table S1) using the fastx_barcode_splitter tool (FASTX-toolkit v0.0.13; http://hannonlab.cshl.edu/fastx_toolkit) allowing no mismatches in the index sequences (--mismatches 0). Demultiplexed reads (14,192,512) were trimmed with cutadapt v1.1 (*25)*, using the short custom adapter sequences (Table S1). Trimming parameters included a maximum error rate of 0.333 (-e 0.333), a minimum read length of 30 nucleotides (-m 30), a maximum read length of 200 nucleotides (-M 200), a minimum read quality Phred score of 20 (-q 20), a minimum of three-nucleotides overlap between the read and the adapter (-O 3), and five iterations of trimming (-n 5). Read quality was visualized using fastQC v0.10.1 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) before and after trimming to make sure the trimming of adapters was efficient. Trimmed reads were then sorted according to 5' Index I (Table S1) using the fastx_barcode_splitter tool (FASTX-toolkit v0.0.13; http://hannonlab.cshl.edu/fastx_toolkit) allowing no mismatches in the index sequences (--mismatches 0). The removal of the five bp 5' Index I resulted in a pool of reads between 25 - 195 bp in length, which were subsequently used for mapping and consensus assembly.

Read mapping and consensus sequence assembly

As we were sequencing elephant bird mitochondrial genomes *de novo*, we had no established reference genome to map against. Previous studies have overcome this limitation by mapping iteratively to a phylogenetically distant reference sequence (*26*, *27*). In the current study we followed this methodological principle using TMAP (https://github.com/nh13/TMAP), which is an assembly tool optimized for mapping IonTorrent data. In the initial round of mapping, only reads from relatively conserved regions of the mitochondrial genome are mapped successfully due to the divergence between the target and the reference. However, the information from these reads is taken into account in the reference for each subsequent round of mapping and acts as a seed for the mapping of reads in more divergent regions. Over multiple iterations, the number of mapped reads increases and the assembly grows outwards

from the original seeds. Iterative mapping continues until either the mitochondrial genome has been completed, or the number of reads added fails to increase.

For *Mullerornis agilis*, a total of 2,877,832 deconvoluted and trimmed reads were mapped to the ostrich (*Struthio camelus*) mitochondrial genome (NC_002785) using TMAP v3.2.2 (https://github.com/nh13/TMAP) with the default parameters for the mapall command. Reads with a mapping quality Phred score >30 were selected using the SAMtools v1.4 (*28*) view command (-q 30), and duplicate reads were discarded using the MarkDuplicates.jar tool from Picard tools v1.79 (http://picard.sourceforge.net). A 50% consensus was generated from the mapped reads, and used as the reference for an additional round of mapping. This process was iterated seven times until no additional reads could be mapped and the quality of the consensus ceased to improve. A final 75% consensus sequence for *M. agilis* (15,731 contiguous bases) was generated from 5,482 unique reads and checked by eye in Geneious v6.1.2 (Biomatters; http://www.geneious.com) before being deposited in GenBank (TBA).

The same mapping protocol was followed for *A. hildebrandti*: 853,102 deconvoluted and trimmed reads were mapped to the *M. agilis* mitochondrial genome (TBA) for seven iterations. A final 75% consensus sequence for *A. hildebrandti* (15,547 contiguous bases) was generated from 2,704 unique reads and deposited in GenBank (TBA). At least one read covered 99.8% of the final *M. agilis* consensus sequence and 92.6% of the *A. hildebrandti* consensus sequence (sites that received no coverage or where there was not a 75% majority base call were coded as IUPAC ambiguities). Mean length of individual reads was 70.6 bp (standard deviation = 26.8) while mean read-depth across the consensus was 24.7x (standard deviation = 8.1) for *M. agilis*; for *A. hildebrandti*, mean length of individual reads was 79.8 bp (standard deviation = 24.9) while mean read-depth across the consensus was 14.5x (standard deviation = 16.3).

Sequence authenticity

We consider the unambiguous, united position of our two elephant bird mitogenomes on the palaeognath phylogeny to be strong evidence for their authenticity. Further, during consensus sequence assembly we observed no evidence of multiple sequence variants (non-duplicate reads that agree with each other but conflict with the consensus, which may indicate contamination) in the final read pile-up for either taxon. However, to more comprehensively test the validity of our consensus sequences and phylogenetic results we: examined the damage profile of mapped reads; compared our consensus sequences to previously published Sanger sequencing data; subjected all mapped reads to a BLAST search; and re-mapped all reads using a different algorithm at even higher stringency and monitored for any change in phylogenetic signal.

We used MapDamage v0.3.6 (*29*) to assess patterns of damage across all mapped reads. Analyses were performed for each taxon on the first (Fig. S5, S7) and final iterations (Fig. S6, S8) of TMAP mapping. In all cases, patterns observed were consistent with degraded ancient DNA (elevated 5’ C-to-T and 3’ A-to-G, and depurination at the position preceding the beginning of the reads), suggesting that contamination of the mapped read pool by modern DNA is negligible. Notably, after the first iteration of mapping elevated levels of all substitutions are observed (Fig. S5, S7). However, this is an artifact resulting from mapping to a phylogenetically distant reference genome. Subsequent iterations of mapping see a marked decrease in this artifact, and it is not observed at all in the final iteration (Fig. S6, S8).
Our consensus sequence for *Mullerornis agilis* is 100% consistent with mitochondrial sequence fragments (AY016018, 391 bp; AY016019, 225 bp) previously published for this same sample (MNZ S38300) (*18*). Our consensus sequence differs from a third published fragment (AY016017, 385 bp) by the presence of two adenosine insertions (sites 7841 and 7851 of our consensus), however these insertions are present in 34/34 (7841) and 31/32 (7851) of mapped reads and may therefore represent sequencing errors or DNA damage in the

previously published data.

All reads used to construct the final consensus sequences for *Aepyornis* and *Mullerornis* were subjected to a BLAST search (blastn as part of blastall 2.2.17 against the full NCBI nucleotide database as of August 2012). The BLAST outputs (20 top hits) were processed with Metagenome Analyzer (MEGAN5)(*30*) using default parameters. As shown on Figure S9, all reads used in the final consensus match a ratite, a higher taxonomic rank within Aves (Neognathae or Passeriformes: likely reflecting regions of the mitochondrial genome highly conserved across all birds), or a higher taxonomic rank in which Palaeognathae is nested. This result indicates that each consensus is built from genuine sequences from the target organism rather than non-specific mapping of exogenous environmental or bacterial DNA. In the absence of established elephant bird reference genomes, it is impossible to conclusively test for contamination by palaeognath taxa, but mapDamage results suggest that any contamination by modern samples must be low. Further, it is highly unlikely that low levels of contamination by ancient palaeognath sequences would be sufficient to mislead our assembly.

To further assess the validity of the consensus sequences of both our reconstructed elephant bird mitochondrial genomes, all reads were re-mapped iteratively to the ostrich mitochondrial genome using a conservative algorithm and higher stringency quality thresholds. Specifically, all reads shorter than 30 bp were discarded (instead of 25 bp with TMAP), and reads were mapped with BWA v0.6.2 (*31*) with published modified parameters for seed length (-l 1024), edit distance (-n 0.01) and gap openings (-o 2) (*32*, *33*). Seven iterations of mapping (building consensus sequences and calling bases as for TMAP above) were performed for *Aepyornis* (resulting in 896 reads covering 32.3% of the consensus; consensus length = 11,220 bp), while nine iterations were performed for *Mullerornis* (resulting in 2,541 reads covering 59.2% of the consensus; consensus length = 14,849 bp). Using BWA, mean length of individual reads was 67.5 bp (standard deviation = 24.8) while mean read-depth across the consensus was 11.7x (standard deviation = 12.0) for *M. agilis*; for *A. hildebrandti* mean length of individual reads was 70.5 bp (standard deviation = 23.1) while mean read-depth across the consensus was 5.5x (standard deviation = 12.9).

Following this more stringent protocol, it is likely that real endogenous reads were discarded as BWA is not ideal for mapping to a phylogenetically distant references and is not optimized for the error profile of IonTorrent data. Consequently, the consensus sequences generated using BWA represent the most conserved regions of the mitochondrial genome. Phylogenetic analyses including the BWA consensus sequences were performed in parallel to analyses using the TMAP consensus sequences (see below) following the exact same protocol. Results are presented alongside the main results in Figure S1. The tree topology is identical to that obtained using the TMAP consensus sequences, and statistical support measures do not differ substantially. Constant phylogenetic signal between analyses confirms that the extra reads mapped using TMAP are most likely endogenous.

Molecular phylogenetics and divergence dating

We added our new mitogenomic data to existing mitogenome sequences from other palaeognaths plus a broad sampling of neognath and crocodilian outgroups (Table S4). Alignment was performed using the ClustalW algorithm in Geneious v6.1.2 (Biomatters; http://www.geneious.com) and checked by eye. Partitionfinder v1.1.1 (*34*) was used to select the partitioning scheme and substitution models for downstream analysis in RAxML (*35*, *36*), MrBayes (*37*) and BEAST (*38*). Each nucleotide partition was assessed for signal saturation using an information entropy index (*40*, *41*) as implemented in DAMBE v5.2.65 (*42*). Significant saturation could be rejected ($p < 0.01$) for each partition, assuming a symmetrical tree. In any case, we took a conservative approach (following *12*) and RY-coded mitochondrial third codon positions to reduce any possibility of bias arising as a result of saturation.

Tree topology (undated) was estimated under maximum likelihood and Bayesian frameworks using RAxML v7.2.8 (*35, 36*) and MrBayes v3.2.2 (*37*), respectively. Our RAxML analysis comprised a maximum likelihood (ML) search for the best-scoring tree (Fig. S1) from 1,000 bootstrap replicates performed on six individually-modeled partitions: H-strand 1st codon positions (GTR+G), H-strand 2nd codon positions (GTR+G), H-strand RY-coded 3rd codon positions (GTR+G), RNA stems (GTR+G), RNA loops (GTR+G) and ND6 (GTR+G). Our MrBayes analysis employed four Markov chains (one cold and three incrementally heated) with default priors. Data was modeled as six separate partitions: H-strand 1st codon positions (GTR+I+G), H-strand 2nd codon positions (GTR+I+G), H-strand RY-coded 3rd codon positions (F81), RNA stems (SYM+I+G), RNA loops (GTR+I+G) and ND6 (GTR+I+G). Each chain ran for 10^7 generations, sampling every 1,000. Convergence in topology was assessed using the average standard deviation of clade (split) frequencies (<0.02), while convergence in individual parameter values was assessed through potential scale reduction factors approaching 1, and through broadly overlapping distributions and effective sample sizes >>200 in Tracer v1.5 (*39*). Sampled trees were summarized as a majority-rule consensus tree by MrBayes after discarding the first 25% of trees as burnin. Bayesian posterior probabilities for clades (labeled on the RAxML tree) are presented in Figure S1. The data matrix for this analysis, and the resulting tree, is available on DRYAD.

Our molecular dating analysis was performed using BEAST v1.7.5 (*38*) using six fossil-based node constraints (Table S5). The branching order was constrained to the maximum likelihood tree topology (Fig. S1); fixing topology facilitated convergence under the complex partitioned clock models used here. Nucleotide substitution models were applied independently to six data partitions: H-strand 1st codon positions (GTR+I+G), H-strand 2nd codon positions (TVM+I+G), H-strand RY-coded 3rd codon positions (F81), RNA stems (SYM+I+G), RNA loops (GTR+I+G) and ND6 (TIM+G). The hypothesis of a global clock was tested and rejected for each data partition so independent relaxed lognormal clock models were used for each partition. Three independent BEAST MCMC chains were run for $2x10^8$ iterations, sampling every 10^4 for a total of $6x10^5$ sampled trees; the first 10% of trees from each chain were discarded as burnin before summarizing to create a maximum clade credibility tree in TreeAnnotator (Fig. S4). Parameter values were monitored in Tracer v1.5 (*39*) to ensure that all parameters converged and had ESSs > 200. The data matrix for this analysis, and the resulting tree, is available on DRYAD.

Testing Sensitivity of Molecular Divergence Dates (mitochondrial and nuclear data)

Palaeognath divergence dates estimated in several recent studies differ substantially. Because the timeline of palaeognath evolution is so important to our understanding of their evolution and biogeography, we sought to explore how taxon sampling and sequence data source (mitochondrial vs. nuclear) contribute to differences in inferred node ages.

We selected taxa from our mitochondrial matrix and a previously published, well sampled 10-locus nuclear dataset such that each mitochondrial and nuclear dataset contained equivalent lineages (Table S6). This necessarily meant that our elephant bird mitochondrial genomes were excluded, as no nuclear loci are available for these taxa. We further subsampled these datasets to exclude: i) crocodilians (due to their huge divergences from all other sampled taxa), ii) tinamous (due to their anomalous evolutionary rates), and iii) tinamous and crocodilians. This resulted in four equivalent taxon sets for both the nuclear and mitochondrial dataset: A) ratites, tinamous, neognaths and crocodilians; B) ratites, tinamous and neognaths (crocodilians excluded); C) ratites, neognaths and crocodilians (tinamous excluded); and D) ratites and neognaths (tinamous and crocodilians excluded). The nuclear and mitochondrial data were analyzed separately under each taxon set.

We performed molecular dating on each of the taxon sets using BEAST v1.7.5 (*38*), with the topology constrained to be compatible with Fig. 1 (allowing for different taxon sampling). Partitioning scheme and model choice were performed for each taxon set using Partitionfinder v1.1.1 (Table S7) (*34*). Independent relaxed lognormal clock models were applied to each partition to estimate branch lengths; the hypothesis

of a global clock was tested and rejected for each partition. Calibrations were implemented as above (Table S5), with the exception that the Alligatorinae and Archosauria constraints were omitted from analyses of taxon sets excluding crocodilians (B and D). For each taxon set, between three and five independent BEAST MCMC chains were each run for 10^8 iterations, sampling every 10^4 for a total of $3x10^4$ to $5x10^4$ sampled trees. Parameter values were monitored in Tracer v1.5 (*39*) to ensure that all parameters converged between chains and had combined ESSs > 200. Mean node ages and 95% highest posterior diversities were calculated for each taxon set by combining the sampled trees from each chain (Fig. S2, Table S8). The first 10% of sampled trees from each chain were discarded as burnin. Data matrices for these analyses, and resulting trees, are available on DRYAD.

Testing Sensitivity of Tree Topology (Mitochondrial, nuclear and morphological data)

To further test the robustness of tree topology, we performed analyses using parsimony, maximum likelihood and Bayesian methods on concatenated mitochondrial (Table S4) and nuclear data (Table S6: taxon set A), with and without a large recent morphological dataset. Further, to test the robustness of our phylogenetic results to taxon sampling, we analyzed each combined matrix (including and excluding morphology): i) without tinamous, ii) without any crocodilian and bird outgroups (i.e. effectively unrooted), and iii) without tinamous or outgroups. Data matrices for these analyses, and resulting trees, are available on DRYAD. All analyses strongly retrieved the kiwi-elephant bird clade/split (Table S9).

The 34 taxa (17 palaeognaths and 17 outgroups; Table S4) in the concatenated mitochondrial and nuclear datasets were scored for morphological data using a dataset derived from Worthy and Scofield (*17*), with additional characters from Johnston (*16*). Fifteen of the seventeen palaeognath taxa could be scored for morphology (only *Apteryx haastii* and *Tinamus major* lacked morphological data), along with three outgroups (Table S4). The addition of this morphological data permitted evaluation of the affinities of several fossil taxa (without any molecular data); addition of these taxa did not change relationships among the "core" taxa, so the discussion first focuses on analyses restricted to taxa scored for both molecules and morphology. The 243 characters were added to the molecular dataset as an additional partition (characters 24901-25143; see data matrices available on DRYAD). They are as follows:

- Characters 1-179 are directly from Worthy and Scofield (*17*).
- Characters 180-202 are characters 2-24 as scored by Johnston (*16*), except *Aepyornis* was scored for character 202 by Worthy (this study).
- Characters 203-208 are characters 26-31 as scored by Johnston (*16*), except moa were scored for character 207 by Worthy (this study).
- Characters 209-219 are characters 33-43 as scored by Johnston (*16*).
- Character 220 is character 55 from Johnston (16), except *Struthio*, *Casuarius* and moa were scored by Worthy (this study).
- Characters 221-243 are characters 63-85 from Johnston (*16*).

Twenty-five of the multistate characters formed morphoclines and were ordered (24 28 32 35 37 38 39 43 49 51 60 66 80 85 88 96 98 99 100 104 111 137 153 167 171); all other characters were unordered. All parsimony analyses used PAUP* (*43*), with heuristic searches involving 100 random addition replicates. Branch support was calculated using nexus batch files constructed with the aid of TreeRot v3 (*44*), and bootstrap frequencies used 200 bootstraps.

The combined data yielded the same single most-parsimonious tree, whether morphology was included (treelength = 38943), or excluded (treelength = 38288). The bootstrap majority-rule consensus was also identical to this tree. Bremer and Bootstrap support for relationships among palaeognath taxa are shown in Figure S2. Addition of morphological traits to the molecular dataset improves Bremer support for six nodes within palaeognaths (Fig. S2), including the kiwi-elephant bird clade: Bremer support increases from 27 to 28 for this clade, while bootstrap support is ~99% in both instances. Previous analyses of the morphological

data in isolation grouped elephant birds with moas (*17*): the combined analysis here reveals that there is a secondary signal in the morphological data that unites elephant birds and kiwis, congruent with the molecular data (see *45*). There are 10 unequivocal (optimization independent) synapomorphies diagnosing the elephant bird + kiwi clade, of which the following are well-sampled across palaeognaths, and relatively conservative:
60: palatine roofing the enlarged choanal fossa (unique within palaeognaths).
80: wide sternum (occurs also in ostriches).
81: sternum with a shallowly concave dorsal surface (occurs in some moa).
171: foramen vasculare distale vestigial (a more extreme state - total loss - occurs in moa).

All clock-free Bayesian analyses used MrBayes 3.2.2 (*37*), on the e-research South Australia computer grid and via the CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway. The Bayesian Information Criterion as employed by Partitionfinder (*34*) was used to choose the optimal partitioning scheme for the concatenated molecular data, and models for each molecular partition. A 17-partition model for the molecular data was chosen (Table S10). The morphological data was included as an 18th data partition and analyzed using the Lewis (*46*) stochastic model; Bayes Factors were slightly higher/better when the gamma parameter for rate variability was included ($\Delta BF = 3.3$ for rooted analyses excluding taxa scored only for morphology; $\Delta BF = 5.5$ for rooted analyses including taxa scored only for morphology). However, inclusion or exclusion of this parameter for morphology did not change topology, and posterior probabilities remained essentially identical. The results shown in Figure S2 are for rooted analyses of the full dataset including the gamma parameter.

Branch proportions ("lengths") were linked across nuclear molecular partitions, and mitochondrial molecular partitions, in both the Partitionfinder tests and the MrBayes analyses. For analyses including only taxa scored for both molecules and morphology, four runs of four chains were used, with default heating parameters. Each run was 2.5×10^7 steps long, sampling every 2×10^4 steps, with a burnin of 20%. Longer runs and burnin were required for analyses that also included taxa scored only for morphology: four runs of four chains were used, with the heating parameter (temp) lowered to 0.08. Each run was 10^8 steps long, sampling every 8×10^4, with a burnin of 30%. Separate branch lengths were allowed for the combined nuclear, combined mitochondrial, and morphological data; different partitions within each of these three datasets (e.g. different nuclear genes) were permitted different overall rates while sharing the same relevant branch proportions (e.g. the nuclear branch proportions). Convergence in topology was assessed by similarity in split (clade) frequencies across runs (<0.05), convergence in numerical parameters assessed using potential scale reduction factor (~1, meaning variance between and within runs is similar) and broadly overlapping parameter samples in Tracer v1.5 (*39*). A majority-rule consensus tree was constructed from the post-burnin samples of all four runs from each analysis.

Maximum likelihood analyses were performed using RAxML v7.2.8 (*35, 36*). The Bayesian Information Criterion as employed by Partitionfinder (*34*) was used to choose the optimal partitioning scheme for the concatenated molecular data, and models for each molecular partition. A 15-partition model for the molecular data was chosen (Table S10). The morphological data was included as a 16th partition, using the MK model option in RAxML. Due to constraints in RAxML, all characters had to be treated as unordered and all polymorphic characters as unresolved for maximum likelihood analyses. Each RAxML analysis comprised a maximum likelihood search for the best-scoring tree from 1,000 bootstrap replicates. Bootstrap support values are shown in Figure S2 for the rooted analyses.

All analyses (both rooted and unrooted) returned very similar topologies. The main difference was the position of the rhea: as sister taxa to all other palaeognaths excluding ostriches in rooted Bayesian analyses (Bayesian posterior probability [BPP] = 1.0, with or without morphology), sister taxa to the casuariids in rooted maximum likelihood analyses (including morphology = 94, excluding morphology = 66), and as sister taxa to the clade comprising moa and tinamous in the parsimony analyses (including morphology: bootstrap = 75, Bremer = 20; excluding morphology bootstrap = 93, Bremer = 23). Using the unrooted dataset, rhea grouped together with the casuariids in the maximum likelihood analysis

(including morphology: bootstrap = 83; excluding morphology: bootstrap = 74) and the Bayesian analysis including morphology (BPP = 0. 74), versus the clade comprising moa and tinamous in the parsimony analyses (including morphology: bootstrap = 83; excluding morphology: bootstrap = 74,) and Bayesian analysis excluding morphology (BPP = 0.96). However, elephant birds grouped with kiwi in all these analyses (Table S9).

Adding eight fossil palaeognath taxa scored for morphology by Worthy and Scofield (*17*), but with no DNA data (*Pachyornis australis, Pachyornis elephantopus, Pachyornis geranoides, Megalapteryx, Euryapteryx curtus, Dromaius baudinianus, Emuarius, Lithornis*) to the Bayesian and parsimony rooted analyses did not change topology among the remaining (core) taxa (Fig. S3). However, the missing data for these fossil taxa greatly reduced Bremer support. Despite this, *Lithornis* emerges as sister to tinamous with moderate support (Bremer 8, bootstrap 87%, PP = 0.9). In Worthy and Scofield (*17*) *Lithornis* was found to be sister to remaining palaeognaths but also with some character support for affinities with tinamous. In the present analysis, where molecular data largely constrain relationships of living palaeognaths to a very different topology, the morphological data strongly place *Lithornis* as a sister group to tinamous. The close relationship between *Lithornis* and tinamous found here was first advocated by Houde and Olson (*47*) based on morphology and was later supported by analyses of *Lithornis* eggshell (*48, 49)*, although on morphology (*48*) the similarities of tinamous and lithornithids were considered to be plesiomorphic within palaeognaths. It is therefore likely that lithornithids are related, or perhaps even ancestors (paraphyletic) to tinamous.

Testing the elephant bird + kiwi relationship against alternative nuclear topologies

Within extant ratites, the greatest phylogenetic uncertainty concerns the position of the rhea. To test whether alternative assumptions regarding the position of the rhea affect the strength of our elephant bird+kiwi clade, we ran additional analyses using our mitochondrial+nuclear matrices (with and without morphology) in which we constrained the position of the rhea to be consistent with the results of recent nuclear studies: rhea+casuariids (Haddrath and Baker's 10 locus phylogeny (*11*)), rhea+kiwi+casuariids (Haddrath and Baker's 27 locus phylogeny (*11*)) and rhea+ tinamous (Smith *et al.*'s 40 locus concatenated phylogeny (*14*); Smith's 40 locus coalescent phylogeny is consistent with our preferred trees Fig. S2, S3). Data matrices for our analyses, and resulting trees, are available on DRYAD. Analyses were run as described above in RAxML v7.2.8, PAUP* and MrBayes v3.2.2 with the addition of backbone topological constraints (see data matrices on DRYAD). Assuming these alternative positions of the rhea still results in the elephant bird+kiwi clade being recovered by all analyses, with high support in the vast majority (Table S9).

Testing the basal position of African/Madagascan ratites

The most concordant position of the elephant birds in regard to vicariant biogeography is a basal position (along with ostriches) in the palaeognath tree (as Madagascar and Africa separate early from all other Gondwanan landmasses). We specifically tested for this phylogenetic topology by running additional analyses using our mitochondrial+nuclear matrices (with and without morphology) in which we constrained the ostriches and elephant birds to fall outside a clade consisting of all other (non-AfroMadagascan) palaeognaths. Data matrices for these analyses, and resulting trees, are available on DRYAD. Rooted and unrooted analyses were run in RAxML v7.2.8, PAUP* and MrBayes v3.2.2 as described above with the addition of topological constraints (see data matrices on DRYAD). The tree resulting from each constrained analysis was compared to the tree resulting from the equivalent unconstrained analysis using the Templeton test (parsimony), SH test (maximum likelihood) and Bayes factors (Bayesian). The unconstrained tree (which grouped elephant bird with kiwi) was significantly better in each case (see Table S9 for value of statistics rejecting basal elephant birds).

Testing for base composition bias

Similarities in nucleotide frequency can potentially cause phylogenetic analyses to spuriously group together otherwise distantly related taxa (e.g. *50, 51*). In order to test whether elephant bird and kiwi group together as a result of shared base frequency we analyzed our combined mitochondrial+nuclear matrix (including all taxa) using the logdet distance model (*52*) in PAUP* (see data matrix on DRYAD). The logdet model explicitly accounts for base composition bias. This analysis still strongly supported a sister-taxon relationship between elephant bird and kiwi (bootstrap = 96%).

Supplementary Text

Kiwi egg

The characteristically large eggs of the ratites now appear to be one of the many morphological characters that have evolved independently in each major ratite lineage. Startlingly, this is apparently true even for the kiwi, which has one of the largest eggs of any bird for its body size. The kiwi's huge egg has long been thought to be a trait inherited from a much larger, flightless ancestor (*53, 54*). However, our results instead suggest that it is most likely an adaptation to predation pressure (increasing chick precociousness) after the arrival of small, flighted ancestral kiwi in New Zealand (*20*).

Early Tertiary distribution and dispersal

Our hypothesis predicts that during the early Tertiary small flighted palaeognaths should have been present on the southern landmasses where modern ratites are distributed. These flighted taxa would have independently given rise to modern flightless lineages, before eventually going extinct (with the exception of tinamous). However, the only known early Tertiary flighted palaeognath fossils are from North America and Europe (*47, 48*). While the lack of flighted palaeognaths in the ex-Gondwanan landmasses initially appears contrary to expectation, it may be more a reflection of taphonomic/sampling bias than of true distribution. North America and Europe have the best-sampled and most comprehensive early Tertiary fossil record, while the southern continents are relatively depauperate (*21*). Only two unequivocal early Tertiary palaeognath taxa are known from the ex-Gondwanan landmasses: a putative stem-rheid (*Diogenornis fragilis*) from the late Palaeocene of Itaborai, Brazil, South America (*55*); and *Eremopezus eocaenus* from the late Eocene of Egypt, Africa (*56*). Both taxa were flightless, but the relationship of *E. eocaenus* to modern palaeognaths is unclear. *Remiornis* (late Palaeocene of France, Europe (*57*)) and *Palaeotis* (middle Eocene of Germany, Europe (*58*)) were also flightless and, like *Eremopezus*, are of uncertain affinity. The ages of these flightless taxa are consistent with our hypothesis of widespread parallel loss of flight following the KPg mass extinction (in the early Palaeocene). Further, the presence of one particular flighted taxon in both North America and Europe contemporaneously (*Pseudocrypturus cercanaxius*, early Eocene (*48*)) indicates a capacity for inter-continental dispersal in early Tertiary flighted palaeognaths. A virtue of our hypothesis is that it offers a more satisfying explanation for the presence of flightless palaeognaths in Europe than does the model of continental vicariance.

Conserved biogeographical history is implicit in the hypothesis of diversification driven by continental vicariance. However, if the distribution of modern palaeognaths is primarily a result of flighted (frequent and potentially long-range) dispersal, then inferring historical biogeography based only on extant taxa is likely to be misleading in the absence of dense fossil data. Unfortunately, the paucity of Palaeocene fossils likely makes this problem intractable for the present. Antarctica is a promising avenue of research that is currently underexplored. While no unequivocal fossil palaeognaths are known from Antarctica (*21*) (but see Tambussi *et al.* (*59*)), its close proximity to the other ex-Gondwanan continents make it likely that at least some taxa were present during the early Tertiary. It is also possible that Antarctica was the source (or one of several sources) of dispersing flighted palaeognath

waves.

Figures

Fig. S1: Best maximum likelihood tree topology. Branch lengths in number of changes per site. Numbers associated with nodes are: Bayesian posterior probabilities (BPP) from analyses of the TMAP / BWA consensus sequences followed by bootstrap percentages (BS) from 1000 replicates for the TMAP / BWA consensus sequences. Nodes receiving maximum support in all analyses (BPP = 1, BS = 100%) are indicated by an asterisk (*). Dashes indicate nodes that do not appear in the Bayesian highest posterior probability tree; MrBayes analyses alternatively favour a clade comprising *Arenaria interpres*, *Haematopus ater*, *Gavia stellata*, *Pterodroma brevirostris*, *Ciconia ciconia* and *Pygoscelis adeliae* to the exclusion of *Apus apus* and *Archilochus colubris* (BPP = 1 TMAP / 1 BWA), and a clade comprising *Pterodroma brevirostris* and *Gavia stellata* (BPP = 0.87 TMAP / 0.71 BWA).

Fig. S2: Relationships among palaeognath birds from parsimony, maximum likelihood and Bayesian analyses of nuclear, mitochondrial and morphological data. Topology and branch lengths (scale=changes per site) are those found in the majority-rule consensus from Bayesian analyses. Values at nodes represent support values for combined / molecular only analyses, in this vertical order: Bremer support (parsimony), parsimony bootstrap, posterior probability (Bayesian inference), maximum likelihood bootstrap. Dash (-) indicates a clade is not present in the parsimony and/or maximum likelihood tree.

Fig. S3: Relationships among palaeognath birds from parsimony and Bayesian analyses of combined nuclear, mitochondrial and morphological data, with inclusion of extinct taxa only scored for morphology (red taxon names). Topology is that returned from the Bayesian (MrBayes) analyses; branch lengths are from the Bayesian analysis of the combined molecular and morphological data. Scale is in changes per site. Values at nodes represent support values for combined analyses, in this vertical order: posterior probability (Bayesian inference), bootstrap frequency / Bremer support (parsimony). Dash (-) indicates a clade is not present in the parsimony tree.

Fig. S4: BEAST time-calibrated phylogeny based on the topology from Figure S1. Time scale is in millions of years. Numbers associated with nodes are mean divergence dates; 95% HPDs are represented by blue bars. Divergence dates among palaeognath taxa are detailed in Table S8 for all analyses

Fig. S5: MapDamage report for the first round of mapping of *Mullerornis* reads against the ostrich mitochondrial genome. The four top panels show the characteristic high frequency of purines immediately before the reads. The two lower panels show the characteristic accumulation of 5’ C-to-T (red) and 3’ G-to-A (blue) misincorporations, and an atypical elevated frequency of T-to-C (orange) and A-to-G (black) across the reads. Other substitutions are shown in grey; insertions and deletions are shown in purple and green, respectively.

Fig. S6: MapDamage report for the final round of mapping of *Mullerornis* reads against the final 75% consensus sequence for *Mullerornis agilis*. The four top panels show the characteristic high frequency of purines immediately before the reads. The two lower panels show the characteristic accumulation of 5’ C-to-T (red) and 3’ G-to-A (blue) misincorporations. T-to-C and A-to-G transitions are shown in orange and black, respectively; other substitutions are shown in grey; insertions and deletions are shown in purple and green, respectively.

Fig. S7: MapDamage report for the first round of mapping of *Aepyornis* reads against the ostrich mitochondrial genome. The four top panels show the characteristic high frequency of purines immediately before the reads. The two lower panels show the characteristic accumulation of 5’ C-to-T (red) and 3’ G-to-A (blue) misincorporations, and an atypical elevated frequency of T-to-C (orange) and

A-to-G (black) across the reads. Other substitutions are shown in grey; insertions and deletions are shown in purple and green respectively.

Fig. S8: MapDamage report for the final round of mapping of *Aepyornis* reads against the final 75% consensus sequence for *Aepyornis hildebrandti*. The four top panels show the characteristic high frequency of purines immediately before the reads. The two lower panels show the characteristic accumulation of 5' C-to-T (red) and 3' G-to-A (blue) misincorporations. T-to-C and A-to-G transitions are shown in orange and black, respectively; other substitutions are shown in grey; insertions and deletions are shown in purple and green, respectively.

Fig. S9: Taxonomy profiles for sequencing reads used to build the *Aepyornis* and *Mullerornis* consensus sequences, as determined using BLAST and MEGAN5. Circles on each cladogram indicate relative frequency of reads assigned to taxa (taxon labels shown only for taxa receiving at least 50 hits). The bar graph for each consensus sequence displays relative frequencies of reads that could be assigned to a "tip" (colored circles).

Tables

Table S1: Structure of DNA sequencing libraries. Components and nucleotide sequences listed in order 5' to 3'.

Table S2: Primer sequences used in library amplification and sequencing preparation.

Table S3: Taxonomy and NCBI accession numbers for data used to create in-solution RNA probe array.

Table S4: NCBI accession numbers for mitochondrial data used in analyses to determine the phylogenetic placement of the elephant birds.

Table S5: Fossil node age constraints used in molecular dating analyses.

Table S6: NCBI accession numbers and taxon sets for the mitochondrial data and the ten nuclear loci used in comparative molecular dating analyses for testing the sensitivity of inferred divergence times to taxon sampling.

Table S7: Partitioning schemes and substitution models for comparative molecular dating analyses for testing the sensitivity of inferred divergence times to taxon sampling.

Table S8: Node ages and 95% HPDs for palaeognath taxa, estimated from molecular dating analyses.

Table S9: Comparison of statistical support for elephant bird + kiwi between total evidence analyses

Table S10: Partitioning schemes and substitution models for combined nuclear and mitochondrial data used in total evidence Bayesian and maximum likelihood analyses.