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SUPPLEMENTARY MATERIALS

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EVOLUTION

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

Kieren J. Mitchell,¹ Bastien Llamas,¹ Julien Soubrier,¹ Nicolas J. Rawlence,^{1*} Trevor H. Worthy,² Jamie Wood,³ Michael S. Y. Lee,^{1,4} Alan Cooper^{1†}

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 that these birds are the closest relatives of the New Zealand kiwi and are 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 toward gigantism and flightlessness was facilitated by early Tertiary expansion into the diurnal herbivory niche after the extinction of the dinosaurs.

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 composed largely of giant terrestrial herbivores and include: the emu and cassowary in Australia and New Guinea, the kiwi in New Zealand, the ostrich in Africa, and

the rhea in South America. In addition, two recently extinct groups included the largest birds known: the moa from New Zealand (height up to 2 to 3 m, 250 kg in weight) (1) and elephant birds from Madagascar (2 to 3 m in height, up to 275 kg in weight) (2, 3). Ratites have been believed to have originated through vicariant speciation driven by the continental breakup of the supercontinent 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 ~80 to 60 million years ago (Ma) (8). However, subsequent studies suggest that kiwi are more closely related

to the Australasian emu and cassowaries (9, 10), whereas 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 the 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 to 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), whereas DNA studies have been hampered by the generally poor molecular preservation of elephant bird remains (18).

We used hybridization enrichment with in-solution RNA arrays of palaeognath mitochondrial genome sequences and high-throughput sequencing to sequence near-complete mitochondrial genomes from both elephant bird genera: *Aepyornis* and *Mullerornis*. Phylogenetic analyses placed the two taxa, *Aepyornis hildebrandti* (15,547 base pairs) and *Mullerornis gigilis* (15,731 base pairs), unequivocally as the sister taxa to the kiwi (Fig. 1 and fig. 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, probably because of the disparate morphology, ecology, and distribution of the two groups. Elephant birds were herbivorous, almost certainly diurnal, and among the largest birds

¹Australian Centre for Ancient DNA, School of Earth and Environmental Sciences, University of Adelaide, North Terrace Campus, South Australia 5005, Australia. ²School of Biological Sciences, Flinders University, South Australia 5001, Australia. ³Landcare Research, Post Office Box 40, Lincoln 7640, New Zealand. ⁴South Australian Museum, North Terrace, South Australia 5000, Australia.
 *Present address: Allan Wilson Centre for Molecular Ecology and Evolution, Department of Zoology, University of Otago, Dunedin, New Zealand. †Corresponding author. E-mail: alan.cooper@adelaide.edu.au

known, whereas kiwi are highly derived omnivores, nocturnal, and about two orders of magnitude smaller. Elephant birds more closely resemble the moa, and analyses of morphology have suggested a close relationship between these taxa (17). However, adding morphological characters to our molecular data set increased support for the relationship between elephant birds and kiwi (figs. S2 and S3) and allowed for identification of several distinctive character states 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 (Fig. 1 and fig. 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 (K/Pg) 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 bird as sister to the kiwi creates a marked discordance between the order of continental breakup (Fig. 3, A and B) and the sequence of palaeognath divergences (Fig. 3C). Instead, it appears that the common ancestor of elephant birds and kiwis was probably flighted and capable of long-distance dispersal, which is supported by a small, possibly flighted kiwi relative from the Early Miocene of New Zealand (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 the 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 phyloge-

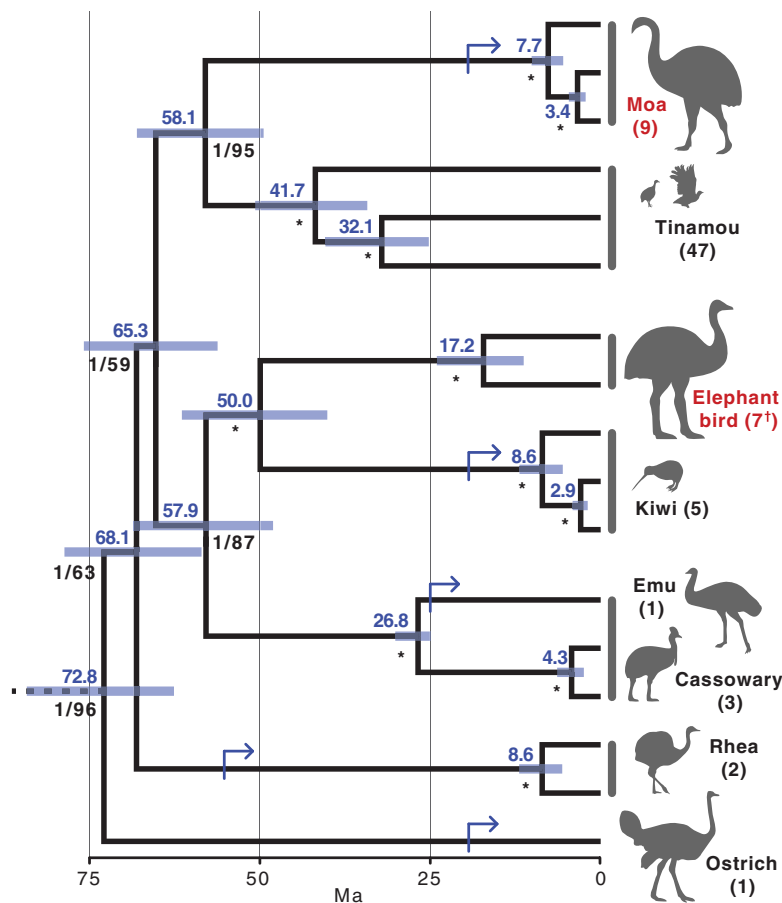


Fig. 1. Phylogenetic position of the elephant birds from mitochondrial sequence data. Bayesian posterior probabilities and maximum likelihood bootstrap are presented in black below each branch; asterisks denote branches that received maximum possible support (bootstrap = 100%, Bayesian posterior probability = 1.0). Divergence dates [blue numbers above branches; blue bars represent 95% highest posterior density (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). The scale is given in millions of years before the present. Silhouettes indicate the relative size of representative taxa. Species diversity for each major clade is presented in parentheses, with extinct groups shown in red. The dagger symbol (†) indicates that the number of elephant bird species is uncertain.

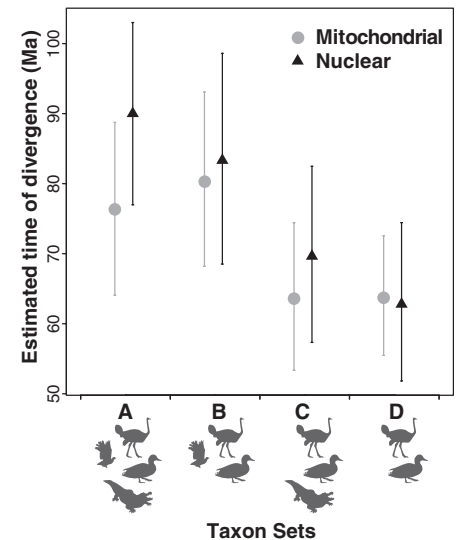
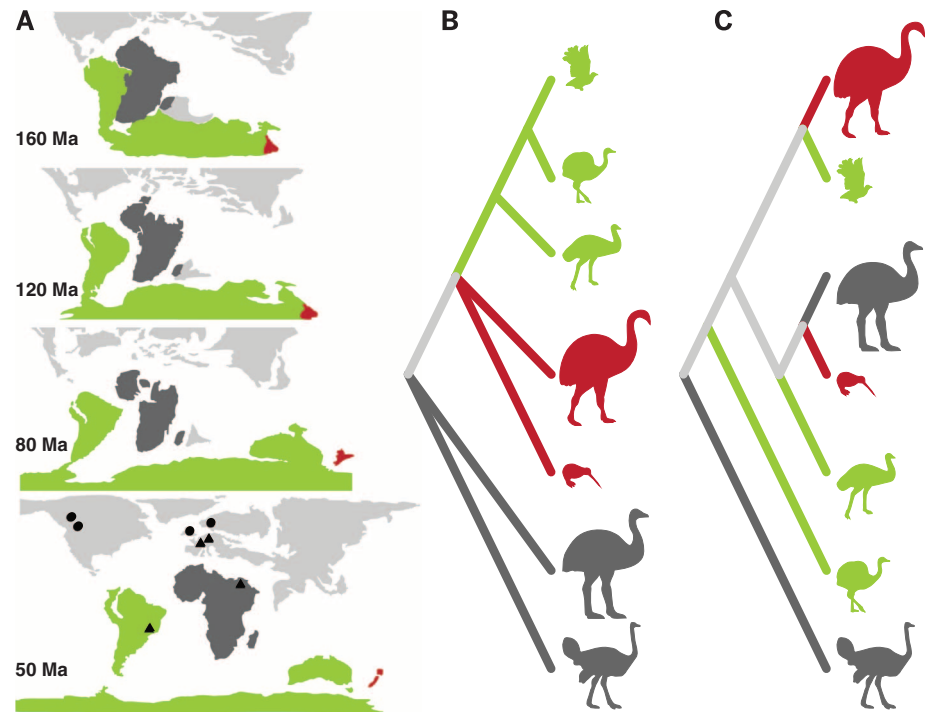


Fig. 2. Sensitivity of palaeognath age estimation to taxon sampling and genetic loci used.

Mean and 95% HPD intervals are displayed for the age (basal divergence) of crown palaeognaths, as inferred under several data set permutations. The y axis represents time before the present in millions of years, whereas age estimates for individual data sets are arrayed on the x axis for both mitochondrial (circles) and nuclear loci (triangles). Results are presented for the following taxon sets: A, a full taxon set including ratites, tinamous, neognaths, and crocodylians; B, ratites, tinamous, and neognaths only; C, ratites, neognaths, and crocodylians only; and D, ratites and neognaths only. Taxon sets A and C are each calibrated with six fossil node constraints, whereas 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 (crocodylians). Analyses excluding the rate-anomalous tinamous (taxon sets C and D) retrieve a young age near the K/Pg 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 gray; 100 to 130 Ma), followed by New Zealand (red; 60 to 80 Ma), then finally Australia, Antarctica, and South America (green; 30 to 50 Ma). Palaeognath-bearing fossil localities from the late Palaeocene and Eocene (21, 22) are represented by 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).



netic inference. Elsewhere, avian gigantism and flightlessness are 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 after 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 from 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 and 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 55 Ma (21) when the tinamous 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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SUPPLEMENTARY MATERIALS

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

Kieren J. Mitchell, Bastien Llamas, Julien Soubrier, Nicolas J. Rawlence, Trevor H. Worthy, Jamie Wood, Michael S. Y. Lee and Alan Cooper

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Ruffling ancient ratite feathers

Biologists have often pointed to the breakup of the supercontinent Gondwana to explain how related species ended up on far-flung continents, but as new research shows, that explanation doesn't fly with ratite birds. Ratite birds are a lineage of large, mostly flightless birds including the African ostrich, the Australian emu, the South American rhea, the diminutive New Zealand kiwi, and the extinct Madagascar elephant bird. Mitchell *et al.* examined the phylogeny of these birds, adding ancient mitochondrial DNA sequences from the extinct elephant bird. It seems that ratites originated from flighted ancestors who evolved large sizes and loss of flight only after flying to their new homes.

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