Extreme reversed sexual size dimorphism in the extinct New Zealand moa *Dinornis*

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The ratite moa (Aves; Dinornithiformes) were massive graviportal browsers weighing up to 250 kg (ref. 1) that dominated the New Zealand biota until their extinction approximately 500 yr ago. Despite an extensive Quaternary fossil record, moa taxonomy remains problematic[1–4] and currently 11 species are recognized. Three *Dinornis* species were found throughout New Zealand and differed markedly in size (1–2 m height at back) and mass (from ~34 to 242 kg)[1]. Surprisingly, ancient mitochondrial DNA sequences show that the three species were genetically indistinguishable within each island, but formed separate North and South Island clades. Here we show, using the first sex-linked nuclear sequences from an extinct species, that on each island the three morphological forms actually represent just one species, whose size varied markedly according to sex and habitat. The largest females in this example of extreme reversed sexual size dimorphism were about 280% the weight and 150% the height of the largest males, which is unprecedented among birds and terrestrial mammals. The combination of molecular and palaeontological data highlights the difficulties of analysing extinct groups, even those with detailed fossil records.

In 1839 Owen predicted the existence of a large flightless ratite bird in New Zealand based on a fragmentary femur[5] (Fig. 1a). The isolation and small size of New Zealand made the claim remarkable, although the unique, terrestrial mammal-free endemic fauna was dominated by a radiation of birds (an estimated 245 species[1]).

Moa were adapted to a terrestrial, principally forest-dwelling habitat[1], and were highly variable morphologically resulting in the descriptions of over 64 species and 20 genera[1]. Current schemes[1,4] divide moa into two families (Emeidae, 8 species; Dinornithidae, 3 species). The short robust emeids differ from the tall and gracile dinornithids in many ways, notably in skull shape and vertebral structure, where the latter have three extra cervicals. The three *Dinornis* species (*Dinornis giganteus* Owen, 1844, *D. novaezealandiae* Owen, 1843 and *D. struthoides* Owen, 1844) show few cladistic differences, and throughout their history have been separated largely on the basis of limb bone size[1–3,6,7] (Fig. 1a, b; see also Supplementary Information). An extensive Quaternary record indicates that *Dinornis* species had overlapping geographical ranges within New Zealand's North and South islands[1] (Fig. 1c).

To investigate the evolutionary history of *Dinornis*, polymerase chain reaction (PCR) primers were designed using complete mitochondrial genome sequences[8]. Rigorous ancient DNA techniques[9] were used to sequence 525 base pairs (bp) of the control region from 32 *Dinornis* specimens, and 1,435 bp of protein-coding/transfer RNA sequences from seven of these (see Methods). Remarkably, despite the enormous morphological size variation there were no consistent genetic differences between the three *Dinornis* species. Furthermore, a strong phylogenetic signal separated all *Dinornis* specimens, irrespective of their size, into simple North Island or South Island clades (Fig. 2). This discrepancy between genetic homogeneity and morphological variation could perhaps be explained by rapid morphological evolution, or differential growth patterns. Rapid dwarfing has been observed in other New Zealand avian groups over the past 10,000 years (10 kyr)[1], but most *Dinornis* sequences in Fig. 2 were from contemporaneous specimens (1–4 kyr), and all specimens were morphologically adult or fully grown (Supplementary Information). Differential growth by geographic area might have contributed some variation but the small *D. struthoides* co-occurred spatially and temporally with larger forms, *D. giganteus* and *D. novaezealandiae* (Fig. 1c).

In the absence of alternative explanations, the possibility that sexual dimorphism might be contributing to the large size variation in *Dinornis* was examined. In ratites, unlike other birds, genetic sexing is complicated because most of the female-specific W chromosome is genetically similar to the Z[10]. Recently, the nuclear-encoded W-specific KW1 locus has been used to sex modern ratite birds through a truncated W-specific copy with distinct sequence differences from multiple Z or autosomal copies[11]. However, most ancient DNA studies have been restricted to high-copy number mitochondrial DNA sequences, and the amplification of single-copy nuclear loci is only expected in a subset of well-preserved ancient specimens[12]. To maximize the number of moa specimens with sufficient nuclear DNA preservation for sex determination, it was necessary to design PCR primer pairs for a short

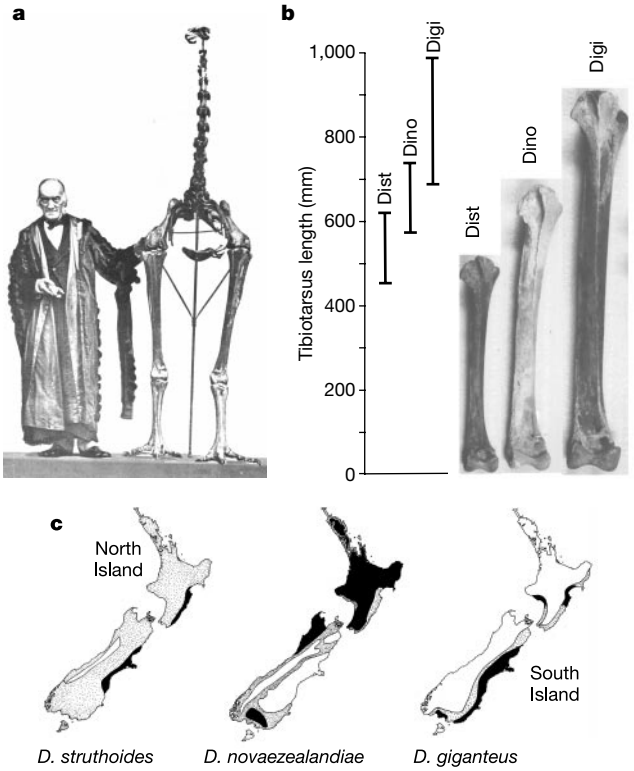

Figure 1 Size and distribution of the three currently accepted *Dinornis* species. **a**, Richard Owen holding the fossil femur fragment used to predict the existence of the moa in New Zealand. The skeleton (*D. novaezealandiae*) illustrates that *Dinornis* is the tallest bird known (ref. 24, pl. 47). **b**, Size comparison of the tibiotarsus of the three *Dinornis* species. The partly overlapping size ranges[1] illustrate the extent of variation in tibiotarsus length within each of the *Dinornis* species (Supplementary Information). Digi, Dino and Dist refer to *D. giganteus, D. novaezealandiae* and *D. struthoides*, respectively. **c**. Distribution of *Dinornis* Holocene palaeontological records within the North Island and South Island of New Zealand. Shading indicates prominence of species in fauna: black, prominent; stippled, regularly present; unshaded, rarely, if ever present.

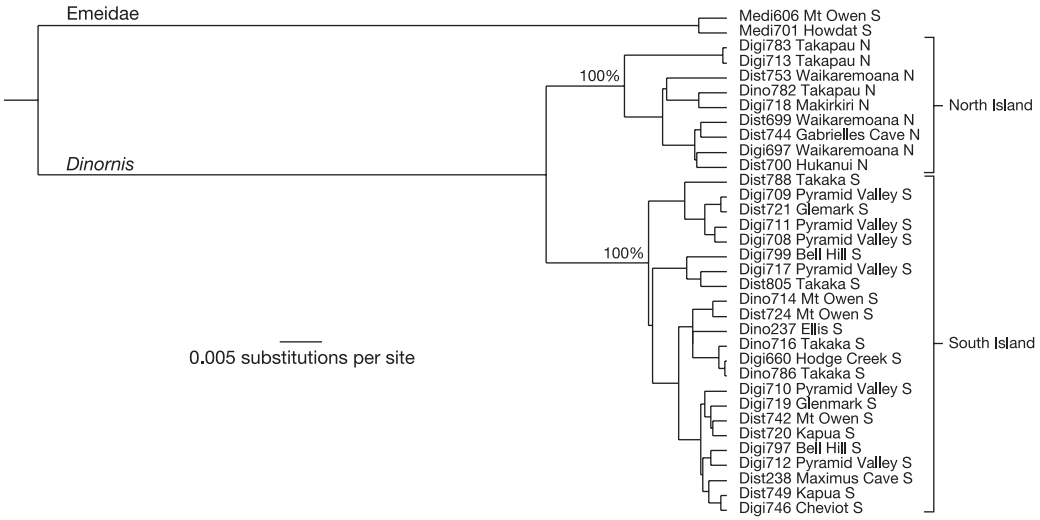

Figure 2 The maximum a posteriori tree of *Dinornis* mitochondrial DNA sequences generated from the posterior distribution using Metropolis–Hastings MCMC and control region sequences (525 bp) of 32 specimens, with protein-coding sequences (1,435 bp) from 7 specimens (see Methods). Two *Megalapteryx* moa sequences were included as out-groups. Morphological species identifications for *D. giganteus* (Digi), *D. novaezealandiae* (Dino) and *D. struthoides* (Dist) are given, along with DNA extract numbers, collection sites and island of origin. All *Dinornis* specimens (irrespective of species) fall into either the North (N) or South Island (S) clade. The split between the islands has a posterior probability of 100%, and is estimated to be mid-Pleistocene in origin (see Supplementary Information).

W-specific (female) product. KW1 sequences of modern rhea, ostrich and kiwi samples were generated and revealed several differences from those previously published[11], as well as several extra Z or autosomal copies (data not shown). The primer pair 112f and 267r (Supplementary Information) consistently produced a 180-bp product from female, but not male, samples of rhea, ostrich and kiwi (Fig. 3a; see also Methods). A similar-sized amplification product was also generated in several well-preserved *D. giganteus* and *D. novaezealandiae* specimens but not in any *D. struthoides* samples. The sequence of the moa 112f −267r product was similar to other ratite W-specific KW1 sequences (Supplementary Information), and was used to design internal primers (185f and 260r) for a short (85 bp) moa W-specific product suitable for amplification from degraded ancient templates (Fig. 3b).

The 185f and 260r KW1 primers were used to screen 34 *Dinornis* specimens, along with a similar-sized control amplification (85 bp) of the nuclear-encoded alcohol dehydrogenase (*ADH*) gene (Fig. 3b). Eight specimens (24%) yielded neither the ADH-positive

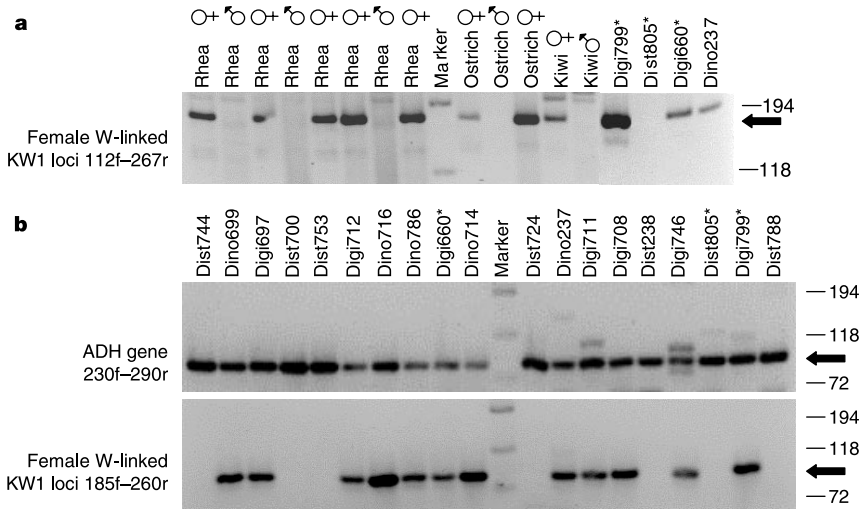

Figure 3 Molecular sex determination of *Dinornis*. **a**, A 180-bp W-linked product was produced from the modern female rhea, ostrich and kiwi samples using the 112f and 267r primer combination. All male samples failed to amplify a product of the correct size (indicated by arrows) or sequence. Similar bands and sequences are shown from two well-preserved *D. giganteus* (Digi799, Digi660) and *D. novaezealandiae* (Dino237) specimens, but not in a well-preserved *D. struthoides* (Dist805) specimen. **b**, Sub-fossil *Dinornis* samples screened for short (85-bp) W-specific and ADH products. ADH PCR products were obtained from 26 specimens (19 shown, upper panel), of which 15 of the 16 *D. giganteus* and *D. novaezealandiae* samples also tested positive for the presence of the 85-bp W-specific amplification product (12 shown, lower panel). Of the 10 *D. struthoides* samples positive for ADH, none gave a W-linked product. The three samples marked with an asterisk were independently replicated in Copenhagen.

	D. struthoides	*D. novaezealandiae*	*D. giganteus*	Ratio Dist: Dino + Digi	
(1) Waitomo Karst	40	31	16	1:1.2	(40:47)
(2) North Is. eastern hills	28	33	13	1:1.6	(28:46)
(3) North Is. Lowland*	34	8	13	1.6:1	(34:21)
(4) North Is. western swamps*	33	59	15	1:2.2	(33:74)
(5) Takaka Hill	14	12	0	1.2:1	(14:12)
(6) Takaka Valley	5	7	1	1:1.6	(5:8)
(7) NW Nelson	22	24	3	1:1.2	(22:27)
(8) Punakaiki	25	24	2	1:1	(25:26)
(9) South Is. Eastern swamps	123	20	173	1:1.6	(123:193)
(10) Bell Hill	9	0	9	1:1	(9:9)
Totals	333	218	245	1:1.4	(333:463)

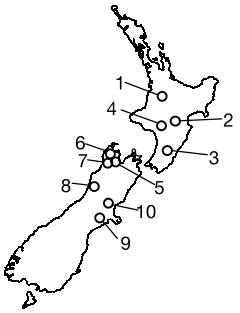

Figure 4 Occurrence of *Dinornis* skeletal remains from swamp and cave deposits across New Zealand. Palaeontological records show that the ratio of *D. struthoides*:*D. novaezealandiae* plus *D. giganteus* (male:female) is 1:1.4 across ten broad geographical areas for which detailed data exists, which is within the range of modern ratites (1:1.1–1.44). Only two swamp-dominated sites (asterisks) differ significantly from the 1:1.4 ratio (χ^2 test; $P > 0.2$), and these may reflect taphonomic biases such as weight-based entrapment or seasonality.

control nor female-specific KW1 products, presumably owing to a lack of amplifiable nuclear DNA. The ADH locus was amplified from the remaining 26 specimens (10 *D. giganteus*, 6 *D. novaezealandiae* and 10 *D. struthoides*), of which 15 of the 16 *D. giganteus* and *D. novaezealandiae* samples were also positive for the female-specific product. In direct contrast, none of the 10 *D. struthoides* samples positive for ADH gave W-specific products (Fig. 3b). Determination of relative numbers of nuclear templates in six extracts by quantitative real-time PCR confirmed *D. struthoides* specimens were among some of the best preserved (Supplementary Information). Consequently, the failure of all the *D. struthoides* specimens to produce female-specific KW1 products seems unlikely to reflect PCR drop-out. The one *D. giganteus* extract (Digi783) that yielded an ADH, but not a KW1 product, showed high levels of PCR inhibition in the quantitative assays, explaining the inconsistent amplification results. Independent analysis of three specimens in Copenhagen replicated the KW1 and ADH results (Fig. 3b).

The mitochondrial data suggest that a single species of *Dinornis* existed on each island, whereas the nuclear sequences indicate that the larger specimens (previously identified as *D. giganteus* and *D. novaezealandiae*) were females and the smaller specimens (*D. struthoides*) were male. A re-examination of the palaeontological data provides further support for such extreme reversed sexual dimorphism (RSD). The distribution of *Dinornis* specimens in well-characterized swamp and cave deposits across New Zealand (Fig. 4) reveals that when *D. giganteus* and *D. novaezealandiae* are considered together (as females), the ratio of putative male (*D. struthoides*) to female (*D. novaezealandiae* and *D. giganteus*) averages 1:1.4 (Fig. 4), within the range of modern ratites (1:1–1.44)[13,14]. Only two swamp deposits depart markedly from this ratio and these may be the consequence of taphonomic biases such as mass-dependent entrapment and/or seasonal variation in swamp size. The size of female *Dinornis* (*D. giganteus* and *D. novaezealandiae*) appears to have ranged from around 1.2–1.9 m in height (at back) and 76–242 kg in mass (Supplementary Information). Both size and mass varied according to habitat, with females averaging 102 kg in northwest South Island and 167 kg in eastern South Island. Males (*D. struthoides*) were significantly smaller, ranging from 0.9–1.2 m and 34–85 kg. Smaller size in both sexes appears to correlate with higher rainfall, more homogeneous closed canopy vegetation, higher altitude, and may relate to available nutritional content of vegetation.

The combined molecular and palaeontological data strongly support RSD in *Dinornis* on a scale unprecedented among birds or terrestrial mammals, with the largest females about 150% the height and 280% the weight of the largest males (Supplementary Information). Other forest-dwelling ratites, such as the kiwi (*Apteryx*) and southern cassowary (*Casuarius casuarius*) exhibit RSD, with females typically 120% and up to 180% the weight of males, respectively[13,15], whereas two species of emeid moa have also been suggested to show sexual dimorphism[16]. Interestingly, RSD is much less evident or non-existent in plain-dwelling ratites. In general, avian RSD is associated with increased investment in egg production[17] and kiwi are a good example. Ecological factors, such as niche partitioning, have also been suggested to exist in *Dinornis* taxa[18,19]. Overall, both the RSD and absolute size variation observed in *Dinornis* seem to represent extreme examples of trends seen elsewhere.

Molecular rate analysis indicates that the North Island and South Island *Dinornis* mitochondrial lineages separated around the mid-Pleistocene epoch, consistent with geological activity in Cook Strait[20], the marine barrier between the islands (Fig. 2; see also Methods). The reciprocal monophyly of the mitochondrial DNA lineages from the two island populations makes it unlikely that substantial gene flow has occurred since then, or that the genetic separation has been caused by geographical lineage sorting[21]. This implies that the existence of RSD on each island represents either convergence or an ancestrally inherited character. Either way, the resolution of all *Dinornis* forms into at most two allopatric taxa explains the lack of cladistic morphological characters separating the previously identified taxonomic groups. The following taxonomy is now advocated: family, Dinornithidae (Bonaparte); genus, *Dinornis* (Owen); species, *D. novaezealandiae* Owen, 1843 (North Island *Dinornis*) and *D. robustus* Owen, 1846 (South Island *Dinornis*). The species *D. giganteus* and *D. struthoides* are placed in the synonymy of *D. novaezealandiae*.

The first molecular sexing of an extinct species has revealed an example of extreme RSD which was previously unsuspected despite an extensive Quaternary record. This combination of molecular and palaeontological data clearly illustrates the potential difficulties of characterizing extinct taxa with less detailed fossil records. □

Methods

Sample information

Sections of cortical bone (about 0.1 g) were collected from moa leg bones and prepared in a physically isolated, ancient DNA laboratory at the Oxford University Museum of Natural History. Museum numbers, provenance, site/bone dates and bone morphology appear in Supplementary Information. Length data for *Dinornis* femora, tibiotarsi and tarsometatarsi were obtained as previously described[7] for all sites with well-characterized material[1,7] (Supplementary Table 1).

DNA extraction, amplification and sequencing

DNA was extracted and amplified as previously described[8,21] using appropriate ancient-DNA techniques[9] in a dedicated laboratory. Multiple negative extraction and amplification controls were included, and selected amplification products were cloned using TOPO TA cloning kits (Invitrogen) to detect contamination and DNA damage. Modern samples and ancient samples subsequent to PCR amplification were analysed in the Zoology Department. Three moa bone samples (Digi799, Digi660 and Dist805) were sent to the University of Copenhagen for independent replication. All PCR reactions were conducted as described[21] using Hi-fidelity Platinum Taq (Invitrogen) to minimize polymerase error. Primers were designed from mitochondrial[8], ADH[22] and KW1[12]

sequences and are given in Supplementary Information. Thermal cycling conditions were 40 cycles of 95 °C/55–60 °C/68 °C (30–45 s each) for mitochondrial amplifications and 40–50 cycles of 95 °C/52–55 °C/68 °C (45 s each) for KW1 and ADH amplifications respectively. PCR re-amplifications were only occasionally required, and used the same primers. Sequences were determined using ABI Big Dye (v.3) on an ABI 310 or 3700 (Applied Biosystems), according to manufacturers instructions.

Phylogenetic methods

Metropolis–Hastings Markov chain Monte Carlo (MCMC) integration was used to jointly estimate the phylogenetic tree and parameters of the substitution model under the assumption of a molecular clock[23]. An HKY + G + I model of substitution was used to characterize the evolution of the concatenated control region and protein-coding sequences (525-bp control region for 32 *Dinornis* specimens, Medi606, Medi701, and 1435 bp of COI, II, tSer, tAsp, tLys, and ATP8 genes for Digi660, Dist700, Dist238, Dino699, Dino237, Digi697, Dist753, Medi606, Medi701). Analysis of the control region sequences alone produced almost identical estimates (Supplementary Information). The tree presented is the maximum a posteriori tree obtained from the MCMC analysis. The benefit of the MCMC approach lies in assessing the uncertainty in the tree through bayesian posterior probabilities of the relevant clades.

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Nuclear DNA sequences detect species limits in ancient moa

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Ancient DNA studies have typically used multi-copy mitochondrial DNA sequences[1,2]. This is largely because single-locus nuclear genes have been difficult to recover from sub-fossil material[3], restricting the scope of ancient DNA research. Here, we have isolated single-locus nuclear DNA markers to assign the sex of 115 extinct moa and, in combination with a mitochondrial DNA phylogeny, tested competing hypotheses about the specific status of moa taxa. Moa were large ratite birds that showed extreme size variation both within and among species[4]. For some taxa, this large variation was hypothesized to represent sexual dimorphism, while for others it was argued to reflect the existence of different species[5]. Our results show that moa were characterized by extreme reverse sexual dimorphism and as a result we have been able to clarify the number of moa species. For example, we show that the three recognized 'species' of *Dinornis* comprised only two monophyletic groups and that two of these 'species' comprised individuals of one sex only. This study also illustrates that single-locus nuclear DNA sequences can be consistently recovered from ancient material.

There are typically hundreds of copies of the mitochondrial genome in most cells, while the nuclear genome is usually present in only two copies[6]. Owing to this difference in copy number, mitochondrial genes are more likely to survive in ancient material[7]. Consequently, there has been an almost complete reliance on mitochondrial DNA sequences in studies of extinct organisms[8–10]. If limited-copy nuclear genes could be consistently recovered, they would provide the opportunity to address a series of previously intractable questions in ancient ecology and evolution. For example, species limits in many extinct vertebrates have been determined by reference to variation in the morphology of skeletal remains[11,12]. However, this approach is problematic in the case of the moa of New Zealand[13], as species delimitation has been confounded by high levels of morphological variation within and among taxa.

Early skeletal analyses, originating with descriptions by Polack in 1838[14] and Owen in 1839[15], suggested that there were very many species of moa, with at least 64 species names being used, together with 20 generic names[16]. Over the last 25 yr the number of recognized species has reduced substantially from 38 to 11 (refs 5, 16). The smaller number is primarily based on the hypothesis that some previously recognized 'species' that differed in size only, actually represented conspecific males and females, with females being the larger sex. For example, in the currently recognized species *Pachyornis mappini*, *Emeus crassus*, and *Euryapteryx curtus* a bimodal size distribution is evident and has been interpreted as intraspecific sexual dimorphism[5]. Other taxa were suggested to represent separate species on the basis of what were regarded as acceptable limits of intra-specific size variation, using living ratite species as a guide. In the heaviest of moa, *Dinornis*, size variation was shown to be exceptionally large and so three morphologically similar, but differently sized species have been designated: *D. giganteus*, *D. novaezealandiae*, and *D. struthoides*. As a result of these osteological analyses, it was concluded that there were, in fact, only 11 species of moa[5,16].

We tested the hypothesis that sexual dimorphism was prevalent in