Moa's Ark or volant ghosts of Gondwana? Insights from nineteen years of ancient DNA research on the extinct moa (Aves: Dinornithiformes) of New Zealand

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Summary

The moa (Aves: Dinornithiformes) of New Zealand represent one of the extinct iconic taxa that define the field of ancient DNA (aDNA), and after almost two decades of genetic scrutiny of bones, feathers, coprolites, mummified tissue, eggshell, and sediments, our knowledge of these prehistoric giants has increased significantly. Thanks to molecular and morphological-based research, the insights that have been obtained into moa phylogenetics, phylogeography, and palaeobiology exceeds that of any other extinct taxon. This review documents the strengths of applying a multidisciplinary approach when studying extinct taxa but also shows that cross-disciplinary controversies still remain at the most fundamental levels, with highly conflicting interpretations derived from aDNA and morphology. Moa species diversity, for example, is still heavily debated, as well as their relationship with other ratites and the mode of radiation. In addition to increasing our knowledge on a lineage of extinct birds, further insights into these aspects can clarify some of the basal splits in avian evolution, and the evolutionary implications of the breakup of the prehistoric supercontinent Gondwana. Did a flightless moa ancestor drift away on proto New Zealand (Moa's Ark) or did a volant ancestor arrive by flight? Here we provide an overview of 19 years of aDNA research on moa, critically assess the attempts and controversies in placing the moa lineage among palaeognath birds, and discuss the factors that facilitated the extensive radiation of moa. Finally, we identify the most obvious gaps in the current knowledge to address the future potential research areas in moa genetics.

Keywords: ancient DNA, moa, Dinornithiformes, ratite evolution, vicariance, palaeognath, molecular clocks

1. Introduction

Although the past 25 years of ancient DNA (aDNA) research have offered many highlights, it has perhaps been the DNA from remains of large charismatic extinct animals (megafauna) that has defined the field, and attracted the attention of molecular biologists interested in the past. Ancient DNA has been extracted and analysed from extinct megafaunal species such as woolly mammoth (*Mammuthus primigenius*) (e.g., Greenwood et al., 1999; Gilbert et al., 2008), woolly rhino (*Coelodonta antiquitatis*) (e.g., Orlando et al., 2003; Willerslev et al., 2009), and cave bear (*Ursus spelaeus*) (e.g., Hofreiter et al., 2004; Stiller et al., 2010), but in terms of the number of publications and DNA-profiled fossils, no other extinct taxon has been studied as intensively with molecular technology as the New Zealand moa (Aves: Dinornithiformes). These giant wingless graviportal birds (Figure 1) have fascinated scientists and the public ever since the famous British anatomist Sir Richard Owen was given the partial shaft of a moa femur in 1839 and predicted the existence of a large flightless Struthious bird in New Zealand (Owen, 1840).

Evidence from both molecular and morphological studies have shown that moa were palaeognaths (Cracraft, 1974, 2001; Cooper et al., 2001; Paton et al., 2002; Slack et al., 2007; Hackett et al., 2008; Harshman et al., 2008; Phillips et al., 2010), which are birds with an archaic paleognathous palate, open ilioischiatic foramen, and rhamphothecal grooves (Bock, 1963; Cracraft, 1974). The distribution of extant palaeognaths is restricted to the Southern Hemisphere, and includes the tinamou (Tinamidae) from South America, and the 'ratites' whose monophyletic relationship has recently been seriously questioned (see Section 3). The extant 'ratites' consist of the ostrich (*Struthio camelus*) from Africa; emu (*Dromaius* spp.) and cassowary (*Casuarius* spp.) from Australia and New Guinea, kiwi (*Apteryx* spp.) from New Zealand, and the rhea (*Rhea* spp.) from South America. All 'ratites' have highly reduced wings, but in moa these have been completely lost (Worthy and Holdaway, 2002). In addition to moa, other extinct palaeognath taxa include the flightless 'ratite' elephant birds from Madagascar (*Aepyornis* spp. *Mullerornis* spp.), several late Tertiary taxa from the Old and New Worlds (Dyke and van Tuinen, 2004; Leonard et al., 2005; Lindow and Dyke, 2006), and a putative 'ratite' from the Eocene of Antarctica (Tambussi et al., 1994; Mayr, 2009).

Like many endemic New Zealand birds, the herbivorous moa exhibited a *K*-selected life history, with delayed sexual maturity (Turvey et al., 2005; Turvey and Holdaway, 2005; Bourdon et al., 2010), and were therefore extremely vulnerable to the hunting and habitat changes that followed the arrival of Polynesians in the late 13th century AD (Anderson, 1989a; Wilmshurst and Higham, 2004; Wilmshurst et al., 2008; McWethy et al., 2009, 2010). Moa hunter sites littered with bones and eggshell fragments are found across New Zealand (Anderson, 1989a), and represent the most compelling evidence that all nine recognised moa species (Bunce et al., 2009; Gill et al., 2010) were hunted to extinction. The model presented in Holdaway and Jacomb (2000a) suggested that moa could have been driven to extinction within a century of human colonisation, although debate still surrounds the exact period of moa-human overlap (Anderson, 1989b, 2000; Holdaway and Jacomb, 2000b).

Although moa have been extinct for centuries, increasingly detailed molecular data are providing new insights into their biology, evolution and ecology (e.g., Wood et al., 2008a; Bunce et al., 2009; Rawlence et al., 2009; Allentoft et al., 2010; Huynen et al., 2010; Oskam et al., 2010). After almost two decades of aDNA research on moa, it seems timely to look back, provide an overview of the achievements, and identify the gaps in our knowledge. This is particularly pertinent because Next

Generation Sequencing (NGS) technology (e.g., Marguelis et al., 2005) is presently revolutionising molecular biology, and this 'revolution' has also affected the field of aDNA (Millar et al., 2008), most notably with the sequencing of whole ancient genomes (Green et al., 2010; Rasmussen et al., 2010; Reich et al., 2010). By assessing previous genetic research on moa, the future directions of aDNA research on these birds can be discussed in light of the potential offered by NGS.

A 'status report' considering only the genetic aspects of moa research would be ignoring the other major contribution to our current knowledge, namely analyses of morphological variation. This review is not aimed at presenting an equivalent overview of morphological-based moa research (reviewed by Anderson, 1989a; Worthy and Holdway, 2002), but realising that molecular biologists and palaeontologists have used vastly different methods to address the same topics, provides an interesting opportunity to assess the interactions arising from such a situation. Reaching a consensus has often been problematic and in some aspects the debate is ongoing. The second and major part of this review will focus specifically on two aspects of moa evolution that have been thoroughly addressed within both research areas: (1) the early evolutionary splits in the 'ratites' and in particular, how moa evolved and when they arrived in New Zealand; and (2) the extensive radiation of the moa lineage in the New Zealand archipelago.

We will review the research from a molecular perspective, but will include results from morphological-based moa research to examine strengths and caveats associated with both approaches.

2. Summarising nineteen years of moa genetics

After 152 years of moa research (see Worthy and Holdaway, 2002), Cooper et al. (1992) published the first molecular study on moa. Since then, many genetic insights have contributed to our knowledge of these birds and Table 1 summarises all the aDNA studies on moa published to date. The publications range from brief critical comments on moa taxonomy that were derived from molecular analyses (Worthy, 2007; Baker, 2007b) to large-scale sequencing projects involving whole mitochondrial genomes (Cooper et al., 2001; Haddrath and Baker, 2001) and DNA sequences from hundreds of individuals (Baker et al., 2005; Bunce et al., 2009; Allentoft et al., 2010). With nine publications appearing in 2009-2011, it is clear that genetic research on moa is still a vigorous area of scientific enquiry. This may seem like a considerable effort expended on investigating the evolution of one particular extinct avian lineage, and it certainly reflects the fascination that surrounds moa. However, it also reflects the abundance of well-preserved moa remains, which, coupled with a relatively recent extinction, means that the retrieval of biomolecules is perhaps slightly less challenging than from other extinct megafauna.

2.1 Substrates

Table 1 documents that moa aDNA has been isolated from a broad range of biological substrates: bone, mummified soft tissue, feather, eggshell, coprolite, and sediment (Figure 2). As was typical for the earliest aDNA studies (e.g., Higuchi et al., 1984; Paabo, 1985), Cooper et al. (1992) focused on soft tissues (muscle and skin) and were successful in amplifying relatively long mitochondrial DNA (mtDNA) fragments (~400 bp). Soft tissue remains of moa are rare though [only ten mummified specimens attributed to species have been found to date (Anderson, 1989a; Worthy and Holdaway, 2002)], but importantly, Cooper et al. (1992) showed that aDNA could also be isolated from a moa rib

bone. This finding considerably broadened the opportunities for further research in the area and since 1992, hundreds of moa bones have been genetically profiled (e.g., Baker et al., 2005, Bunce et al., 2009, Allentoft et al., 2010).

Highly optimised aDNA isolation protocols (e.g., Nohland and Hofreiter, 2007) have led to a generally high success rate in retrieving moa DNA. Exemplifying this, Allentoft et al. (2010) obtained positive genetic species identifications for 267 of 268 (99.6 %) sampled bone elements. Bone has indeed been the preferred substrate of moa because of an abundance of well preserved specimens. However, DNA from moa feathers (Rawlence et al., 2009), coprolites (Wood et al., 2008a) and to a lesser extent sediments (Willerslev et al., 2003; Haile et al., 2007), has been amplified with considerable success as well. The ability to assign the DNA in such samples to species level has yielded significant new insights into moa biology, to an extent that today we have detailed information on aspects such as sex ratios among adults and juveniles (Allentoft et al., 2010), moa diet (Wood et al., 2008a), and intra- and inter-specific plumage variation (Rawlence et al., 2009). Recently refined extraction protocols (Oskam et al., 2010) have resulted in successful DNA isolation from ancient avian eggshell, yielding DNA from a range of depositional environments (Huynen et al., 2010; Oskam et al., 2010). Moa eggshell fragments are, like bones, frequently encountered in the New Zealand fossil record at natural nesting localities (Wood, 2008) and also in archaeological sites namely ovens and middens used by the early Maori (Keepax et al., 1981). Interestingly, eggshell appears to exhibit a lower microbial load compared to bone, making it more suitable to the indiscriminate 'shotgun' sequencing that characterises NGS technology (Oskam et al., 2010).

To utilise the full temporal potential in aDNA research, it is essential to maximise the recovery of suitable template molecules and at the same time effectively remove polymerase chain reaction (PCR) inhibitors (see Nohland and Hofreiter, 2007). However, even the most suitable DNA isolation methods have little effect if the DNA is highly fragmented. The intensity of hydrolytic and oxidative processes, which are largely responsible for long term post mortem DNA fragmentation, are closely correlated with temperature (Paabo, 1989; Lindahl, 1993a, b; Hoss et al., 1996; Paabo et al., 2004). Hence, substrates from cold environments are preferable in aDNA studies, and explain why the present age limit of reliably recorded aDNA preservation (450,000-800,000 years) was achieved with aDNA isolated from Greenlandic ice cores (Willerslev et al., 2007). Most parts of New Zealand have a temperate climate and it is highly unlikely that amplifiable moa DNA has survived for hundreds of thousands of years. Yet, fossil sites such as limestone-buffered, anoxic peat deposits (Wood et al., 2008b; Allentoft et al., 2010; Allentoft et al., In Press; Rawlence et al., 2011) and numerous cave sites (e.g., Bunce et al., 2009) have provided conditions that are favourable for long term DNA preservation, explaining the general success in obtaining moa DNA despite the absence of optimal permafrost environments. The oldest securely dated moa specimen from which aDNA has been obtained is a ~19,000 year old bone (MNZ S28184, *Megalapteryx didinus*, Te Ana Titi; Bunce et al., 2009). This taxonomic affiliation of the oldest moa DNA is not surprising given that *M. didinus* was an alpine, cold adapted species that had a high relative fossil abundance during the Otiran Glaciation (70,000-10,000 years ago) around Te Ana Titi.

2.2 Target DNA sequences

Mirroring a general trend in the field of aDNA, there has been a substantial preference for targeting mtDNA sequences in genetic research on moa (Table 1). This is because mtDNA loci are more

abundant than nuclear loci, since each cell contains multiple copies of the mitochondrial genome but only one nuclear genome. Ancient DNA research is effectively a 'numbers game' and the probability of success is higher when multiple copies of a suitable template molecule are present.

The majority of the current 758 moa entries on GenBank (assessed late-2010) represent hyper-variable region 1 of the mtDNA control region (CR). Because of its high mutation rate (estimated to 8.7 % per million year for moa, Bunce et al., 2009), this region is highly variable and thus suitable for species identification and for inferring inter- and intra-specific phylogeographic patterns (e.g., Baker et al., 2005; Bunce et al., 2009). Other mtDNA genes such as NADH dehydrogenase subunit 6 (ND6), cytochrome oxidase I (COI), and ribosomal RNA subunit 12 (12S) have been amplified successfully on several occasions (Table 1) for a range of objectives, including clarification of the 'ratite' phylogeny and moa species identification (dealt with in later sections). In 2001, the complete mitochondrial genomes (16,997 bp) of two moa species (*Dinornis robustus* and *Emeus crassus*) were sequenced (Cooper et al., 2001). These were the first mtDNA genomes of extinct species to be sequenced, allowing a detailed molecular analysis of 'ratite' evolution. Shortly after, Haddrath and Baker (2001) repeated the effort on two additional specimens (*Anomalopteryx didiformis* and *Em. crassus*).

For the more challenging nuclear DNA, the achievements are less impressive. Amplification of moa nuclear DNA is currently restricted to: (1) the c-mos gene, applied in 'ratite' phylogenetics (Cooper, 1997); (2) the sex-identification markers [W-chromosome specific KW1 locus, alcohol dehydrogenase (ADH), and chromo helicase DNA (CHD) binding protein], presented in Bunce et al., (2003), Huynen et al. (2003), and used again in Allentoft et al. (2010) and Huynen et al. (2010); and (3) six characterised polymorphic microsatellite markers, developed specifically for moa (Allentoft et al., 2009, 2011). As of yet, no detailed study of genetic diversity in the moa nuclear gene pool has been published, despite the potential.

3. Placing the moa lineage

An obvious challenge when describing an extinct species is to establish its taxonomic affiliation. For moa, this topic has been studied extensively utilising morphological and molecular methods. Based on comparisons with avian, reptilian, and mammalian bones, Owen (1840) realised that the large, flightless, extinct moa was closely related to the ostrich. However, further resolution of the phylogenetic relationships within 'ratites', including the position of moa, has proven extremely challenging despite a considerable amount of morphological research on this topic (reviewed by Bledsoe, 1988; Houde, 1988; Anderson, 1989a; Sibly and Ahlquist, 1990; Cooper et al., 1992; Cracraft, 2001; Worthy and Holdaway, 2002; Dyke and van Tuinen, 2004; Harshman et al., 2008; Phillips et al., 2010).

Although doing little justice to a long standing debate with many interesting aspects, the two opposing theories regarding the evolution of moa can be summarised as: (1) moa and kiwi are sister-taxa, evolving from a common ancestor that was isolated on proto New Zealand (hereafter referred to as Zealandia) with the breakup of the ancient supercontinent Gondwana (Figure 3). This is the vicariance theory, or the 'Moa's Ark' theory (as coined by Bellamy et al., 1990); and (2) moa and kiwi are not sister-taxa and represent different colonisation events, for example by volant Gondwanan ancestors (the dispersal theory).

In addition to elucidating the taxonomic affiliation of an extinct avian lineage, the evolution of moa and other palaeognaths may provide key information towards understanding the role of continental drift in shaping the distribution of the world's biota. With the acceptance of plate tectonics theory in the 1960's, the vicariance model of allopatric speciation offered an elegant explanation for many otherwise mysterious cross-continent distribution patterns (e.g., Gibbs, 2006). In a landmark paper, consolidating 'ratites' as prime examples of vicariance, Cracraft (1974) conducted a cladistic analysis on 25 skeletal characters of 'ratites' (including moa) and found that they were monophyletic with a Gondwanan origin. It was suggested that the flightless 'ratite' ancestors had been isolated on the different landmasses in the Southern Hemisphere as they slowly drifted apart from the mid Jurassic. The proposed phylogeny placed tinamou as most basal, then moa/kiwi, elephant birds, cassowary/emu, ostrich, and finally rhea. Cracraft's (1974) cladistic analysis supported the long favoured hypothesis (e.g., Mivart, 1877; Fürbringer, 1888; Parker, 1895; Pycraft, 1900; Meise, 1963) that moa and kiwi were closely related or sister taxa and concluded that a common ancestor was isolated on Zealandia as it separated from eastern Gondwana (the Moa's Ark theory) (Figure 3). Although this sister taxa relationship has been supported by some subsequent morphological studies (Dyke and van Tuinen, 2004; Bourdon et al., 2009), others have argued against it (Zelenitsky and Modesto, 2003; Grellet-Tinner, 2006; Livezey and Zuzi, 2007). A cladistic analysis of 88 postcranial characters, for example, concluded that moa were basal in the 'ratite' phylogeny, then elephant birds/ostrich/rhea, followed by kiwi/cassowary/emu (Bledsoe, 1988). Adding to the confusion, Worthy and Holdaway (2002) criticised studies both in favour (Cracraft, 1974) and against (Bledsoe, 1988) a moa-kiwi sister taxa relationship. It was suggested that an unusually modest level of homoplasy appeared in the Cracraft (1974) data given the convergence expected in a group of large flightless birds, whereas it was noted that 28 of the 88 characters used in the analysis of Bledsoe (1988) were from the pectoral girdle, which is lacking in moa, and may therefore bias the analysis because of missing data (Worthy and Holdaway, 2002).

In 1992, the field of aDNA entered the debate by showing that a simple vicariance model was not applicable to New Zealand 'ratites' (Cooper et al., 1992). The 12S rDNA sequence was amplified from four different moa species, and representatives of each genera of extant 'ratites', and demonstrated that kiwi and moa were not sister taxa (Cooper et al., 1992). Kiwi formed a more recent Australasian clade with emu and cassowary, whereas moa were older and more closely related to the South American rhea. This result was inconsistent with a simple Gondwanan vicariant history and supported earlier work based on DNA-DNA hybridisation results from extant 'ratites' (Sibley and Ahlquist, 1981, 1990). Cooper et al. (1992) concluded that a common moa-kiwi ancestor could not have been isolated on Zealandia by continental drift. Rather, two independent colonisation events had taken place. Because of the relative branching order, it was suggested that the ancestors of moa were isolated on Zealandia when it separated from eastern Gondwana (Figure 3), whereas the kiwi, diverging later, had arrived in New Zealand by swimming or island-hopping (also suggested by Sibley and Ahlquist, 1981). The early molecular work on moa was extended in several other studies adding additional mitochondrial and nuclear DNA sequences (Cooper 1993, 1997) (Table 1). Although the topology changed slightly and the exact position of moa varied, moa and kiwi were never recovered as sister taxa. Advocating the new molecular data in favour of morphology, Cooper (1997) commented that earlier morphological studies (Cracraft, 1974; Bledsoe, 1988) used many of the same morphological characters, but achieved different phylogenies altogether, showing that subjective decisions about character states and polarity were influencing morphological studies. However, as the sections below will show, equivalent problems can apply to molecular phylogenetics.

A milestone in aDNA research was reached with the sequencing of complete mitochondrial genomes of two species of moa, *D. robustus* and *Em. crassus* (Cooper et al., 2001). Mitochondrial genomes from extant 'ratites' and two species of tinamou were also included, and the study confirmed that 'ratites' were monophyletic, with rhea basal, followed by moa, ostrich, and the Australasian clade (emu, cassowary and kiwi). To calibrate a molecular clock and estimate a mutation rate for mtDNA, the divergence of moa from all other 'ratites' was fixed at 82 million years ago (Mya), when Zealandia was believed to have separated from Gondwana (Cooper et al., 2001) (Figure 3). By doing so, it was estimated that all 'ratite' taxa except kiwi had a Gondwanan vicariant history and diverged during the Late Cretaceous, followed by the subsequent dispersal and speciation of kiwi about 65-72 Mya.

The results were confirmed in a largely similar study of ratite mtDNA genomes, except that moa was recovered as basal among 'ratites' (Haddrath and Baker, 2001). By using an estimated split between emu and cassowary at 35 Mya (Boles, 1992) as a calibration point, the divergence time of moa was estimated at 78.9 Mya and 62 Mya for kiwi. Interestingly, despite 'problems' with kiwi (and ostrich as well) branching off too late to match a classical Gondwanan vicariance hypothesis, both these studies (Cooper et al., 2001; Haddrath and Baker, 2001) interpreted their results in a vicariance context, using considerable effort to explain the observed deviations from the predominating view. Importantly, these whole mtDNA genome studies consolidated for a while the ~80 Mya evolutionary split of moa, thereby favouring the Moa's Ark theory (Figure 3), although this ark of Zealandia obviously did not include kiwi.

However, recent research has suggested that the story is likely to be more complex. When detangling prehistoric dispersal events on an evolutionary time scale, one has to consider that dispersal capability is not a static measure.

3.1 The volant moa?

With the advent of phylogenomics, increasingly detailed molecular analyses of 'ratite' evolution have been undertaken. In two comprehensive analyses of multiple nuclear loci from numerous bird species, Hackett et al. (2008) and Harshman et al. (2008) showed that the volant South American tinamous (Tinamidae), previously considered as the sister group of 'ratites', are nested within the 'ratite' clade. Harshman et al. (2008) focused specifically on palaeognaths and sequenced 20 independent nuclear loci from all extant 'ratites', and representatives of Tinamidae, Neoavian and crocodilian out-groups. They found that ostrich were basal, followed by rhea, tinamous, and the Australasian clade, showing that 'ratites' are not monophyletic as previously thought (e.g., Cracraft, 1974, 2001; Sibley and Ahlquist, 1990; Cooper et al., 1992; Lee et al., 1997; Cooper et al., 2001; Haddrath and Baker, 2001; Paton et al., 2002; Dyke and van Tuinen, 2004; Pereira and Baker, 2006; Livezey and Zusi, 2007). It should be noted though that some morphological studies of cranial and eggshell characters had disagreed with 'ratite' monophyly (Bock and Buhler, 1990; Elzanowski, 1995; Grellet-Tinner, 2006). In that context it was suggested that crania were less affected by morphological convergence caused by flightless, cursorial lifestyles, and could therefore contain more reliable characters (see Phillips et al., 2010).

A topology showing volant bird species within lineages of flightless ones (Figure 4) requires either that flight was lost several times, or that flight was lost in an archaic palaeognath and then regained among the tinamous. Harshman et al. (2008) advocated the former hypothesis, citing as supporting

297 evidence the multiple losses of flight in other avian lineages, especially on islands, but no evidence of
298 flight ever being regained. Although offering significant new insights on 'ratite' evolution by
299 rejecting the vicariance theory, Harshman et al. (2008) did not include moa sequences in their
300 analyses, and the placement of this lineage remained unclear.

301 The most recent molecular contribution was based on complete mtDNA genomes of 'ratites',
302 including moa (also involving two kiwi mtDNA genomes and several outgroup taxa), and provided a
303 solid independent confirmation that 'ratites' are paraphyletic (Phillips et al., 2010), although the exact
304 topology differs from the other recent molecular efforts (see Hackett et al., 2008; Harshman et al.,
305 2008). By applying complex phylogenetic analyses with model partitioning between different genes
306 and codon positions, moa and tinamou were recovered as sister taxa within the 'ratites' (Figure 4)
307 (Phillips et al., 2010). It is perhaps unexpected that the closest relative to New Zealand's wingless
308 megafauna should be found within a family of small (relatively speaking), volant birds from South
309 America that were not even considered as 'ratites'. Moreover, the results suggested that at least four
310 losses of flight happened during palaeognath evolution (Phillips et al., 2010) (Figure 4).

311 These new findings have severe implications for assessing the timing of the palaeognath radiation.
312 Because open water cannot be regarded as an efficient barrier to a volant ancestor, the suggested
313 divergence between moa and other 'ratites' by the separation of Zealandia from eastern Gondwana at
314 82 Mya (Figure 3), seems at least conceptually fallacious. Thus, Phillips et al. (2010) used a new suite
315 of fossil calibrations (associated with Cretaceous-Tertiary extinction events and the break-up of
316 southern Gondwana) to calculate divergence times within palaeognaths. With this approach, moa and
317 tinamou diverged c. 60 Mya (Figure 4) (95 % highest posterior density, HPD 38.3-81.6 Mya), and it
318 was therefore suggested that a common ancestor of moa and tinamous arrived in New Zealand by
319 flight (from South America or Antarctica) about 60 Mya, dispelling the Moa's Ark theory (Phillips et
320 al., 2010) (Figure 3).

321 Following the breakdown of the 'ratite' vicariance theory, it was suggested that Gondwana might not
322 be the geographical origin of palaeognaths (Phillips et al., 2010), which is perhaps supported by the
323 finding of archaic 'ratite'-like fossils from the Northern Hemisphere (Houde, 1986; Houde and
324 Haubold, 1987; Dyke, 2003; Dyke and van Tuinen, 2004; Leonard et al., 2005; Lindow and Dyke,
325 2006). Unfortunately, the fossil record of New Zealand does not shed much light on proto-moa
326 morphology and the timing of their arrival. The earliest known fossils (bone fragments and pieces of
327 eggshell) attributable to moa are dated at 16-19 Mya (Tennyson et al., 2008; Tennyson, 2009), which
328 seems relatively young considering a suggested 60 Mya arrival of proto-moa. This 'discrepancy' can
329 probably be ascribed to the general paucity of terrestrial Tertiary fossil localities in New Zealand
330 (Tennyson et al., 2008).

331 To complicate matters even more, recent research has proposed that the opening of the Tasman Sea
332 was more complex than a single split at 82 Mya. Schellart et al. (2006), based on sophisticated
333 geological and computer modelling, argues that the separation of Zealandia from eastern Gondwana
334 was initiated in the south (around present day Antarctica) about 85 Mya, but did not occur in the north
335 (through the Lord Howe Rise, Norfolk Ridge and present day Queensland) until c. 60-55 Mya (Figure
336 3) (Haynes and Ringis, 1973; Kennett et al., 1975b; Gaina et al., 1998a, b; Norvick et al., 2001). If
337 correct, we cannot dispel the Moa's Ark theory, because the result implies that already flightless moa
338 and kiwi ancestors could have independently walked to Zealandia to become isolated around 60 Mya
339 (Figure 3), which is in fact the current best estimate of moa and kiwi speciation (Phillips et al., 2010),
340 with their subsequent isolation in New Zealand following the drowning of the Lord Howe Rise and

Norfolk Ridge during the Oligocene (Cooper and Millener, 1993; Waters and Craw, 2006; Trewick et al., 2007; Landis et al., 2008). Clearly, more research is needed on the subject, and we encourage future phylogenetic studies on palaeognath evolution to consider this possibility.

3.2 Advanced genetic modelling

The analytical approaches in recent avian molecular studies (Hackett et al., 2008; Harshman et al., 2008; Phillips et al., 2010) offer suggestions as to why previous work has consistently maintained 'ratite' monophyly. Exemplifying state-of-the-art molecular phylogenetics, Phillips et al. (2010) partitioned the data at several levels rather than treating the entire mitochondrial genome as a single unit. For example, RNA-coding genes were analysed in a separate partition to protein-coding genes. Such partitioning allows different evolutionary models and mutation rates to be applied for DNA regions under different selective constraints. Importantly, all the protein-coding sequence data were further partitioned into first, second, and third codon positions with the third codon position being RY-coded (assigning each nucleotide as a purine or pyrimidine). This procedure can reduce the effects of signal saturation, owing to 'multiple hits' in the more frequently mutating third codon position. Saturation effects can be particularly pertinent when analysing sequences across very old evolutionary splits. RY-coding was shown to effectively improve the phylogenetic signal among these basal avian lineages (Harris et al., 2004; Phillips et al., 2010).

Moreover, many molecular studies are now applying relaxed phylogenetics (e.g., Drummond et al., 2006), allowing the mutation rates to differ among branches of the phylogenetic tree. Because previous molecular analyses of palaeognath evolution have suggested an unusually high mutation rate among tinamous, compared to the other palaeognaths (e.g., Sibley and Ahlquist, 1990; Paton et al., 2002), it proved essential to account for rate heterogeneity across sites and lineages. Ignoring this matter, the tinamou lineage was attracted towards the base of the tree, resulting in apparent 'ratite' monophyly (Harshman et al., 2008; Phillips et al., 2010). Phillips et al. (2010) mention that even when accounting for rate bias, tinamous would in some occasions still be basal in the tree, but only when moa were excluded from the analyses. This situation is equivalent to earlier attempts of sorting the basal placental mammalian splits (e.g., Kretteck et al., 1995). For example, hedgehog DNA sequences have, like tinamous, evolved at a very high rate (Lin et al., 2002) and were therefore artificially attracted to the base of Placentalia in earlier molecular phylogenetic analyses. It was not until the hedgehog DNA was accompanied by sequences from a close relative (a slower evolving shrew), that molecular analyses displayed hedgehogs as nested well within Placentalia (Lin et al., 2002).

Perhaps the most important improvement in the recent analyses compared to some of the earlier efforts (e.g., Cooper et al., 2001; Paton et al., 2002; Pereira and Baker, 2006), was that they did not enforce 'ratite' monophyly in their analyses (Harshman et al., 2008; Phillips et al., 2010). Hence, the analyses and interpretations were probably less biased by assumptions about the taxonomic relationships than previous attempts. Interestingly, Phillips et al. (2010) admit that moa sequences were excluded from some previous analyses (Harrison et al., 2004) because the resulting topology did not conform to the tinamou-'ratite' sister-group hypothesis prevailing at the time. Similarly, some of the phylogenetic analyses conducted by Haddrath and Baker (2001) placed tinamous inside of 'ratites', but were discarded because they went against the established theory. These are examples of how molecular analyses can be subjected to equivalent selective and subjective interpretations that have been highlighted as problematic for morphology-based phylogenetics (e.g., Cooper, 1997).

According to the latest data, the evolutionary splits within palaeognaths occurred between 104 and 36 Mya (including 95% HPD's), with the exception of a more recent separation between cassowary and emu (Figure 4) (Phillips et al., 2010). We note that even the most complex evolutionary models are likely to be simplified versions of the actual changes in genomes through time, and when analysing the timing and order of very early phylogenetic splits, small inaccuracies in the applied mutation model may lead to highly biased results. However, despite some controversy on the exact topology and the timing of the evolutionary events, all molecular analyses on this subject have concluded that moa and kiwi could not be sister taxa, irrespective of the methodology, taxa or genes analysed, or size of the dataset.

3.3 A controversy revisited

From a morphological viewpoint, the challenges have been equally profound. Worthy and Holdaway (2002) state that resolving the evolution of 'ratites' is complicated because of their primitiveness (clustering at the base of the avian phylogeny) and at the same time extreme modification as large, flightless, cursorial birds, which makes it difficult to identify the derived, phylogenetically informative characters. Most morphological assessments have agreed on 'ratite' monophyly and a close moa-kiwi relationship, and the most recent morphological contribution supports these theories. Bourdon et al. (2009) analysed 129 morphological characters (77 not previously assessed), and identified 13 synapomorphies for moa and kiwi, suggesting a sister group relationship between the New Zealand 'ratites', and all other 'ratites' (Figure 4). This is in stark contrast to all molecular studies. Bourdon et al. (2009) further conclude that 'ratite' evolution fits very well with the classic Gondwanan virariance model. It is beyond our objectives (and competences) to assess in detail the informativeness of the 129 characters used in their study. However, two central aspects attract our attention in the Bourdon et al. (2009) study. First, the use of Tinamidae and *Hesperornis*, [a highly derived extinct flightless aquatic bird from the Late Cretaceous (Marsh, 1880)], as fixed outgroups in the cladistic analyses. If Tinamidae is indeed placed well within the 'ratites' as the molecular analyses suggest, and *Hesperornis* exhibited a highly derived aquatic lifestyle, it may have complicated the identification of informative, morphological characters in their analyses. Second, 24 of the 129 characters are from the pectoral girdle (sternum and scapulocoracoid) and wings (humerus, ulna and carpometacarpus), which are modified or missing (e.g., moa) in 'ratites', raising questions of potential biases due to homoplasy and missing data [see Worthy and Holdaway (2002) on their concerns about Cracraft (1974) and Bledsoe (1988)]. Unfortunately, the morphological results of Bourden et al. (2009) were published almost simultaneously with the new avian molecular studies (Harshman et al., 2008; Hacket et al., 2008; Phillips et al., 2010), probably explaining why these highly conflicting results did not lead to any immediate confrontations in the literature.

To summarise this section, it seems that a consensus among molecular biologists has been established that moa and tinamou are sister taxa. This implies that the traditional 'ratite' clade is paraphyletic and suggests that several events of flight-loss have occurred among palaeognaths, perhaps causing the breakdown of the Gondwanan vicariance theory. These conclusions are, however, not supported in the most recent morphological contribution on the subject (Bourdon et al., 2009). Moreover, it is still highly speculative what factors caused the speciation of moa and kiwi, and how and when they arrived in New Zealand.

4. Moa diversity and radiation

4.1 Species diversity

Since the first description of moa (Owen, 1840), their taxonomy has been in constant review with as many as 64 species in 20 genera proposed over the past 170 years (see Worthy and Holdaway, 2002), before reaching the present consensus of nine species (e.g., Bunce et al., 2009; Gill et al., 2010). There are several reasons for this long history of taxonomic over-splitting and rearrangements. Irrespective of the exact number of taxa, it is evident that Dinornithiformes represents a relatively speciose order, and to discriminate between multiple, sympatric 'ratites' was challenging for early palaeontologists working without large comparative collections. Moa range in size from the smallest 12-15 kg turkey-sized, North Island form of *Euryapteryx curtus* to the female of *D. robustus*, which weighed >200 kg and was two meters high at the back (Worthy and Holdaway, 2002; Huynen et al., 2003; Bunce et al., 2003; Worthy et al., 2005). However, moa display significant sexual size dimorphism, and intraspecific allometric size variation among fossils from different glacial/interglacial periods and geographic regions (Worthy, 1987; Worthy and Holdaway, 1996, 2002). This extensive intraspecific morphological plasticity is responsible for a continuum of overlapping adult body sizes, constituting a true taxonomic minefield.

There has been considerable debate into the importance of sexual dimorphism in explaining allometric size variation in moa (e.g., Owen, 1844a, b; Hutton, 1875, 1892; Cracraft, 1976a, b, c; Millener, 1981; Worthy, 1987; Holdaway and Worthy, 1997; Worthy et al., 2005). These papers suggested among many things that observed bimodal distributions in leg bone lengths or length/width ratios could be explained by sexual dimorphism. Despite these speculations, it was not until 2003 that solid evidence of sexual dimorphism in moa was finally obtained. Two independent aDNA studies (Bunce et al., 2003; Huynen et al., 2003) showed that the genus *Dinornis*, which was previously separated into three species on the basis of size: *D. giganteus*, *D. novaezealandiae* and *D. struthoides* (Worthy, 1994a; Worthy and Holdaway, 2002), comprised reciprocally monophyletic North (*D. novaezealandiae*) and South (*D. robustus*) Island clades. By targeting DNA sequences on the female specific W-chromosome, it was demonstrated that larger *Dinornis* individuals (previously classified as *D. giganteus* and *D. novaezealandiae*) were females, whereas smaller individuals (previously *D. struthoides*) were males. Huynen et al. (2003) extended their study to examine six proposed moa species that Cracraft (1976a, b, c) had argued (based on morphological data) represented only three sexually dimorphic species. In each case, Huynen et al. (2003) proved Cracraft right by showing that the larger morphs represented females and that the smaller ones were males. These molecular studies on sexual dimorphism in moa (Bunce et al., 2003; Huynen et al., 2003) are 'text book' examples of the potential caveats in relying solely on morphological data when establishing species boundaries.

It is beyond the scope of this paper to address the morphological features that separate moa species. Diagnostic characteristics of the skull, sternum, pelvis and leg bones (femora, tibiotarsi and tarsometatarsi) have been published (see Worthy, 1988a; Worthy and Holdaway, 2002; Worthy et al., 2005), and as a result, present day specialists will rarely misidentify well-preserved bones from adult moa (Scofield et al., 2005) despite suggestions of the opposite (Baker et al., 2005; Huynen et al., 2008). It is clear however that in some occasions these morphological criteria do fail to distinguish between moa species. The results presented in Allentoft et al. (2010) demonstrated that a molecular approach represents the most secure way of identifying juvenile material and severely damaged bones, when they lack diagnostic features. By using molecular sexing techniques, it was also shown that a fraction of bones from the (smaller) males of four moa species had previously been morphologically misidentified as smaller moa taxa (Allentoft et al., 2010).

Indeed, aDNA has provided a powerful alternative for species identification. In genetic research on moa it is common practice to compare mtDNA CR target sequences against the >300 moa CR sequences available on GenBank (an approach known as 'Blasting'). Although some variation exists in the exact procedure for genetic species assignment of moa remains (e.g., Rawlence et al., 2009; Allentoft et al., 2010), 'Blast' searches will normally provide clear answers, showing a high percentage similarity against reference DNA sequences of the species in question.

Despite a consensus of nine species, it seems likely that moa taxonomy will be subject to further revisions. The taxonomy of the genus *Megalapteryx*, for example, has been heavily debated (e.g., Owen, 1883; Haast, 1884, 1886; Archey, 1941; Worthy, 1988b, Baker et al., 2005; Bunce et al., 2009). Originally, three *Megalapteryx* species were suggested based on allometric differences, but with the advent of radiocarbon dating in the 1980's, it was subsequently suggested that *Megalapteryx* was a single species, exhibiting temporal and altitudinal size variation (Worthy, 1988b, 1993, 1994b; Worthy and Holdway, 2002). The molecular results have not provided clear answers on this matter, showing that some clades within *Megalapteryx* exhibit 4-5% sequence divergence in their CR sequences (Lambert et al., 2005; Bunce et al., 2009). This is more than what separates well-established moa species such as *Em. crassus* and *Eu. curtus*, questioning whether these genetic distances represent deep phylogeographic structuring or different species (Baker et al., 2005; Baker, 2007; Bunce et al., 2009). The genus *Pachyornis* is also problematic. Currently three species are recognised: *P. geranoides*, *P. australis*, and *P. elephantopus*. Both *P. geranoides* and *P. elephantopus* exhibit deep phylogeographic structuring in their mtDNA gene pools, and the position of *P. australis* is still uncertain (Huynen et al., 2003; Baker et al., 2005; Bunce et al., 2009), despite robust morphological characters that argue that *P. australis* is a distinct species (Worthy, 1989).

With the development and promotion of DNA barcoding technology (e.g., Hebert et al., 2004), it was investigated whether this approach could elucidate moa taxonomy (Lambert et al., 2005). DNA barcoding uses a short fragment of the mitochondrial COI gene to identify specimens to known reference species based on a pre-defined threshold for DNA sequence similarity. Using the COI barcode, Lambert et al. (2005) matched the results of previous CR-based moa research (e.g., Cooper et al., 1992; Cooper and Cooper, 1995; Cooper, 1997), providing a preliminary confirmation that the divergence in COI was applicable as species identifier. Based on the barcoding approach, it was then proposed that each of 14 observed lineages in moa (Baker et al., 2005) be raised to species status (Baker, 2007a). However, the potential impact of this study was diluted by problems with small sample sizes in some lineages, and problems with the taxonomy and proposed nomenclature (Baker, 2007b; Worthy, 2007).

By not relying solely on genetic distances between clades, Bunce et al. (2009) took a more conservative approach towards recognising species boundaries. They recognised three moa families (Megalapterygidae, Dinornithidae, Emeidae) (Figure 5), six genera, and nine species, and rejected the use of a strict bar-coding approach to moa taxonomy. Their arguments against bar-coding focused on the difficulties in distinguishing between temporal, geographic and reproductive genetic barriers when investigating DNA from closely-related extinct animals (Bunce et al., 2009). Also, if female moa held a home range but males dispersed more widely (Allentoft et al., 2010), inferences based on maternally inherited mtDNA could overestimate the level of intraspecific genetic divergence between geographic regions, resulting in taxonomic over-splitting. Moreover, it is important to note that reciprocal monophyly is not necessary expected in closely related species. In fact, mitochondrial paraphyly is evident in 44% of non pelagic continental Australian-Papuan biological avian species surveyed (Joseph and Omland, 2009). Hybridisation, incomplete lineage sorting, and retention of ancestral

polymorphism can blur the genetic species boundaries, making it necessary to use multiple criteria to assess species limits in closely related taxa (e.g., de Queiroz, 2007; Sites and Marshall, 2004).

Because of all these unresolved problems, the present consensus of six genera and nine species (Figure 5) reported in Tennyson and Martinson (2006), Bunce et al. (2009), and Gill et al. (2010) is not based exclusively on genetics, but on a combination of molecular, morphological, palaeoecological and phylogeographic data that have been compiled since 1840.

Lastly, it should be noted that a recent publication has ignited the debate again (Huynen et al., 2010). Although originally described as two separate species, *Euryapteryx curtus*, a small endemic North Island moa, and *Euryapteryx gravis* (previously *Eu. geranoides*, see Worthy, 2005), a large graviportal southern North Island/South Island moa, a uniform mitochondrial gene pool suggests that these species are synonymous and represent a single species (*Eu. curtus*), showing pronounced temporal, sexual and geographic allometric size variation (Bunce et al., 2009). However, Huynen et al. (2010) has now advocated for two sympatric *Euryapteryx* species based on eggshell thicknesses: Class I (Thick, 0.98-1.6 mm) and Class II (Thin, 0.74-0.98 mm), and two accompanying base changes in a small fragment of the mtDNA CR sequence. Although potentially important, we argue that further evidence is required to establish these minute differences as consistent and taxonomically diagnostic.

4.2 The radiation of moa

With nine proposed species of moa, only tinamous show greater species diversity among palaeognaths. This observation has promoted considerable research into the events responsible for such an extensive radiation on the New Zealand archipelago.

Historically, moa were separated into two families based on shared morphological characteristics: Dinornithidae (containing the genus *Dinornis*) and Emeidae (containing the genera *Pachyornis, Euryapteryx, Emeus, Anomalopteryx* and *Megalapteryx*) (reviewed in Worthy and Holdaway, 2002). However, Worthy and Holdaway's (2002) morphological phylogenetic analysis showed there was some support for *Megalapteryx* being basal in the moa phylogeny (65% bootstrap support), rather than belonging to Emeidae. This was confirmed in the most recent morphological phylogenetic analysis, which showed that *Megalapteryx* is basal (defined by four synapomorphies), followed by *Dinornis, Pachyornis, Anomalopteryx*, and *Emeus/Euryapteryx* (Bourdon et al., 2009).

The first genetic studies on moa (Cooper et al., 1992; Cooper and Cooper, 1995; Cooper, 1997) had already suggested that *Megalapteryx* was basal to all other moa genera, and this was confirmed by more comprehensive molecular studies (Baker et al., 2005; Lambert et al., 2005; Bunce et al., 2009). Thus, though the exact number of moa species is debatable, geneticists and morphologists agree on the taxonomy and branching order above species level (Figure 5).

Genetic research has also provided opportunities to study the timing of moa radiation. The time dependency of molecular rates (Ho et al., 2005; Ho and Larson, 2006) combined with inappropriate calibration points (Graur and Martin, 2004; Ho et al., 2008) implies that the use of molecular clocks can be misleading, and results should always be interpreted with caution, particularly when deep phylogenetic splits are involved. However, if applied cautiously, the estimation of mutation rates

provides a tool to investigate the temporal dimension of evolutionary processes in greater detail than can be achieved from morphological data alone.

The main contributions on the timing of the moa radiation analysed mtDNA sequences from hundreds of individuals (Baker et al., 2005; Bunce et al., 2009). To investigate the divergence times between moa taxa, Baker et al. (2005) first estimated a mutation rate based on available 'ratite' trees (Cooper et al., 2001; Haddrath and Baker, 2001). The percentage sequence divergence between lineages was calculated, and by fixing the basal divergence of moa at the often applied 82 Mya (when Zealandia was thought to have separated from eastern Gondwana), a mutation rate could be estimated. The rate was then applied within the moa phylogeny to date the basal divergence of *Megalapteryx* to around 18.5 Mya. This divergence proved relatively soon after the Oligocene drowning maximum 22 Mya, when only an estimated 18 % of New Zealand's current landmass was above sea level (Fleming, 1979; Stevens, 1985; Cooper and Millener, 1993; Cooper and Cooper, 1995). Baker et al. (2005) therefore suggested that this initial moa radiation occurred after the Oligocene bottleneck. According to further analyses, a series of lineage splitting occurred 4-10 Mya, which largely coincided with the formation of the Alpine Fault (causing uplift of the Southern Alps on the South Island) (Campbell and Hutching, 2007), and the opening of Cook Straight, separating the North and South Islands of New Zealand, suggested to have occurred ~5 Mya (Baker et al., 2005). It was argued that the reshaping of the New Zealand landmass during this period resulted in geographic isolation and ecological specialisation of moa populations and species, facilitating an extensive radiation (Baker et al., 2005). While these are plausible interpretations, the applied molecular clock rate is likely to be incorrect as a result of using the 82 million year (My) Gondwana-Zealandia split – this calibration point has subsequently been shown to be invalid because of the suggested volancy of ancestral 'ratites' (Harshman et al., 2008; Phillips et al., 2010), or because the final separation of the two landmasses may not have occurred before 60-55 Mya (e.g, Schellart et al., 2006) (Figure 3).

To account for these novel insights on palaeognath evolution, Bunce et al. (2009) re-analysed the available moa data but included additional specimens sampled in a systematic manner, new geological information (such as the formation of Cook Straight about 450 Kya, rather than ~5 Mya), and applied a combination of two independent molecular clock analyses. One analysis was based on external calibration points, excluding the controversial 82 My calibration in favour of other well accepted avian fossil data. The second molecular clock analysis was based on internal calibration points from a large number of radiocarbon-dated moa specimens associated with the aDNA sequences (Bunce et al., 2009). In these analyses, *Megalapteryx* was once again recovered as the basal moa genus, but the split was now estimated to have occurred just 5.8 Mya (Figure 5), in contrast to the 18.5 Mya estimated by Baker et al. (2005).

Clearly, this result has significant implications for the interpretation of the events responsible for the moa radiation. Because the basal moa split occurred so recently, it was argued that the ancestors of the Quaternary moa lineages could not have been present on both the North and South Island remnants during the Oligocene drowning c. 22 Mya (Bunce et al., 2009). This does not necessarily imply, however, that moa were absent from one of the drowning landmasses, but rather that lineages from only one island contributed to the Quaternary moa species diversity. Bunce et al. (2009) argued that moa ancestors survived on the South Island and then re-colonised the North Island about 1.5-2 Mya, when the two islands rejoined after 30 My of separation. This is supported by observing that (1) North Island moa lineages have a more derived position in the phylogeny (Bunce et al., 2009), (2) the oldest identified moa remains (fossilised eggshell and bone fragments) date to 16-19 Mya on the South Island (Tennyson et al., 2008; Tennyson, 2010), and (3) the suggested timing of the moa radiation

coincided with the formation of the Southern Alps, as noted previously (Baker et al., 2005) (Figure 5). Bunce et al. (2009) concluded that the observed highly complex phylogeographic inter- and intraspecific genetic structuring in moa was caused by the formation of the Southern Alps <6 Mya, the separation of the North and South Islands around 450 Kya, and the habitat fragmentation on both islands resulting from Pleistocene glacial cycles, volcanism, and landscape changes.

Assuming that these hypotheses are correct, the following question is pertinent: Is it possible that moa did not diversify between their suggested colonisation of New Zealand ca. 60 Mya (Phillips et al., 2010) and the 5.8 Mya separation of the basal moa, *Megalapteryx* (Bunce et al., 2009)? The fossil record may provide the answer. Thickness assessments of fossil moa eggshell from the Saint Bathan's Miocene fauna indicate that at least two moa species were living in the South Island 16-19 Mya (Tennyson et al., 2010; Tennyson, 2010). This finding does not imply a conflict between morphology and molecular data. Bunce et al. (2009) specifically state that although the Quaternary moa fauna stem from a single lineage with the first radiation occuring ~5.8 Mya (Figure 5), the genetic data could just be recording the latest of several moa radiations, with earlier lineages having gone extinct before ~5.8 Mya. Extinction of these early moa lineages is not unexpected or unprecedented because crocodilians, turtles, swiftlets (Aves: Apodidae), cracticids (Aves: Artamidae), palaelodids (Aves: Phoenicopteriformes), endemic bat families and terrestrial mammals known from the Miocene Saint Bathan's Fauna have all become extinct (Worthy et al., 2006, Tennyson, 2010). .

In summary, the overall taxonomic and phylogenetic relationships at family and genus level in moa are now fully resolved (Figure 5), but it seems likely that the taxonomy at the species level will be revised in the future, perhaps once nuclear data can contribute to the debate. The examples discussed above demonstrate how molecular data have provided significant advances in studying the nature and timing of very complex radiation events, but at the same time it is clear that different molecular methods can produce vastly different results (in this case the diversification date of *Megalapteryx* differing by ~13My) depending on the information that is incorporated into the analysis.

5. The future of moa genetics

The last 19 years of aDNA research on moa has generated as many new questions as answers and there are many aspects of moa evolution and biology that are still unresolved. Even at the most basal level, regarding the early avian evolutionary splits and the radiation of palaeognaths, we have probably not heard the last. For example, sequencing of the extinct elephant bird mtDNA genome is likely to be possible given the recent encouraging results from elephant bird eggshell (Oskam et al., 2010), and may result in additional rearrangements of the palaeognath phylogeny. In addition, Next Generation Sequencing platforms are responsible for enormous amounts of data becoming available, and ever more sophisticated methods for handling and analysing genetic data could also revise our current knowledge of palaeognath evolution. Although the most recent molecular publications on this matter appear to be approaching a consensus (Ericson et al., 2008; Harshman et al., 2008; Phillips et al., 2010), it is not shared among some morphologists (Bourdon et al., 2010), and more independent morphological analyses are required to shed light on the discrepancies.

Many questions concerning moa radiation and taxonomy also remain to be answered. For example, if the molecular dates for the radiation of moa at 5-6 Mya are correct, what happened to the two moa lineages that appear to be represented by fossilised eggshell and bone around 16-19 Mya? Resolving

644 this problem, however, will require more fossil finds from this period rather than more aDNA
645 analyses of the Quaternary moa fauna.

646 Moreover, species level taxonomy is highly problematic, including the taxonomy and branching order
647 in the genera *Euryapteryx*, *Megalapteryx* and *Pachyornis*. Next Generation Sequencing may have a
648 significant role to play in clarifying moa taxonomy – for example by sequencing a large number of
649 full mtDNA genomes and nuclear genes from all the identified moa lineages. This could provide a
650 strong basis for a re-evaluation of the taxonomy. The potential of this technique is demonstrated by a
651 recent phylogeographic study on killer whales that generated a highly informative dataset by
652 sequencing the complete mtDNA genomes of 139 individuals (Morin et al., 2010). Although an
653 equivalent study on fragmented moa DNA would be a much bigger challenge, there is little doubt that
654 it can be done with the technology available. This is exemplified by the 18 complete mammoth
655 mtDNA genomes sequenced in Gilbert et al. (2008). In time, the complete nuclear moa genome might
656 be sequenced, as has recently happened with three ancient hominids (Green et al., 2010; Rasmussen et
657 al., 2010; Reich et al., 2010). However, before a nuclear genome from an extant palaeognath has been
658 mapped to work as a scaffold, the problems might easily overshadow the benefits in doing a full *de*
659 *novo* assembly on moa sequences.

660 As Table 1 shows, the extraction of new and valuable information from extinct taxa does not
661 necessarily require whole genomes and massive sequencing efforts. Simple genetic identifications of
662 feathers (Rawlence et al., 2009), coprolites (Wood et al., 2008), eggshells (Huynen et al., 2010;
663 Oskam et al., 2010) and bones (Allentoft et al., 2009) have led to new insights on moa biology and
664 will continue to do so in the future. Eggshell represents a novel substrate in an aDNA context, and
665 because moa eggshell is often found in archaeological sites of the earliest Polynesian settlements
666 (Keepax, 1981; Gill, 2010), they may represent a valuable and largely untapped molecular resource
667 towards studying the human-moa interactions and extinction process (Oskam et al., 2010).

668 The potential for studying population level dynamics is rarely encountered in the field of aDNA, but
669 the wealth of sub-fossil material from moa can allow the effects of climate and habitat change on the
670 temporal population demographics of moa to be investigated in a similar fashion to studies on bison
671 (*Bison priscus*) (Shapiro et al., 2004; Drummond et al., 2005), musk ox (*Ovibos moschatus*) (Campos
672 et al., 2010) and mammoth (*Mammuthus primigenius*) (Barnes et al., 2007; Debruyne et al., 2008).
673 Having access to a large number of well preserved moa fossils from a large number of well
674 characterised sites offers an opportunity unmatched elsewhere, to build and explicitly test models of
675 megafaunal population demography against empirical data.

676 Gemmell et al. (2004) represents the only published attempt to determine moa population sizes from
677 genetic data. Based on DNA sequences from GenBank, and a simple theoretical prediction between
678 genetic diversity and effective population size, Gemmell et al. (2004) estimated the population size of
679 *Dinornis* at 300,000-1.4 million individuals. By assuming that the other moa species had similar
680 population sizes, the total standing census population size of moa was estimated at 3-12 million.
681 Comparing this estimate to Holdaway and Jacomb's (2000a) ecological estimate of 158,000 moa at
682 the time of Polynesian arrival, Gemmell et al. (2004) concluded that moa were in severe decline
683 before Polynesians colonised New Zealand, perhaps due to increased mortality from introduced avian
684 diseases and widespread volcanism. First of all, it seems that the genetic and ecological estimates of
685 moa population size apply to the same timeframe, so it is not intuitively clear why the observed
686 discrepancy is interpreted as a population decline. Secondly, aside from challenges such as obtaining
687 a reliable mutation rate, and accurate information on maturity and sex-ratios of an extinct taxon (all

688 factors used in the calculations), the population size estimate of Gemmell et al. (2004) also relied on a
689 relatively small sample size (Forsyth et al., 2010; Lee et al., 2010), and a potential problematic
690 assumption of panmixia through the extensive temporal and spatial distances covered by the *Dinornis*
691 data. The population size estimate of Gemmell et al. (2004) have been criticised by Forsyth et al.
692 (2010) and Lee et al. (2010) as being too high. Rather, these studies suggest that moa population
693 densities were comparable to extant ratites (at 0.3-0.6 individuals per km^2). Moreover, the disease
694 hypothesis [originally proposed by MacPhee (1999), and MacPhee and Marx (1999)] seems illogical
695 because moa fossils were in many areas very abundant in late Holocene deposits and there are no
696 other well-documented declines of taxa in the Late Quaternary fossil record outside of major episodes
697 of climate and habitat change (Worthy and Holdaway 1994, 1995, 1996; Worthy 1997, 1998a, b,
698 1999). The population size estimate of Gemmell et al. (2004) was based on the information and
699 methods available at the time, but since then more data has become available (e.g., Bunce et al. 2009)
700 and more advanced analytical tools have been developed to handle heterochronous data in
701 demographic analyses (e.g., Drummond et al., 2005, Anderson et al., 2006). For that reason a re-
702 assessment of moa population sizes based on genetic data seems very timely.

703 Finally, as a novelty for aDNA research, polymorphic microsatellite loci, ideal for population level
704 investigations, have recently been isolated and characterised for moa (Allentoft et al., 2009; Allentoft
705 et al., 2011). By analysing highly informative microsatellite data, in combination with all the available
706 mtDNA sequences, it should be possible to generate a detailed image of the genetic diversity that
707 characterised moa populations during the Late Quaternary. This could result in more accurate
708 assessments of population level structuring and demographic history than attempted previously for an
709 extinct taxon**.**

710

711 **6. Concluding remarks**

712 This review has highlighted the benefits of applying a multidisciplinary approach to taxonomic
713 research, with the most illustrative examples drawn from the studies of sexual dimorphism (Bunce et
714 al., 2003; Huynen et al., 2003; Worthy et al., 2005) and species diversity (e.g., Bunce et al., 2009).
715 The insights from these investigations and the resulting taxonomic rearrangements were only
716 achieved by combining genetic data with morphological assessments and palaeoecological
717 information of species distributions. Combining data across scientific disciplines seems highly
718 advisable when studying the taxonomy of closely related extinct taxa, where the level of reproductive
719 isolation is very difficult to assess.

720 Despite these cross-disciplinary benefits, clear contradictions between morphological and genetic
721 research were also documented. In particular, the deep splits in palaeognath evolution have remained
722 controversial (e.g., Bourdon et al., 2009; Phillips et al., 2010) (Figure 4). Recent molecular studies
723 point strongly towards 'ratites' as paraphyletic, and suggest that palaeognath evolution could have
724 been driven by long range dispersal rather than Gondwanan vicariance, although several dispersal
725 scenarios are possible (Figure 3). In addition, the molecular evidence for moa and kiwi not being
726 sister taxa is overwhelming. To our knowledge, only a single morphological study in recent times
727 claims otherwise by supporting vicariance and a common ancestor of the New Zealand 'ratites'
728 (Bourdon et al., 2009). Although this review is not aimed at resolving the controversy, we argue that
729 more supporting morphological evidence is required (e.g., not using Tinamidae as a fixed outgroup) to
730 'tip the balance' back in favour of a strict vicariance hypothesis.

Our review has also showed that subjectivity is not limited to morphological studies but can affect genetic research as well, even if the data analyses are highly complex. While it is near impossible to misinterpret character states in a clean DNA sequence (as each site is represented by either A, C, T, or G), subjectivity has instead been introduced at the analytical level. Examples of this include enforcing 'ratite' monophyly, as has occurred in some of the earlier research (both molecular and morphological), and the exclusion of DNA sequences that resulted in a topology that differed from the predominating view. In that sense, genetic analyses are not necessarily less influenced by subjectivity than analyses of morphological characters.

Detailed information from several scientific disciplines has increased the knowledge on moa to an unprecedented level for any extinct taxa – indeed our knowledge of genetic diversity in moa is now greater than that of most extant palaeognaths. However, these genetic insights could not have been achieved if they had not been considered in the context of 150 years of morphological and palaeontological achievements. In that sense, the history of moa research is a shared inter-disciplinary triumph.

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222 **Table 1:** A chronological summary of 19 years of aDNA research on moa

Reference	Title	Substrate	Target DNA	Objectives and/or findings
Cooper et al. (1992)	Independent origins of New Zealand moas and kiwis	Skin, muscle, bone	mtDNA (12S)	The first moa aDNA paper. Demonstrates that moa and kiwi are not monophyletic, so two independent 'ratite' colonisation events of New Zealand must have taken place.
Cooper (1993)	DNA from museum specimens	As above + feather	mtDNA (12S)	Paper discusses in detail the methods applied in Cooper et al. (1992) to successfully isolate DNA from ancient moa remains.
Cooper and Cooper (1995)	The Oligocene bottleneck and New Zealand biota: genetic record of a past environmental crisis	As in Cooper (1993)	mtDNA (12S, ND6)	Moa contain less genetic variability than other avian lineages. The same pattern is apparent in kiwi, and NZ wrens, suggesting a massive prehistoric bottleneck - perhaps facilitated by the Oligocene drowning.
Vickers-Rich et al. (1995)	Morphology, myology, collagen and DNA of a mummified upland moa, *Megalapteryx didinus* (Aves: Dinornithiformes) from New Zealand	Skin, muscle	Moa DNA, no target	Discusses aDNA extraction from a mummifed moa and shows in hybridisation experiments that the extracted DNA is not contamination.
Hickson et al. (1996)	Conserved sequence motifs, alignment, and secondary structure for the third domain of animal 12s rRNA	GenBank	mtDNA (12S)	12S rRNA moa sequences were included in a large meta study to investigate conserved domains and the secondary structure of the mitochondrial 12S sequence in animals.
Cooper (1997)	Studies of avian ancient DNA: From Jurassic Park to modern island extinctions	As in Cooper et al. (1992)	mtDNA (12S + ND6)	A re-analysis of the data presented in Cooper et al. (1992). Additional data are included and confirm that moa and kiwi are not monophyletic.
Cooper and Penny (1997)	Mass survival of birds across the Cretaceous-Tertiary boundary: molecular evidence	GenBank	mtDNA (12S)	DNA from many birds (including moa) was analysed to demonstrate that at least 22 avian lineages survived the Cretaceous-Tertiary boundary, which was the end for many other vertebrates.

Cooper et al. (2001)	Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution	Bone	mtDNA (whole genomes)	First mtDNA genomes of extinct taxa. Used to clarify 'ratite' evolution. The analyses suggest that rhea is basal and that the moa lineage splits off next.
Haddrath and Baker (2001)	Complete mitochondrial DNA genome sequences of extinct birds: ratite phylogenetics and the vicariance biogeography hypothesis	Bone	mtDNA (whole genomes)	A 'ratite' evolution study based on whole mtDNA genomes. The analyses suggest that moa is basal and that the rhea splits off next.
Braun and Kimball (2002)	Examining basal avian divergences with mitochondrial sequences: model complexity, taxon sampling, and sequence length	GenBank	mtDNA (whole genomes)	18 whole avian mtDNA genomes (including moa) are analysed to examine the basal phylogenetic splits in birds, and to address the impact of certain biases, introduced by the data and the analyses.
Paton et al. (2002)	Complete mitochondrial DNA genome sequences show that modern birds are not descended from transitional shorebirds	GenBank	mtDNA (whole genomes)	Two moa mtDNA genomes are included in a big dataset, to reject a hypothesis of shorebirds as ancestors to modern birds. Results show palaeognaths as sister group to all other modern birds (incl. shorebirds).
Bunce et al. (2003)	Extreme reversed sexual size dimorphism in the extinct New Zealand moa *Dinornis*	Bone	Nuclear sex markers + mtDNA (CR)	Female moa were much larger than males and previous morphological taxon designation in Dinornithidae simply reflects different genders.
Huynen et al. (2003)	Nuclear DNA sequences detect species limits in ancient moa	Bone	Nuclear sex markers + mtDNA (CR)	Shows that moa display extreme reverse sexual size dimorphism, and this genetic insight is used to define new species boundaries.
Willerslev et al. (2003)	Diverse plant and animal genetic records from Holocene and Pleistocene sediments	Sediment	mtDNA (CR + 12S)	First study documenting that aDNA can be extracted from ancient sediment. The study includes moa sequences amplified from New Zealand sediment samples.
Gemmel et al. (2004)	Moa were many	GenBank	mtDNA (CR)	A genetic estimate of moa population size is compared to a previous estimate based on ecological data, and the difference is interpreted as evidence that moa were in decline prior to human arrival in New Zealand.

Lambert et al. (2005)	Is a large-scale DNA-based inventory af ancient life possible?	Bone	mtDNA (CR + 12S + COI)	Investigates whether the COI gene is suitable for barcoding in moa. The presented levels of intra- and inter-specific genetic differentiation appear to support the applicability of COI barcoding.
Baker et al. (2005)	Reconstructing the tempo and mode of evolution in an extinct clade of birds with ancient DNA: the giant moas of New Zealand	Bone	mtDNA (CR + more)	Applies a molecular clock to date moa radiation. Detects 14 monophyletic lineages. First taxon to split off is *Megalapteryx* approximately 18.5 Mya. Further radiation in moa occurred 4-10 Mya.
Scofield et al. (2005)	Recent claims for more moa and huge errors in museum collections - cutting through the spin	NA	NA	A criticism of Baker et al. (2005). Argues that the large morphological identification error rates of moa remains suggested in Baker et al. (2005) (based on genetic species ID's) are not scientifically justified.
Baker (2007a)	Molecular advances in the study of geographic variation and speciation in birds	GenBank	mtDNA (CR + 12S + COI)	Discusses geographic genetic variation and speciation for a range of New Zealand taxa. Advocates full species status for all 14 moa lineages identified in Baker et al. (2005).
Worthy (2007)	Moas and phylogenomics: How nomenclatural errors do a disservice to the understanding of moa taxonomy	NA	NA	A critique of the nomenclature applied in Baker (2007a) in his advocacy for full species status of all 14 genetic lineages of moa.
Baker (2007b)	Nomenclatural errors in moa taxonomy: a reply to Worthy	NA	NA	A reply to the critique in Worthy (2007).
Haile et al. (2007)	Ancient DNA chronology within sediment deposits: are paleobiological reconstructions possible and is DNA leaching a factor?	Sediment	mtDNA (CR)	Shows that moa DNA can be extracted from sediment. The study suggests that downwards DNA migration happens for sheep DNA (potentially mediated by large volumes of urine) but not for moa DNA.
Huynen et al. (2008)	Genetic identification of moa remains recovered from Tiniroto, Gisborne	Bone	mtDNA (CR)	Confirms that mtDNA can be extracted from moa bones and bone fragments.

Wood et al. (2008)	Coprolite deposits reveal the diet and ecology of the extinct New Zealand megaherbivore moa (Aves, Dinornithiformes)	Coprolites	mtDNA (CR)	Ancient DNA was extracted to identify 24 moa coprolites to species. Plant contents were analysed microscopically, to show a diverse diet of low shrubs. No obvious signs of dietary niche separation between species were documented.
Allentoft et al. (2009)	Identification of microsatellites from an extinct moa species using high-throughput (454) sequence data	Bone	nuclear STRs	A 'proof of concept' paper, demonstrating how microsatellite markers can be developed from aDNA templates.
Bunce et al. (2009)	The evolutionary history of the extinct ratite moa and New Zealand Neogene paleogeography	Bone	mtDNA (CR + more)	Establishes the current consensus of nine moa species. Phylogeography and NZ geology are discussed. The radiation of moa did not begin until 5.8 Mya with *Megalapteryx* splitting off.
Rawlence et al. (2009)	DNA content and distribution in ancient feathers and potential to reconstruct the plumage of extinct avian taxa	Feather	mtDNA (CR)	Shows that aDNA can be extracted from very old (moa) feathers. The level of color preservation is investigated, followed by attempts to reconstruct moa plumage.
Allentoft et al. (2010)	Highly skewed sex ratios and biased fossil deposition of moa: ancient DNA provides new insights on New Zealands's extinct megafauna	Bone	Nuclear sex markers + mtDNA (CR) + nuclear STRs	A moa population study. Extreme excess of females among fossils from adults, but not among juveniles. Large compositional differences (taxa, sex, and maturity) between fossil sites despite their proximity in space and time.
Oskam et al. (2010)	Fossil avian eggshell preserves ancient DNA	Eggshell	mtDNA (CR)	Shows that aDNA can be extracted from ancient eggshell, including eggshell from five moa species.
Huynen et al. (2010)	Ancient DNA reveals extreme egg morphology and nesting behaviour in New Zealands's extinct moa	Eggshell, bone	mtDNA (CR) + nuclear sex markers	Authors suggest that egg morphology (thickness) can be used to identify moa eggshell fragments to species. Also, the DNA on the eggshells could indicate that male moa were incubating.
Kan et al. (2010)	Characterization of the complete mitochondrial genome of the Rock pigeon, *Columba livia* (Columbiformes: Columbidae)	GenBank	mtDNA (whole genomes)	The complete mtDNA genome of rock pigeon was mapped and the base composition bias was calculated from mtDNA genomes of 30 bird species, including moa.

Phillips et al. (2010)	Tinamous and moa flock together: mitochondrial genome sequence analysis reveals independent losses of flight among ratites	GenBank	mtDNA (whole genomes)	By removing the often enforced monophyly of 'ratites', it is shown that ostrich is basal in the phylogeny and that (volant) tinamous and moa are sister taxa. This implies that flight was lost several times among palaeognaths.
Allentoft et al. (2011)	Profiling the dead: generating microsatelite data from the fossil bones of extinct megafauna - protocols, problems, and prospects	Bone, eggshell	Nuclear STRs	Presents six polymorphic microsatellite markers for moa and establishes strict methodological protocols to ensure high data fidelity. The first microsatellites to be developed exclusively for an extinct taxon.
Allentoft et al. In Press	A molecular characterisation of a newly-discovered megafaunal fossil site in North Canterbury, South Island, New Zealand	Bone, eggshell	mtDNA (CR)	Uses aDNA and morphology to determine the species composition and preservation of moa bones from the Rosslea fossil deposit, discovered in 2008.

Legends

Table 1
Chronological summary of 19 years of aDNA research on moa. Not all the publications reported here have generated or directly analysed aDNA sequences, but they do at least relate to, or somehow rely on, aDNA sequences from moa in reaching their conclusions. All studies on moa (molecular or not) published since 1992 have been drafted with the most current genetic information available at the time, so it is not easy to draw a definite line between DNA-based and non DNA-based moa research. Among the contributions in this grey area (not listed in Table 1), are two books on the extinct New Zealand avifauna (Worthy and Holdaway, 2002; Tennyson and Martinson, 2006), the most recent checklist of New Zealand birds (Gill et al., 2010), and two recent review papers on molecular phylogeographic patterns in New Zealand (Wallis and Trewick, 2009; Trewick and Gibb, 2010). The notion 'GenBank' implies that the study has not generated the sequence data but downloaded it from GenBank. *Abbreviations*: mtDNA, mitochondrial DNA; CR, control region; 12S, ribosomal RNA Subunit 12; ND6, NADH Dehydrogenase Subunit 6; COI, Cytochrome Oxidase I; STRs, short tandem repeats (or microsatellites); aDNA, ancient DNA.

Figure 1
Reconstruction of a moa. The image depicts an adult *Pachyornis elephantopus* with the speckled plumage as identified in Rawlence et al. (2009). Image courtesy of Jamie R. Wood.

Figure 2
Biological substrates used in genetic research on moa. Clockwise from top: Bone (femur) of *Pachyornis australis*; mummified soft tissue (neck) of *Emeus crassus*; coprolites; feather of *Pachyornis elephantopus*; and eggshell. Images courtesy of NJR (feathers, bone), Charlotte L. Oskam (eggshell), Jamie R. Wood (coprolites), and Otago Museum, Dunedin, New Zealand (neck).

Figure 3
Three hypotheses explaining how moa may have arrived in New Zealand. *Left:* The Moa's Ark theory where proto moa was isolated on Zealandia as it separated from eastern Gondwana. This event was originally estimated to have occurred c. 80 Mya (e.g., Cooper et al. 2001; Haddrath and Baker, 2001). *Middle*: The hypothesis of Phillips et al. (2010) was based on advanced genetic modelling and showed that moa and the volant tinamou have a common ancestor, implying that the moa ancestor was probably volant. The moa-tinamou split was estimated at 60 Mya, suggesting that proto moa arrived by flight on the already separated Zealandia. Following this isolation moa lost their wings. *Right*: An 'updated' Moa's Ark theory based on new geological data, which suggest that Zealandia did not fully separate from eastern Gondwana before 60 Mya (e.g., Schellart et al., 2006). If correct, it implies that an already flightless proto moa could have walked to New Zealand until 60 Mya, potentially compromising the previous interpretations on this subject. We note that other scenarios are possible depending on the timing of the moa–tinamou split, the timing of the break-up of Gondwana, and the palaeognath topology; all aspects that are still debated in the literature. Dark grey shading represents shape of present day coastlines. Figure based on information from Worthy and Holdaway (2002), and Schellart et al. (2006).

Figure 4

The latest opposing theories on the evolution of 'ratites'. A: Based on the analysis of morphological characters by Bourdon et al. (2009), 'ratites' are monophyletic and the sister-group to tinamous (fixed as outgroup), suggesting a single loss of flight in ratite evolution. Kiwi and moa are sister taxa within 'ratites'. B: Based on the genetic analysis of Phillips et al. (2010) 'ratites' are paraphyletic, with moa and tinamou as sister taxa. Disregarding the possibility of tinamous regaining flight, at least four instances of loss of flight must have occurred. Loss of flight is indicated with dots on the branches. The displayed time scale, estimated by molecular clock approaches applies only to phylogeny B. The confidence intervals of the divergence times can be seen in Phillips et al. (2010).

Figure 5

The phylogeny and timescale of moa evolution, based on Bunce et al. (2009). Numbers in brackets represent the number of species within each genus. For *Pachyornis*, only *P. elephantopus* is sketched. For *Dinornis* only *D. robustus* is sketched but represented by both sexes. The confidence intervals for the divergence times can be seen in Bunce et al. (2009). Moa sketches courtesy of Colin Edgerley and New Zealand Geographic.

Figure 1

Figure 2

Figure 3

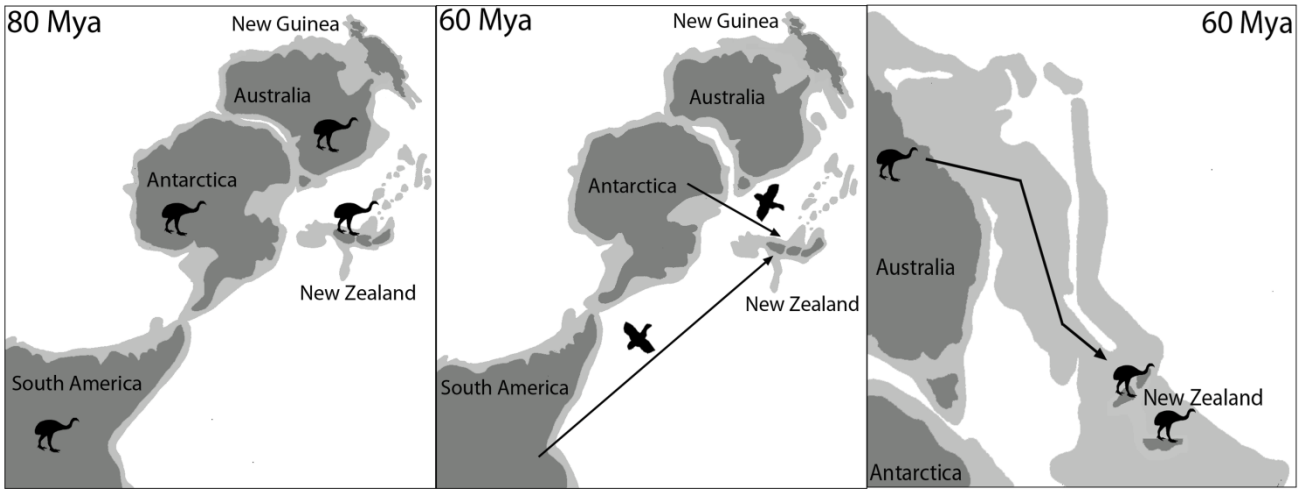


Figure 4

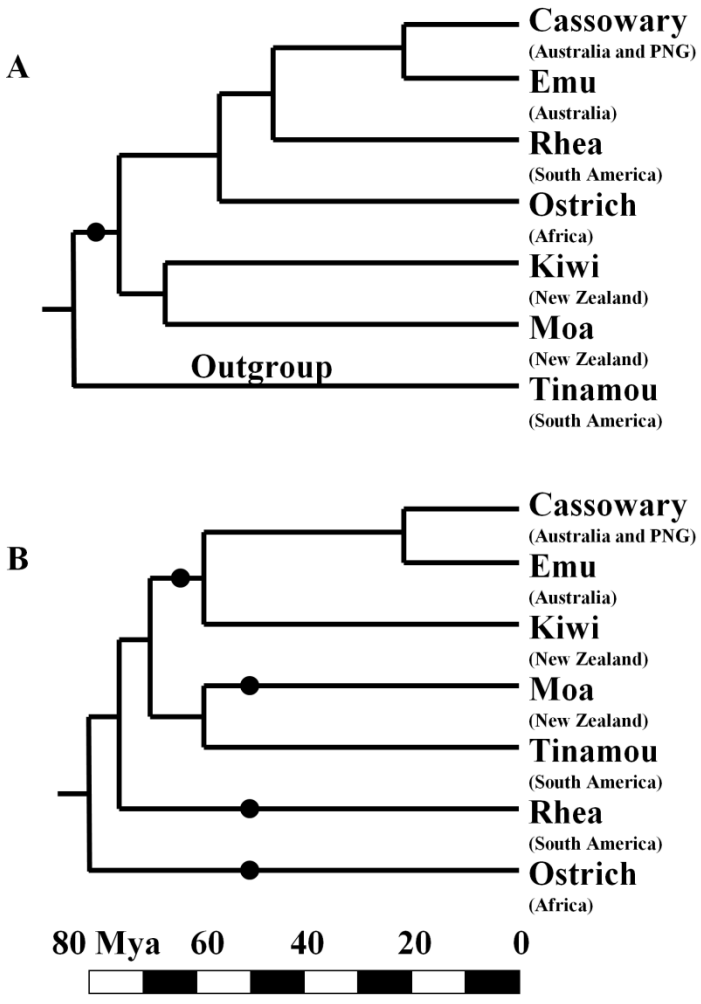
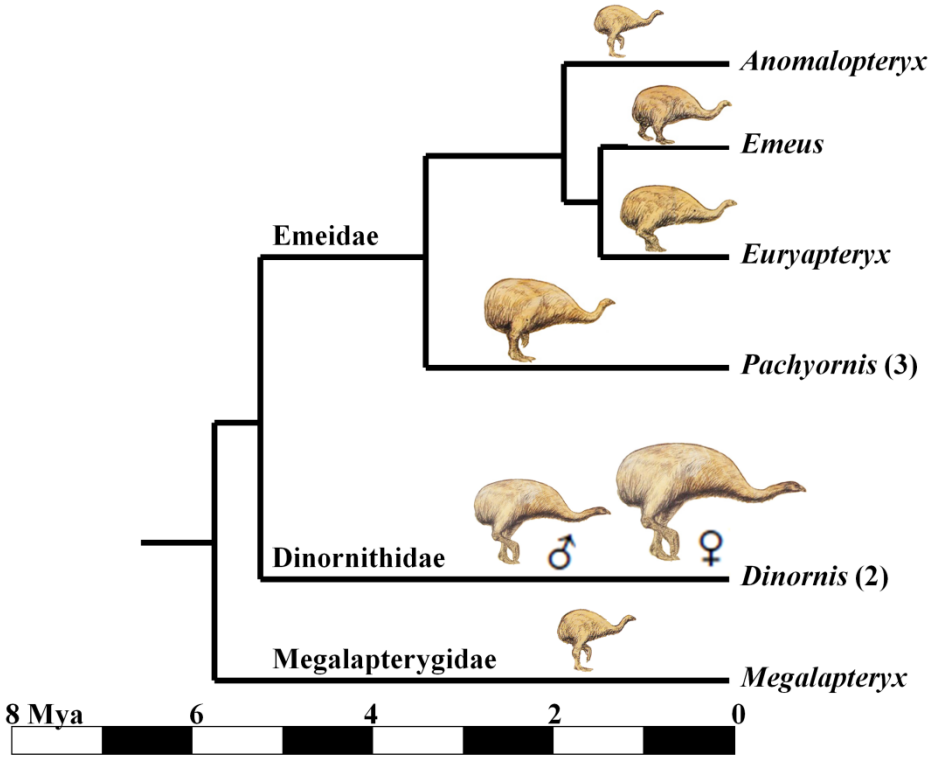


Figure 5


Anomalopteryx
Emeus
Euryapteryx
Emeidae
Pachyornis (3)
Dinornithidae
♂
♀
Dinornis (2)
Megalapterygidae
Megalapteryx
8 Mya
6
4
2
0