Ancient DNA of New Zealand's Extinct Avifauna

Using next-generation sequencing (NGS) to research the paleoecology of the megaherbivore moa (Aves: Dinornithiformes †) and the phylogenetics of the enigmatic gruiform *Aptornis* (Aves: Aptornithidae †)

A thesis submitted in fulfilment for the requirements for the degree of Master of Philosophy

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"... a symphony of 'the most tunable silver sound imaginable'. Aotearoa's multitudes of birds performed that symphony each dawn for over 60 million years. It was a glorious riot of sound with its own special meaning, for it was a confirmation of the health of a wondrous and unique ecosystem. To my great regret, I arrived in New Zealand in the late twentieth century only to find most of the orchestra seats empty. Walking through the ancient forest, whose still-living trees were once browsed by moa, I heard nothing but the whisper of leaves blowing in the wind. It was like the rustle of the last curtain fall on an orchestra that will be no more" Tim Flannery (*The Future Eaters*)

Image: skulls of *Aptornis defossor* (South Island adzebill, top) and *Pachyornis elephantopus* (heavy-footed moa, bottom) from Richard Owen, *Memoirs on the extinct wingless birds of New Zealand, with an appendix on those of England, Australia, Newfoundland, Mauritius, and Rodriguez,* 1879

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Abstract

As a direct result of human activity and other environmental changes, considerable ecological changes and species extinctions have occurred on most terrestrial environments during the last 50,000 years. Therefore, correctly interpreting naturally occurring biogeography or ecology is limited without including historical information. Detailed insights into past environments or extinct species can be provided by ancient DNA (aDNA), yet this field has long been limited by the degraded nature of ancient genetic material. Recent developments and the increasing availability of next-generation sequencing (NGS), is now allowing for a breakthrough in aDNA studies. It is now possible to obtain entire genomes from long-extinct organisms, and sequence high-depth 'environmental DNA' representative of prehistoric environments.

New Zealand (NZ) was the last of the large landmasses (except Antarctica) to be colonised by humans, approximately 700 years ago. Furthermore, the long isolation of NZ from Gondwana (from which it separated between ~85-52 Ma) resulted in an ancient, ecologically complex and biogeographically unique biota. Due to a near-absence of mammals this biota was dominated by a diverse array of endemic bird species, of which nearly 50% are now extinct due to human activity. Nonetheless, the recent age of NZ's extinctions, combined with a temperate climate, has permitted an unparalleled degree of preservation of the pre-human ecosystem. By use of aDNA and NGS methods, NZ's biota may now be understood in relative completion.

This thesis uses aDNA NGS methods to research a select number extinct bird species. Chapters Two and Three investigate environmental DNA of avian coprolites (paleofaeces), most of which originate from four species of the megaherbivore ratite moa (Dinornithiformes). It is identified for the first time that the extinct moa consumed and dispersed species of mycorrhizal fungi important in natural forest dynamics. Phylogenetics of sequenced moa parasite DNA found rare evidence for a higher taxonomic level co-extinction event: between moa, and an identified genus or family of moa-specific nematodes. Finally, differences in parasite, plant and fungal DNA assemblages greatly support divergent behaviour strategies between each moa species. I also analyse shotgun DNA and DNA-RNA hybridization enrichment data on avian coprolites representative of rich deposits in semi-arid areas of the central South Island. I find that these samples have been greatly affected by thermal-based DNA damage and/or formation of organic compounds inhibiting DNA amplification. Future approaches aiming to resolve these issues are discussed.

Chapter Four investigates the phylogeny one of NZ's most poorly understood large bird taxa, the adzebills (*Aptornis*, Aptornithidae). Sequencing of near-complete complete mitochondrial genomes of both recently extinct species, resolves placement of these birds within the superfamily Ralloidea (Gruiformes). This in turn elucidates evolutionary details into the global radiation of Ralloid birds and the *Aptornis* lineage in NZ.

The final Fifth chapter summarizes the results of these diverse studies and how they interrelate, and concludes the thesis by discussing future related aDNA projects to be undertaken on NZ's ancient ecosystem.

Contents

CHAPTER ONE

General Introduction

1.1 Foreword and Background

Such a severe loss of biodiversity has occurred over the past 50,000 years that this period is now often described as the 'sixth mass extinction'[1,2], an event comparable to the end of the "age of the dinosaurs" 65 million years ago [3–5]. This sixth extinction event began with the worldwide extinction of megafauna in the Late Pleistocene and continues with the ongoing Holocene extinction and ecological crisis today [6–11]. Whether this recent extinction has occurred as a result of human activity alone is heavily debated. However, it is clear nonetheless that current biodiversity is modified and impoverished. As a result, it is essential that we obtain detailed information from ancient ecosystems or recently extinct organisms in order to understand recent ecological, biogeographical and evolutionary events [12–17].

Classical palaeobiology has generally relied on osteological, morphological or microfossil studies, which have considerable limitations when making robust ecological or phylogenetic conclusions [18]. The relatively new and technically complex field of ancient DNA (aDNA) has built upon and even directly refuted long-established ideas by sequencing genetic information from long dead organisms [19–21]. However, aDNA has long been limited by the application of Sanger-sequencing technologies. Ancient DNA is typically in low abundance, fragmented and especially vulnerable to contamination [19–21]. As a result, amplification of ancient genetic material has generally been laborious and restricted to only short fragments of DNA.

Only in very recent years, the field of next-generation sequencing (NGS) has become widely available and has revolutionised aDNA research. NGS can provide massive amounts of DNA data at the cost of short sequenced fragment length. However, as aDNA is already highly fragmented, this limitation is of little consequence. Further, the sequencing depth provided by NGS can greatly overcome the impacts of contaminating DNA. Finally, NGS amplicons are not by necessity constrained by a predestined sequence length, which can assist capture of especially short DNA fragments. As a result, NGS is extremely well suited for use on ancient organisms. It is now becoming feasible to sequence entire genomes (mitochondrial or even nuclear) [20,21]. Furthermore, NGS has revolutionised research of 'environmental DNA' (including metagenomic and metabarcoding), where entire communities of

organisms can be sequenced in single reactions from modern or ancient environmental samples [22–28].

New Zealand is extremely well-suited for the application of new ancient DNA technologies for palaeobiological reconstruction. New Zealand has been isolated as an archipelago of oceanic islands since it separated from the supercontinent Gondwana between ~52-85 million years ago [29–31]. As a result, its biota has developed a complex, poorly understood and presumably deeply co-evolved ecology, containing bizarre and evolutionarily distinct taxa [32–34]. For example, terrestrial mammals were not a part of the modern biota until the arrival of humans, and so a diverse array of birds comprised the large vertebrate fauna. This avifauna included the tallest birds ever known, the largest known eagle, mice-sized flightless 'wrens', gigantic flightless nocturnal parrots and many others [35–38]. However, this unique ecosystem has suffered considerably from human activity: nearly 50% of New Zealand's endemic birds are now extinct [35–39]. Importantly for palaeobiological reconstruction, this extinction occurred much more recently than similar events on other continents, occurring shortly after the relatively late arrival of Polynesians ~700 years ago [40–42]. The recent age of New Zealand's extinctions, combined with a temperate local climate, has resulted in an exceptionally well-preserved prehistoric biota.

The usefulness of New Zealand as a laboratory for aDNA studies has long been realised: both the first ancient DNA from an extinct bird and the first mitochondrial genome sequenced from any extinct species were from New Zealand's moa [43,44]. However, the use of NGS-based ancient DNA methods to research New Zealand's 'lost world of the moa' [35] remains minimal and there are numerous outstanding questions to be answered. In this thesis I use NGS derived aDNA data to investigate the evolution and ecology of several prehistoric bird species from New Zealand. In my three research chapters I explore several NGS approaches: "shotgun" sequencing, metabarcoding, and hybridisation enrichment. By combining the results of these approaches I am able to provide new insights into New Zealand's extirpated avifauna as a whole, and how it functioned and evolved.

1.2   New Zealand's lost avifauna

When the first Europeans arrived in the remote islands of New Zealand, they encountered a rich avian fauna unlike anywhere else on earth. Botanist Joseph Banks aboard the Endeavour anchored off New Zealand in 1770, gives the oldest written account of the Islands' living birdlife: "*…I was awakd by the singing of the birds from ashore from whence we are distant not a quarter of mile, the numbers of them were certainly very great... their voices were certainly the most melodious wild musick I have ever heard, almost imitating small bells but with the most tuneable silver sound imaginable…* " [45]. Sadly today, several of the birds likely heard or seen by Banks are extinct, and only brief and unsatisfying descriptions of their songs remain. Yet even then, Pacific rats introduced by the Maori centuries ago would have been ubiquitous, and huge areas of the once almost unbroken forest had been burned and cleared [35,40]. Oral histories and legends of Maori contain only tantalising hints of the animals they may have once encountered.

By the early 19th century New Zealand had seen few naturalists since Banks. Yet in 1839 an unusual bone fragment from New Zealand was delivered to the prominent English anatomist Sir Richard Owen, founder of the Natural History Museum in London. Although he initially dismissed the fragment, Owen later correctly identified it as part of a femur from an unknown bird, famously surmising "*…there has existed, if there does not now exist, in New Zealand, a Struthious bird nearly, if not quite, equal in size to the Ostrich*" [46]. Four years later Owen acquired enough bones to reconstruct an enormous, complete skeleton, poised to its full height of over three meters. Owen named it *Dinornis novazealandiae* ('terrible' bird of New Zealand) [47]. Gradually, a wide variety of differently shaped and sized 'moa' (taken from the Polynesian word for 'fowl') were discovered. Owen and other naturalists during the next century would propose dozens of species [35].

More large bones first described and identified as a moa by Owen, instead gradually revealed a large, flightless, rail-like bird with an enormously reinforced skull and beak, later redescribed as *Aptornis* (the 'adzebill') [35,47–50]. Owen also described an enormous, flightless goose (*Cnemiornis*), almost twice the weight of the largest swans [35,36,51]. Future discoveries included an eagle larger and more powerful than any known (Haast's eagle, *Aquila moorei*), the largest-known harrier (Eyle's

harrier, *Circus teauteensis*), a raven, three small 'wrens' (Acanthisittidae), an owlet-nightjar, a parrot, two penguins, a petrel, five species of duck and five species of rail [35–38,52,53]. As with moa, no accounts of live birds, or collected skins of these particular bird species are known. Only black swans and (possibly vagrant) Australian pelicans which also became extinct in New Zealand, survived elsewhere (black swans have since been re-introduced) [35].

European naturalists were first documenting this vanished bird community during a period when a wide diversity of other recently extinct species from around the world were being discovered and described. When Owen received his enigmatic moa bone fragment, he had just named giant marsupials from Australia and bizarre hoofed animals from South America, none seen alive [54–57]. Only since the 1790's had animals such as mammoths, mastodons, cave bears, woolly rhinos or ground sloths been scientifically described. Perhaps as a result of this, New Zealand's avifauna has been commonly included as a component of these 'megafaunal extinctions' [58,59], the cause of which is still a source of debate. Most frequently, it is either proposed that these extinctions were a result of early human hunting, or climatic/environmental changes during the last glacial maximum [7,8,60]. New Zealand's extinctions were eventually revealed as much more recent than those elsewhere. Nonetheless, links between New Zealand's extinctions and the remaining global 'megafaunal extinctions' have often been proposed. It has been frequently suggested that moa and other New Zealand birds have been in natural decline for thousands of years, or that some species had been long extinct before any human contact of New Zealand [35,36,61–65].

Evidence from linguistic, genetic and oral history suggests that the ancestors of the New Zealand Maori arrived from central Polynesia (near Tahiti or the Cook Islands) [66–68]. Controversial research proposing a ~2 ka introduction of the pacific rat (*Rattus exulans*), based on radiocarbon dates and apparent identification of bones below the Hapete tephra (a massive eruption of the Taupo volcano ~232 AD [69]) were initially accepted by much of the scientific community [70,71]. However, these are now generally rejected. Systematic dating of bones, charcoal, vegetation changes and presence indicators of pacific rats now unanimously support an initial colonisation date of around 1280 AD [40–42]. Archaeological evidence of widespread bird hunting since this time is so prevalent, that the early phase of Maori history is generally referred to as the "moa-hunter" period [35,63,72,73]. The afore-mentioned Pacific rats

introduced by Maori, are hypothesised to have briefly formed a devastating and unimaginable 'rat plague' persisting until food stocks of native invertebrates, birds and seeds were depleted [35,74]. Based on radiocarbon dates and statistical modelling, moa and several other birds appear to have been entirely extinct by around 1500, from only roughly 200 years of human cohabitation [64,75]. These data strongly support a recent, entirely human-mediated extinction event. Evidence of similar, later extinction events can be found on the Chatham Islands, ~680km to the east of New Zealand, believed to have been colonised by early Maori around 1500 AD (who would develop into the distinct and isolated Moriori culture) [53,76–79].

Human effects would only continue. At the time of European settlement in the early 19th C., New Zealand's forests still contained rich and abundant native bird-life. Over the 19th and 20th centuries, huge stretches of remaining forest were burned or cleared, swamps and wetlands drained, and the abundant fauna were extensively exploited for foot, sport or by the demand of collectors [36,80]. Most destructive of all, more animals were introduced, including brown rats, black (ship) rats, mice, cats, ferrets, stoats, weasels, Australian brush-tail possums, deer, pigs, numerous new bird species and European wasps [80]. Together these preyed on, or competed with, remaining native species. Bird populations declined rapidly and many more species became extinct, including several species of snipes, rails, songbirds (including two more 'wrens'), a quail, an owl, a shore-plover and a merganser [36,37]. Many once-abundant species now survive in vastly reduced populations, some of which are effectively 'functionally extinct' or found only on offshore islands cleared of predatory mammals [81].

As with other island biotas, New Zealand's animals tend (or tended) to be large, flightless, and/or have unusual diets compared to related species [82–86]. They were also maladapted to predation by the otherwise-absent mammals, which they arguably emulated. Birds filled various 'mammalian' niches, including often-quoted analogues of 'deer', 'giraffes', 'tigers', 'badgers', 'rabbits', 'squirrels', 'sheep', and 'mice' [87,88]. Unusual examples included the New Zealand wrens (Acanthisittidae) of which five of the seven species are extinct. Four of these are believed flightless (avian 'mice'), comprising four of only five known flightless passerines, and the smallest flightless birds known [35,36,89–91]. The top predators were also birds, and the huge (max 17.8 kg) Haast's eagle is supported by fossil evidence to have preyed on even adult

giant moa [35,92–95]. The bizarre and heavily built adzebills are supported by isotopic evidence of being carnivorous or omnivorous [35].

The moa themselves are widely believed to have been herbivorous, fulfilling the roles of avian 'deer' and 'giraffes'. Moa have always been acknowledged as a type of 'ratite', which include the extant and herbivorous emu, cassowary, ostrich and rhea (the other surviving lineage, and an important anomaly, are the hen-sized, flightless, insectivorous, nocturnal kiwi also from New Zealand). Large gastroliths ('gizzard stones'), characteristic of those consumed elsewhere by herbivorous birds, are frequently found with moa remains [96]. Variation of beak shapes, localities of remains, and physical morphology supports adaptations to a variety of environments, vegetation types and diets [35]. Finally, coprolites (paleofaeces) believed to have originated from moa, and gizzards still found within some moa remains, support a diet of fruit, leaves and fibrous vegetation [97–100]. Although other birds such as the native geese (*Cnemiornis* spp.) likely grazed or browsed on vegetation, the various species of moa were clearly the primary megaherbviores in New Zealand [35,101].

The impact this diversity of birds had on the ecology is hard to estimate, and presumably must have provided important roles in nutrient recycling, predation, seed dispersal, pollination and habitat modification [35,74,102]. Bizarre life strategies of several plants exist, including 'divarication' (dense, 'twiggy', growth with small leaves) and 'heteroblasty' (different morphologies through a plant's ontogeny), often argued as representing adaptations countering bird herbivory [103]. For example, species of the understory tree genus *Pseudopanax* initially develop long tough leaves as juveniles, switching to more typical broad leaves at ~3m (the height of the tallest moa) [104]. Several fungi found throughout New Zealand's forests are brightly coloured, and lack a mechanism for forcible spore discharge, strongly suggestive of dispersal by ground-dwelling, herbivorous birds [105,106].

Another extremely important avian community were the seabirds. While breeding colonies numbering thousands of individuals still occur on offshore islands, few remain on the mainland; either being small, widely dispersed, or restricted to high latitudes [35]. However they have a major impact on the ecosystems where they occur [107,108]. Although few species are entirely extinct, evidence supports that large seabird colonies occurred throughout the country [35,102]. Outside of Antarctica, New Zealand would have formed by far the largest mammal-free landmass available worldwide for breeding birds such as petrels, prions, albatross and penguins. This unimaginable loss

is best described by Worthy and Holdaway [35] "*It is probably no exaggeration to say that there would have been hundreds of millions, if not billions, of petrels in the New Zealand region before humans made contact… A higher understanding of the paleoecology of New Zealand will rest on our ability to estimate the impact of the loss….".* The effect these birds had, on both terrestrial and marine ecosystems, was likely staggering.

Constructing a complete cohesive picture of how this complex ecosystem functioned, evolved and interacted with other species continues to be problematic. Questions raised since Owen's time still remain elusive, and many have only recently been answered. The first DNA sequence from an extinct bird in 1992 [44] and the first complete mitochondrial genome from an extinct animal in 2001 [43], were both from moa. While moa are confirmed as 'paleognaths' (sister group to most birds, and inclusive of all ratites), they are now confirmed as the sister taxon to the volant and hen-sized tinamous of South America, finding 'ratites' to be polyphyletic [109,110]. Another remarkable discovery was that several smaller moa 'species' represented the male forms [111,112]. For example, females of the giant moa (*Dinornis* spp.) could be as much as 2-3 times the weight of males (the now defunct '*D. struthiodes*'), the greatest reverse sexual-dimorphism known for any bird. After considerable revision, and evidences from aDNA, the number of moa species now stands at an accepted total of nine found in three families and five genera [112–118]. This remains the greatest single radiation of large, herbivorous birds known.

Ancient DNA has provided other, more indirect revelations. Population genetics has now confirmed moa were not naturally declining prior to human arrivals, finally putting to rest old concepts of a natural moa decline [59]. DNA from shed feathers has even helped resolve the plumage of different moa, revealing several to have been reddish-brown, and others to be dark with a mottled or specked appearance [119]. Eggshells could be assigned to species, determining giant moa had proportionately the thinnest and most fragile of all bird eggs; and while most moa eggs where white, the genetically divergent upland moa had eggs coloured an olive-green [117,120]. DNA from the eggshell surface appeared to represent only male moa [117], possibly supporting male-specific incubation and parental care as in all other paleognaths [121]. Bones of moa from Maori middens were much more likely to come from the smaller, and possibly incubating, males, providing clues into the mechanism of moa extinction [122]. And finally, coprolites from across the country could be assigned to the exact host

species using DNA, and even the containing taxa could be sequenced [123–130]. This has strongly supported niche partitioning among moa species, and even that different moa may have supported distinctive parasite communities.

Despite these advances, only a small fraction of the extinct avifauna has been surveyed using ancient DNA. Even then, most of these bird species have only been investigated using Sanger sequencing, the same technology used for the first sequences over 20 years ago. For example, the North Island takahe (*Porphyrio mantelli)*, piopios (*Turnagra* spp.), New Zealand geese (*Cnemiornis* spp.) and adzebills (*Aptornis* spp.) have only had short lengths of their degraded genomes sequenced, and have not been revisited since the late 90s [101,131–133]. In addition, the DNA preserved in many coprolites is so damaged that it cannot be sequenced whatsoever using conventional PCR methods [20]. As a result, NGS may provide new insights into the evolution and ecology of New Zealand's birds and bring fresh evidence to debates that have persisted for almost 200 years.

1.3   New Zealand's natural history

New Zealand and its native biota have a long and complex history, which today continues to be heavily debated. Despite its small size and isolation, the island group is geologically of continental Gondwanan origin. As a result, biogeographically, New Zealand has characteristics of both a continental landmass and an oceanic island [32,33,134,135]. While many endemic lineages do appear to be relictual and deeply ancient in origin, others evidently have more recent origins congruent with oceanic dispersal [32,34]. Furthermore, New Zealand's isolation has prevented or limited colonisation by many groups normally abundant elsewhere, which has resulted in a niche vacuum and some extraordinary examples of macroevolution. Nonetheless, understanding the correlations between New Zealand's biogeography, ecology and geological history, have been far from straightforward. DNA research has provided valuable insights into New Zealand's prehistory and the evolution of its endemic biota. Yet this work is far from comprehensive, and exacerbated by the recent extinction of so many of its native species.

CHAPTER ONE: General Introduction

The island group today contains a 'mainland' of two large islands separated by the narrow Cook Strait (North and South Islands) and several smaller islands (for example Stewart Island, Great Barrier Island, Codfish Island and others), with a temperate, wet, oceanic climate supporting a largely forested ecosystem. These islands formed a single landmass during Pleistocene glaciations (resulting in lowered sea levels), most recently ~12,000 years ago [136]. These periods were also accompanied by forest retreats and expansions of herb-fields and grasslands [136]. The New Zealand region also sits astride a subducting boundary between the Pacific and Indo-Australian tectonic plates, resulting in uplifting and submergence of land, mountain building, and volcanism. One direct effect is a network of much more distant, young, oceanic islands, including five subantarctic island-groups to the southeast, the Chatham Islands ~680 km to the east, and the highly volcanic Kermadec Islands ~800-1,000 km to the northeast. Together these islands cover a vast area, from 29° S (Raoul Island) to 52° S (Campbell Island), over 2,500 km in latitude, from the subtropics to the subantarctic. In addition, the Australian territories of Norfolk Island ~1,000 km to the northwest, and Macquarie Island ~1,500km to the south (which have native taxa more typical of New Zealand, such as *Cyanoramphus* parakeets and *Hemiphaga* pigeons) also share strong biogeographic links [36,137,138].

The gondwanan origin of New Zealand is clearly demonstrated by a terrestrial fossil record extending to the Permian [139–142], and by an extinct mid-ocean ridge in the central Tasman Sea (supporting a prolonged period of sea floor spreading) [143]. Modern New Zealand represents a small portion of the submerged subcontinent 'Zealandia' (only 7% remains above sea level), forming a complex of underwater rises, ridges and plateaus as well as islands, roughly 3.5 million km^2 (roughly the size of India) [32]. It is proposed most of Zealandia was once above sea level (a terrestrial 'Zealandia continent'), and began to rift from the eastern edge of a highly fragmented late-stage Gondwana (comprised of South America, Australia and Antarctica) around 85 Ma [29–32,143]. The timing of separation is still uncertain, but appears to have been complete by 52 Ma [30,31]. Submergence of Zealandia was rapid (reduced largely to a 'proto-New Zealand' by around 65 Ma) due to an absence of tectonic/volcanic activity, erosion, and tectonic submergence of the crust [32,144,145]. Only during the Miocene (beginning ~23 Ma) did mountain-building activity lead to the emergence of modern New Zealand [146]. This landmass comprised at least two large islands (precursors to the modern North and South Islands) separated by the large 'Manawatu

Strait', which closed around 1.5-2 Ma [93,147]. The modern Cook Strait is a much more modern feature appearing around ~0.45 Ma [148]. Other areas of Zealandia above sea level (such as New Caledonia, Lord Howe Island, and New Zealand's oceanic islands) have primarily volcanic origins [149].

The connection between the biotas of modern, emergent 'New Zealand' and the original gondwanan 'Zealandia continent' is still unclear, and several authors have proposed the two should be viewed as separate biogeographic entities [32,33,144,145]. Prior to Miocene tectonic activity, Zealandia reached a peak level of oceanic submergence and was reduced to at most a few small islands during the Oligocene (33.9-23.03 Ma), an event known as the 'Oligocene drowning' [134,150]. Most examined plant and animal lineages appear to have radiated into species groups only since this time [150-153], and it has even been proposed that no emergent land connectivity between New Zealand and the 'Zealandia continent' occurred whatsoever [144,145]. Furthermore, since the Pliocene (<5.3 Ma), New Zealand has been subject to extensive climatic cooling and glaciation events, resulting in additional extinctions, likely to have included many surviving pre-Oligocene lineages [154]. For example, post-Oligocene fossils of typically 'gondwanan' plants and animals not found in New Zealand today are well known (including eucalypts, *Araucaria*, mekosuchine crocodiles, meiolaniid turtles, and even a terrestrial mammal) [70,155–157]. Paleogene aged fossils pre-Oligocene (Paleocene-Eocene 66-33.9 Ma) in age, are extremely rare [158,159], and reconstructing the biogeographic history during this mysterious period is largely reliant on genetic information [148].

Despite this dynamic history, numerous 'ancient' lineages still persist in New Zealand, and endemicity is astonishingly high [32,70]. Two of the most striking endemic lineages are the relictual tuatara (the last surviving sphenodontid, otherwise extinct worldwide since the Cretaceous) and leiopelmatid frogs (among the most primitive of all living frog families). However, both groups have early Mesozoic origins long predating New Zealand's formation [160–162]. Other taxonomically important, or typically 'gondwanan' groups include ratite birds (represented by as many as nine moa and five kiwi species), Acanthisittidae wrens, Strigopoidea parrots, conifers (including Araucariaceae, Podocarpaceae and callitroid Cupressaceae), southern beech (Nothofagaceae), and vast numbers of invertebrates (including particular families of earthworms, molluscs, crustaceans, insects, spiders and velvet worms) [70]. Nonetheless, endemicity is almost entirely restricted to the species and genus level

(totally so in plants), and there are relatively few endemic animal families [32,34,70,163] (at least for a landmass of 'gondwanan' age). In many more respects, New Zealand is more biologically characteristic of recently formed oceanic islands, and most species appear to have arrived comparatively recently from nearby landmasses such as Australia and New Caledonia.

Genetic evidence has provided some fascinating and conflicting insights. For example, southern beech are confirmed to have arrived from Australia recently, and the original 'Zealandian' southern beech are long extinct [163–165]. The New Zealand kauri (*Agathis australis*) has been found to represent by far the most genetically distinct member of its (possibly Mesozoic-aged) genus, yet the molecular age of the taxon remains controversial with dates ranging from pre-Cretaceous to post-Oligocene [166–168]. Similar examples can be found among the birds. Examples include the endemic Strigopoidea parrots and Acanthisittidae wrens, confirmed as the basal members of the diverse and widespread Psittaciformes and Passeriformes, respectively [169,170]. Molecular date estimates for these taxa, (at least those independently calibrated of a Gondwanan separation) are only weakly congruent with the latest split of New Zealand (>52 Ma) [31]. New Zealand's ratite moa have ages more compatible with vicariant origins (kiwi do not, yet have pre-Oligocene ages), nevertheless the now-flightless ratites are widely accepted to have dispersed by flight across Gondwanan fragments than by walking as has been long proposed [109,110]. New Zealand's other bird taxa mostly share strong affinities with Australia or New Caledonia; although it is plausible a few other endemic lineages pre-Oligocene in age may persist [171–175].

Understanding the biological history of New Zealand is essential to infer its ecology. For an oceanic island, New Zealand is among the few in the world with a history tens of millions of years in age or more. Madagascar, which similarly rifted from Gondwana, remained close enough to Africa to permit colonisation by terrestrial mammals [176,177]. Other continental islands, for example islands of Malesia, were connected to major landmasses during ice age glacial events [178,179]. Distant oceanic island groups, such as Hawaii, are of recent volcanic origin, and have been entirely colonised by long-distance dispersal in the past few million years [180,181]. As a result, New Zealand is unique from an evolutionary and biogeographic perspective. Due to the paucity of so many plant and animal groups, the long age of the biota, and the persistence of taxonomically unusual lineages, New Zealand's ecosystem functioned

and evolved like no other. Presumably, not only did phylogenetically isolated lineages persist or evolve; strange and deeply ancient species interactions have also most certainly developed.

A clear consensus on the history of New Zealand's plants and animals is sorely lacking. Classically held and cherished concepts, in particular the 'gondwanan' nature of New Zealand's biota are now being increasingly challenged [32], yet a much more exhaustive phylogeographic survey of the endemic biota is needed. An important limitation of New Zealand is the severity of its recent extinctions and the transformation of its ecosystems; yet, alone among 'continental' landmasses, these changes occurred only centuries ago. As a result, this biota can potentially be understood in relative completion. Ancient DNA has, and will continue to, provide incredibly valuable insights into the evolution and origins of New Zealand's biota.

1.4   Aims, objectives and outline of this thesis

The research I present in this thesis uses ancient DNA to investigate a number of prehistoric New Zealand bird species and is divided into several research chapters, each of which is in preparation for submission to peer-reviewed journals. These projects draw on environmental DNA and phylogenetic data to understand the ecology, evolution and biogeography of the New Zealand fauna. Major species covered are members of the larger avian megafauna that became extinct during the initial prehistoric extinction phase roughly 500 years ago. This includes four moa species: the little bush moa (*Anomalopteryx didiformis*), South Island giant moa (*Dinornis robustus*), the upland moa (*Megalapteryx didinus*) and the heavy-footed moa (*Pachyornis elephantopus*). The final taxa, and the target of Chapter Four, are the mysterious adzebills (*Aptornis defossor*, and *A. otidiformis*). The extant, but critically endangered kakapo (*Strigops habroptilus*) also features prominently. However, several other bird taxa, although not directly investigated, are discussed throughout this thesis.

Although bird coprolites from New Zealand have previously been investigated for aDNA, they have never yet been researched using NGS technology. In Chapter Two, I aim to more fully investigate moa diet and parasite diversity in order to test the

hypothesis that moa consumed a wider range of plants and fungi than previously identified. To this end, I examine a total of 23 ancient bird coprolites from seven different field sites using the locus 18S rRNA, with universal primers designed to specifically target eukaryotes (animals, plants, fungi and protozoans). Based on the limitation of previous aDNA studies to moa coprolites on Sanger-sequencing, and limited physical preservation of various taxa (including plants, animals and fungi) groups, I hypothesise moa diet and parasitic communities are more diverse than previously realised. Many fungi in New Zealand's forests produce fruiting bodies with clear adaptations for bird-based dispersal [105,106], therefore these may have been consumed by moa. Furthermore, due to the geographic and phylogenetic isolation of moa in New Zealand, it is likely that moa parasitic communities were complex and unique, and underwent a high degree of co-extinction.

In Chapter Three I use NGS methods to further research New Zealand avian coprolites using a metagenomic shotgun sequencing approach. The major objective of this chapter is to investigate the content of endogenous DNA in coprolites from semi-arid areas of New Zealand's central South Island. Rich deposits of prehistoric avian coprolites have been identified from these areas, yet have not had DNA amplifiable by previous studies.

Chapter Four investigates the evolution and phylogenetics of the adzebills (*Aptornis*, Aptornithidae). These massive (up to 25kg), bizarre flightless birds were entirely endemic to New Zealand, and have long been an ecological and taxonomic enigma. This chapter sequences near-complete mitochondrial genomes from both recently extinct species. By utilising a comprehensive dataset of other bird taxa (including new data for the South American trumpeters *Psophia*), we are able to conclusively resolve the relationship of adzebills to other bird taxa. Hypotheses regarding the ecology and macroevolution of these unusual birds are presented and discussed based on my results.

The final chapter (Chapter Five) is a synthesis of the findings of the preceding three chapters and a discussion of their implications for our understanding of New Zealand's pre-human ecosystem. I also outline the problems encountered throughout this thesis and propose solutions. Finally, I identify outstanding questions and describe potential future research projects.

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CHAPTER TWO

Metabarcoding ancient metagenomes of animal coprolites: revealing the lost ecological interactions of New Zealand's extinct ratite moa and critically endangered kakapo parrot

Statement of Authorship

Title of Paper	Metabarcoding ancient NGS DNA from animal coprolites: revealing the lost ecological interactions of New Zealand's extinct ratite moa and critically endangered kakapo parrot.
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Contribution to the Paper	Received initial processed data (in biom data format); filtered data; performed bioinformatics analyses, high-resolution taxon identifications and phylogenetic analyses. Identified major results, designed and illustrated figures, and wrote paper.		
Overall percentage (%)			
Signature		Date	11/06/2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Alan Cooper		
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Contribution to the Paper	Assisted with paper and study design, provided edits and supervised research. Assisted with paper outline. Performed early DNA laboratory work (DNA extractions). Provided samples.
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2.1   Significance

The large-scale ramifications of key species extinctions remain largely unexplored, due to a lack of technical availability. We use NGS-based metabarcoding of ancient DNA from New Zealand extirpated bird species coprolites (paleofaeces) to reconstruct disease, diet, and past species interactions. Our new approach yielded a diversity of species interactions in exceptional detail, demonstrating new evidence extinct moa species regularly ate mosses and fungi and served as a key dispersal agent in ancient New Zealand. Tracking dietary information and parasite dispersal across species and through time also reveals behavioural information of extinct species. Similar use of ancient-DNA based metabarcoding on other less well-studied prehistoric ecosystems has the opportunity to elucidate loss niche interactions in a modern world.

2.2   Abstract

Due to widespread species declines and extinctions during the late Quaternary, a complete understanding of natural, unaltered ecosystem function, community structure, and species interactions depends on high-resolution paleoecological data. Animal coprolites provide a unique source of such information, including detailed insights into trophic interactions, behaviours, gut parasites and microbiotas, that can allow us to better understand the wider ecological impacts of extinctions and can potentially guide contemporary conservation and ecological restoration efforts. Here, we use high-throughput (HTS) next-generation sequencing (NGS) of eukaryotic DNA from animal coprolites to reconstruct past ecological interactions, as well as provide high resolution on past disease, diet, and behaviour of four species of extinct moa and a critically-endangered parrot from New Zealand (kakapo). While ancient DNA (aDNA) from moa coprolites has previously been studied, our results reveals evidence that moa and ancient kakapo consumed fungi mycorrhizal with several key New Zealand forest tree species, suggesting that these birds may have played an important role in this aspect of New Zealand's natural forest dynamics. We also identify the first genetic evidence that moa consumed ferns and moss species. Finally, we also find the first genetic evidence for the existence of several endemic clades of moa parasites, confirming that the extirpation of moa led to substantial biodiversity loss through co-extinction. Ultimately,

our study demonstrates that NGS sequencing of DNA from coprolites is a powerful new tool for paleoecological studies, providing insights unattainable by conventional analyses.

2.3   Introduction

The late Quaternary through to present day is characterised by terrestrial faunal extinctions and population declines, most striking being the widespread extinction of megafauna due to anthropogenic and/or climate change over the past ~50,000 years [1–6]. The extirpation of a single species can reverberate throughout an ecosystem, as all species are involved in dynamic and complicated ecological interactions (e.g. dispersal, competition, nutrient cycling, commensal, mutualistic and parasitic interactions) [7–13]. Assessing these wider impacts of species extinction requires detailed information into past species interactions and ecosystem function, which is difficult to ascertain from fossil evidence alone. Coprolites (paleofaeces) from the late Pleistocene contain both microfossils and ancient DNA. Consequently, analyses of data from coprolites can provide valuable information about past environments by revealing the identity, diet, behaviour, microorganism community, and parasite load of the depositing species [14–23]. These data can in turn be invaluable for contemporary conservation and ecological restoration efforts.

Previous attempts to analyse ancient DNA from coprolites have yielded valuable insights into ecosystem function, but have been limited to Sanger-sequencing and cloning methods to assess the diversity of taxa contained [14–23]. Next-Generation Sequencing (NGS) approaches that target a barcoding locus such as 18S or 16S rRNA, now allow for vast numbers of unrelated species to be identified from individual samples [24–26]. Critically, this approach can identify taxa even when DNA is degraded, in low abundance, or (due to sequencing of obscure loci) is otherwise unidentifiable via conventional shotgun sequencing approaches [24–26]. Despite these considerable advantages, metabarcoding studies on coprolites have thus far been restricted to ancient microbial communities from human samples [27–30], and dietary plants of Pleistocene arctic megafauna [31].

New Zealand is a large (268,021 km^2) South Pacific island group, which separated from the super-continent Gondwana 85-55 Ma [32–35]. As a result of New Zealand's long isolation, its native biota is generally considered much greater in age, diversity and ecological complexity than that of most other oceanic islands [36]. The native terrestrial fauna lacks

mammals and was once dominated by birds, including nine species of extinct, herbivorous ratite moa (Dinornithiformes), with adults ranging between 9-242 kg in body mass and up to 3.6m in height [35,37–39]. Human settlement of New Zealand during the 13th C. [40] resulted in drastic ecosystem changes, including the extinction of moa within 200 years [38] and the loss of 41% of endemic bird species [35,37,41]. Many of the remaining species are now endangered and range-restricted, resulting in a major loss of ecosystem diversity and function. Importantly, these ecological changes transpired very recently, and the cold climate in NZ is ideal for the preservation of prehistoric fossil material, providing an excellent opportunity to examine past ecosystem interactions via ancient DNA analysis. Thousands of coprolites, up to >6,000 years old, have been recovered from New Zealand [23]. Of these, almost 100 have been identified by aDNA, as one of five moa species [16–20,22,23], or the critically endangered kakapo parrot (*Strigops habroptilus*) [21]. Both kakapo and moa coprolites have been extensively researched for plant and parasite content using microfossil and Sanger-sequencing approaches, [16–23,42,43]. However a number of important questions still abound. For example, New Zealand's forests contain a diversity of mycorrhizal fungi with clear adaptations for dispersal by mycophagous birds, despite the extensive extirpation of the endemic ground dwelling avifauna. Additionally, the phylogenetics and community structure of moa parasites or pathogens is limited to individual sequences from a small number of samples. An NGS analysis would permit a holistic capture of the broader diversity structure, and provide new insights into past ecosystems in New Zealand.

In the present study we apply a NGS barcoding approach to ancient coprolites from four species of extinct moa and the endangered kakapo parrot to reconstruct paleoecological interactions in New Zealand. Using eukaryote-specific universal primers to amplify a ~100bp barcoding fragment of the 18S small-subunit ribosomal RNA (18S rRNA) gene, we are able to identify a wide-range of New Zealand fungi, plants, animals and protozoans from 23 ancient NZ bird coprolites. We verify our methodology against previous microfossil and Sanger sequencing approaches, and are able to reconstruct previously unknown species interactions, including the first direct evidence for ingestion and dispersal of native fungi, first DNA evidence of dietary fern and moss species, and a previously unreported diversity of parasitic taxa. We further demonstrate that New Zealand's megafaunal birds played a specific, now absent, role in ecosystem dynamics, and their extinction has impacted a diversity of other species. Overall, we strongly support that metabarcoding ancient DNA from materials such as coprolites can provide exceptional detail into past species interactions, and should be applicable to other, less-well resolved prehistoric ecosystems elsewhere.

2.4   Results

2.4.1   Site and sample information

In the present study we analysed DNA data from a total of 23 coprolites. These coprolites were collected from eight different New Zealand sites, which were dominated by either southern beech forest (Nothofagaceae) or arid shrubland / grassland (Fig. 1). Radiocarbon dating confirmed that the coprolites ages range between 124 to 1,557 years before present (Table S1). Sanger DNA sequencing had previously confirmed that each coprolite belonged to either the critically endangered kakapo (*Strigops habroptilus*) or one of four moa species: little bush moa (*Anomalopteryx didiformis*), South Island giant moa (*Dinornis robustus*), upland moa (*Megalapteryx didiformis*), or heavy-footed moa (*Pachyornis elephantopus*) [16,19,22]. While coprolites from all four of these moa species were concurrently identified at one site (Dart River Valley), from most sites we sampled only a single species (Fig. 1). From past studies, these species have been identified as having a variety of dietary and habitat preferences. Giant moa had generalised diets and occupied a variety of habitats, upland moa were typically found at higher altitudes (and were the only alpine moa), little bush moa were specialist forest browsers of woody vegetation, and heavy-footed moa found in more open habitats and swamp margins [16–20,22,23,44]. Kakapo were found in a variety of forested habitats across New Zealand, and are strictly herbivorous [42,45–47]. Many of the samples were used in a prior aDNA study (see Table S1), allowing for a comparative methodological analysis.

The 18S ribosomal RNA (rRNA) region specific to eukaryotes was successfully amplified and sequenced on an Illumina HiSeq with read depth falling between 26,102 – 257,186 reads per sample (Table S2) For comparison, fecal remains of a range of captive zoo animals, (from Muegge *et al.* [48]) comprising a range of mammals and two species of extant ratites (North Island brown kiwi *Apteryx australis* and ostrich *Struthio struthio*), were also analysed in subsequent analysis.

2.4.2   Total Diversity

Taxa were identified by clustering reads into operational taxonomic units (OTUs) in QIIME [49], and comprised a diversity of eukaryotes including fungi, land plants and parasites (Figs 1, S1-S4). A large proportion of OTUs could not be identified to a eukaryotic kingdom, likely due to lack of appropriate reference data and ancient DNA damage, and were subsequently removed from downstream analyses. Ancient moa coprolites demonstrated significantly higher diversity than any other sample set, especially those obtained from captive species (p=0.003) as expected [48]. All bird samples, both modern and ancient, showed more heterogeneity between samples, in contrast to relatively homogenous and uniform modern zoo mammalian samples dominated by common gut eukaryotes, namely *Rhodotorula* and *Blastocystis* (Fig. S1). Significant differences in alpha diversity were not apparent between moa, kakapo or modern ratites (Fig. S5*a*). Within moa coprolites, the highest diversity was found in samples from southern-beech sites, in contrast to shrubland / grassland sites (p=0.03, Fig S5*b*). Within moa species, upland moa had the highest OTU diversity, while heavy-footed moa had the lowest, although the differences were non-significant (Fig. S5b).

Overall diversity between samples was compared using unweighted distance values (Fig. S6). Jackknifed UniFrac distances found Zoo mammals grouped (100%) to the exclusion of bird groups (Fig S6, S7*a*). Bird samples mostly fell within two distinct groups; all kakapo and moa from southern beech sites clustered to the exclusion of a second group that consisted of moa from shrubland / grassland sites and modern birds (86%) (Fig. S6, S7*bcd*). However, both ostrich and kiwi failed to cluster with either group (Fig S6, S7*bcd*). Host class explained most variation within samples, i.e. mammals vs. birds (Fig. S6, S7*a*), although variation within coprolites was explained by location and environment type (Fig. S7*bcd*), indicating that almost all coprolite diversity is geographically dependent.

2.4.3   Fungal aDNA and evidence of dietary fungi

Fungi, which include potential dietary and parasitic taxa, dominated eukaryotic diversity (see Fig. 2, 3, S1, S2). A conservative approach was taken to identify fungal species that were not likely to have arisen from post-depositional contamination. 'Sac-fungi' (Ascomycota) and a variety of yeast taxa (Saccharomycetes, Microbotryomycetes,

Tremellomycetes) were common in most samples including zoo animals; however, with the exception of common gut flora types found in the modern mammals, none of these taxa could be confirmed as pre-depositional or pathogenic. For example, all yeast taxa (including species of *Cryptococcus*) were phylogenetically similar to common soil forms, and the only confirmed macrofungal (and potentially edible) Ascomycota (*Lasiobolus, Ascobolus*) are coprophilous [50] (Fig. 3).

In contrast to environmental or coprophilous taxa, dietary macrofungi were also identified among Basidiomycota reads, which (except for microbotryomycete yeasts) were dominated by Agaricomycetes (which include all 'true' mushrooms, bracket fungi, and puffballs). Agaricomycetes were only abundant in coprolites from southern-beech forest sites, likely a due to higher moisture and the rich biodiversity of mycorrhizal mushroom species found in this forest type [51] (Fig. S2). Notable mushroom-forming genera identified included the mycorrhizal *Cortinarius* (2 OTUs) and *Inocybe* (1 OTU) and the plant parasite *Armillaria* (1 OTU), all found from southern beech site samples only. These mushroom taxa were also only found in kakapo (confirmed mycovores) [42,47] and upland and giant moa, the only two moa species believed to most frequently feed on forest-floor vegetation [16,18–20]. These species of Basidiomycota are not known to be coprophilous, and have distinct nutrient requirements (i.e. non-saprobic/decomposers and require symbiosis with tree roots), making hyphal growth into hard coprolite material or cave sites highly improbable. In addition, the aerobic settlement of spores post-deposition is also unlikely, as these taxa were observed in high abundance and were sample and species specific, rather than site dependent. Therefore, these Basidiomycota are likely to represent a previously unexplored portion of the moa diet. Additional mushroom forming taxa were also identified (Fig. 3, S2); however, all are saprobic and therefore cannot be ruled out as post-depositional contamination. Nonetheless, this finding is the first evidence of mycophagy in moa.

2.4.4 *Plant aDNA, including fern and moss taxa*

Although fungi likely formed a component of moa diet, previous aDNA and subfossil analyses of moa coprolites support a moa subsistence strategy high in plant material, although previous DNA sequencing strategies have only targeted edible angiosperms [16,18–20]. These studies revealed that little bush moa browsed largely on forest shrubs, ferns and trees (such as southern beech and podocarps), whereas heavy-footed moa fed on vegetation characteristic of

open habitats (especially *Coprosma* shrubs, *Phormium* 'flax' leaves, dicot herbs and grasses) [16,18,20,44]. In contrast, giant and upland moa were comparative generalists that fed on forest herbs, fruits, seed pods, and in the case of upland moa, aquatic plants (notably the watermilfoil *Myriophyllum*) [16,18,43,44].

In this study, we confirm these previous findings using high-throughput DNA sequencing, identifying plant species from all coprolites and modern faeces, except, remarkably, from all kakapo samples (Fig. 2, 3, S3). Kakapo plant diversity may be limited due to preservation bias within their relatively small and fragile coprolites and/or intensive food-processing by this species [52], which would predispose sequencing to be limited to robust parasite cysts and fungal spores, as observed (Fig. 2). Plant reads were generally abundant only in southern beech forest sites (Euphrates and Dart River), which also may be why no gymnosperms were identified, as conifers are not common in these areas. The majority of identified OTUs were angiosperms, including shrubs, trees and herbs native to New Zealand, and also represented taxa found in moa coprolites from past studies [16,18–20,22]. This includes Saxifragales from upland moa and heavy-footed moa (aquatic watermilphoil and the shrub *Gonocarpus*), and Myrtales from upland moa only (similarly only found in upland moa in past studies, represented by the rata genus *Metrosideros*, and the Rohutu *Neomyrtus*) [18,19,43]. Overall, read depth varied between samples, but was the highest in giant and upland moa from southern beech sites (Table S2), again suggesting that plant dietary diversity is likely geographically distributed.

Although moa coprolites have been extensively studied for plant dietary components, our approach allowed us to sequence non-angiosperms thus revealing several novel food sources. Despite low taxon resolution due to lack of reference data, especially in New Zealand flora, we were able to confirm the presence of three moss (bryophytes), and three fern (pteridiophytes) taxa in moa coprolites. Mosses were found only in upland moa from southern beech forest sites (except extremely low numbers of reads from a single *Dinornis*), while ferns were found in all taxa, except heavy-footed moa (Fig. 3, S3). This is the first aDNA evidence of these taxa from moa, and supports both the known ecology of each species and fossil data [18,19,22,43], although limited evidence suggests that heavy-footed moa may have also occasionally consumed ferns [18,22]. Despite the current limitations of 18S barcoding of plants, our results confirm microfossil and Sanger sequencing studies, and, importantly, reveal new information regarding moa diet (fern and moss data) that suggest dietary niche partitioning amongst these species is strongly apparent.

2.4.5   *Parasite aDNA and moa-parasite co-extinction*

Parasitic eukaryotic taxa amongst the 18S amplicon sequencing data were investigated to examine host-parasite co-extinction, as Wood *et al.* [17] previously identified both subfossil parasite eggs and parasite DNA of moa. Here, we utilize many of the same coprolites specimens to examine the diversity of parasites via this new high-throughput approach. From these specimens, Wood *et al.* previously identified a species of the single-celled Apicomplexa (Eimeriidae), one trematode (Notocotylidae), and two species of nematode (Heterakoidea and Strongylida), while three different nematode egg types were also described (Heterakoidea, Trichinellidae, and an unidentified ascarid). While our results closely matched these observations, we also identify new parasitic taxa and expand the diversity of moa parasites from approximately six species in six families to 24 species in eight families. Phylogenetic and BLASTn methods identified highly abundant Eimeriidae, nematodes (Ascaridida: Heterakoidea) and trematodes (Notocotylidae), as well as more rare nematodes (Strongylida). Interestingly, we also identified multiple Eimeriidae and Heterakoidea OTUs (nine each), rather than single sequences, suggesting a much greater interspecies diversity than previously detected. Comparative phylogenetic and nucleotide distance analyses support that these OTUs represent multiple unique species (Fig. S8, S10). Additionally, we identify three unique taxa not previously found, including single OTUs of an additional Apicomplexa (Sarcocystidae), and two additional Ascaridida nematodes (superfamily Ascaridoidea, and family Seuratidae). These new data demonstrate the robust nature of a metabarcoding sequencing approach, and demonstrate significant improvements over current methodologies used to monitor co-extinction events.

Several of the taxa identified by Wood *et al.* were only found in specific moa species, and while we do find host-specific associations, our observations differ considerably, in part due to comparisons with other ratite species and zoo mammals. Wood *et al.* identified several parasitic taxa only from upland moa (single Strongylida, trematode, and Eimeriidae species) that were reported to be moa specific. However, firstly the Strongylida OTU found in moa (OTUAJJ920349 see Fig. S12) was also present in in zoo mammals. Furthermore, several Eimeriidae previously identified only in upland moa from Dart River Valley were not found to be host specific, as Eimeriidae taxa were widely distributed and shared between moa species and the extant kiwi (Fig. S12). Trematodes and some nematodes (Heterakoidea, Seuratidae) were also more widely distributed than expected. For example, a single trematode species, previously thought to be only in upland moa, was also present in smaller

number in heavy-footed and giant moa (Fig. 3), and all moa species in southern beech sites, except heavy-footed moa, shared very similar nematode communities. While few unique OTUs were found for any moa species, many OTUs were shared amongst species at a single site, demonstrating that different moa species likely interacted within their niche. However, comparisons of genetic divergence between taxa found in coprolites and in extant New Zealand species, it seems likely that several taxa were specific to moa as a whole and represent a co-extinction. For example, heterakoid nematodes were only found in moa species, and we could identify no close relatives. From our comparison with previous study, these metabarcoding results demonstrate a considerable improvement in resolution over previous methods.

2.5   Discussion

2.5.1   Improvements of NGS metabarcoding over Sanger sequencing

The present study demonstrates that metabarcoding analyses using a single locus are an efficient, robust technique to obtain paleoecological information. Other methods, such as microfossil analysis, DNA cloning, or Sanger sequencing, previously applied to moa coprolites are constrained by considerable logistical difficulties and may perform poorly in detection of rare taxa and total species diversity. Although moa are now one of the most thoroughly studied groups of extinct species, revisiting moa with this method provides several critical findings, absent after years of analysis utilizing alternative methods: a) detecting a wider species diversity in a single analysis, i.e. fungi, plants, and parasites, and b) identified rarer taxa, which included entirely new dietary components and eukaryotic parasite lineages and c) provided resolution within closely related taxa, i.e. Emeriidae. Together, our results have demonstrated considerable advances over previous methods, and have provided novel insights into moa ecology, behaviour, and diet that were previously unknown.

2.5.2 *New dietary taxa, niche partitioning and dispersive roles of moa*

In this study, we find the first evidence that moa and ancient kakapo consumed native fungi, and provide the first DNA evidence to support the consumption of moss and fern by multiple moa species. In these findings, niche partitioning was strongly supported. For example, the two moa species believed to be dietary generalists (upland and giant moa) were the only moa species observed to consume fungi. Niche partitioning was also supported by the ingestion of aquatic plants (Saxifragales) by upland and heavy-footed moa, as well as the presence of a trematode, usually transmitted by consumption of aquatic vegetation or pond-snails [17–19,43]. Furthermore, considerable differences were demonstrated in co-occurring moa at Dart River Valley (for example moss DNA was almost totally restricted to upland moa in this location), supporting that different species played different roles in New Zealand's ancient ecosystem and exhibited variable feeding strategies.

The conservation of moss DNA within upland moa samples (all upland moa samples from Dart River and Euphrates Cave), and near-absence from other species, was especially striking. In moa coprolites, moss fragments have been found in upland moa coprolites from Euphrates cave [19], confirming these results. However, in a study of 49 Dart River Valley moa coprolites, moss fragments were not found in upland moa (which included three of the four upland Dart River samples sequenced in this study) [18]. Conversely, in this same previous study, moss fragments were found in coprolites one each from heavy-footed, giant, and little bush moa. Moss fragments have also been identified in gizzards from heavy-footed, giant, and eastern (not included in this study) moa species that were preserved in swamps [44], which may be a taphonomic artefact (e.g post-mortem introduction of moss fragments, or trapped moa feeding on surrounding vegetation). Furthermore, these fossil results failed to identify ferns, from which we found DNA of in many of the same samples. These conflicting results do indicate that moss and fern structures generally fail to physically preserve, thus requiring DNA sequencing to identify. Our results also suggest that while most moa species may have occasionally consumed mosses (perhaps unintentionally with other foods or from water sources), upland moa may have been the only species to have fed on them regularly.

In this study, the consumption of edible mycorrhizal fungi was supported in upland and giant moa. This is may be of some importance as Southern beech (Nothofagaceae) dominate over half of New Zealand's forests, and are entirely co-dependent with a functioning mycorrhizal network [51]. This close association between Southern beech trees and mycorrhizal fungi may have proved crucial in determining biogeographic patterns in New

Zealand, such as several enigmatic 'beech gaps' across the country [51,53]. Today, many modern herbivorous birds frequently consume fungi, including kakapo [42,47] and modern ratites [54,55]. Specifically, one genus identified in moa coprolites (*Cortinarius*) is one of numerous New Zealand mycorrhizal lineages to independently evolve 'sequestrate' mushrooms (caps enclosed preventing spore release), which typically require animal-dispersal (generally by mammals) for ecological success [56–59]. New Zealand's sequestrate mushrooms tend to be unusually colourful – a classical indication of bird-dispersal, despite the fact that most likely native mycophagous birds are extinct or endangered [35,37]. Although introduced mammals in New Zealand, including deer and possums, consume and disperse mycorrhizal fungi, recent research has found that native fungal species do not survive and germinate after dispersal, whereas introduced fungi associated with invasive tree species do [60]. Together, our findings suggest that moa and other native birds provided a potentially irreplaceable dispersive role for ecologically essential fungi. Furthermore, given that extirpated birds consumed both seeds and fungal species [16,19,43,44], they may have provided a unique mechanism for co-dispersal of beech and mycorrhizal fungi, which is now lost. This is potentially alarming in light of current species reductions and local extinctions, and further illustrates that New Zealand's native birds have no ecological analogues among introduced species.

2.5.3 *Indirect evidence for moa interactions and migratory behaviour*

Direct information on extinct animal behaviour such as migration or social interaction tends to be extremely difficult to resolve [61]. However, behavioural observations can be inferred from tracking parasite prevalence among moa species. In Dart River Valley, coprolites of all moa, except heavy-footed (3/4 species), showed extremely similar Ascaridida nematode communities, and also shared several Apicomplexa (Fig. 2, S12). These parasites are generally transmitted via food that has been recently contaminated by infected faecal material [62], thus these three moa species were likely in close contact (rather than sequentially using these sites). As a corollary, this also supports behavioural differences amongst heavy-footed moa. Overall, upland moa also had an especially unique parasite community, including large numbers of trematode (notocotylidae) taxa irrespective of site or ecology, despite the fact that some taxa were shared with extant host species and other moa. The Notocotylidae found in upland moa typically infest a diversity of semi-aquatic animals [63], requiring ingestion of larvae (cercariae) encysted on infected water plants or snails [63]. As a

result, this finding suggests that upland moa may have consumed larger amounts of aquatic plants relative to other moa. Alternatively, the more alpine-dwelling upland moa were uniquely exposed to infested, still-water ponds in the form of alpine tarns[17,43]. As upland moa were infected regardless of site altitude, this finding would provide evidence for a seasonal altitudinal migration of upland moa, as frequently suggested [19,43].

2.5.4   *Extinction of host-specific parasites*

Co-extinction of hosts and parasites is increasingly featured in conservation debates and likely represents one of the greatest mechanisms of biodiversity loss [64,65]. However, direct evidence for this remains limited. While conservation of specific Eimeriidae taxa to individual moa species was previously shown [64], we identified a five-fold higher diversity of Emeriidae taxa within moa. In addition, we identified several emeriids in the extant kiwi, indicating the survival of this parasite clade in modern species. This result is unsurprising as emeriids are known to be flexible in regard to their host species [66]. In contrast, heterakoid nematodes identified in this study were entirely restricted to moa and not shared with other New Zealand birds or mammalian species. The only native heterakoids in New Zealand (except species of *Ascaridia* from parakeets and migratory cuckoos [67]), are relict and rare species identified from similar 'gondwanan' taxa: the kiwi and the tuatara (the proposed 'Kiwinematidae') [17,68,69]. Putative heterakoid eggs from moa coprolites also share morphological similarities with these species, and a relationship was suggested by Wood *et al.* [17]. Unfortunately, DNA or specimens of extant Kiwinematidae species are unavailable. However, this study also showed that the moa heterakoids were highly diverse, even more so than that found between most related host families. Given this inference, along with their apparent abundance in moa and the low diversity and rarity of relatives in modern birds, it is likely that these heterakoid taxa have undergone co-extinction with moa. Furthermore, this increased level of genetic divergence may demonstrate a rare observed example of two clades of taxa becoming co-extinct. At the very least, we clarify co-extinction within moa, and provide evidence towards the depth of biodiversity loss accompanying host-parasite extinctions elsewhere.

2.5.5   Conclusions

In this study, we apply a metabarcoding technique to ancient coprolites as a novel approach to efficiently produce robust and diverse paleoecological information, and identify several surprising findings regarding diet, behaviour, and disease in a classic extinct species group. While 18S provided a useful 'snapshot' study, deeper sequencing, longer-read lengths developed from other sequencing platforms, targeting additional taxon-specific loci, such as ITS (for fungi), or rbcL or trnL (for plants), and collecting robust reference data will provide further insight into paleoecological interactions of extinct species. Nevertheless, our metabarcoding analysis provides a unique window into ancient New Zealand ecology, and provides critical information applicable to current conservation efforts. This study suggests that the disappearance of moa and other native New Zealand bird species had significant ecological consequences, effecting both forest ecosystem function and greatly reducing parasite diversity. Further NGS studies of animal coprolites from less well-studied ancient ecoystems, such as those recovered from extinct ground-sloths of the North-American Pleistocene [15] will provide invaluable new insights into lost interactions in different ecological contexts. As extinction and biodiversity change is an ongoing process, relevant and detailed historical information is urgently required. Quantifiable, high resolution data, such as the type found in this study, is vital to determine ecoystem change, identify the impacts of extinction and biodiversity loss and assess the depth of past species interactions, in turn invaluable for contemporary conservation and ecological restoration efforts.

2.6   Methods (note)

See supplementary information for comprehensive methods (complete manuscript will comprise a reduced methods section in the main text).

2.7   Figures

Figure 1. Map of New Zealand's south Island with localities of coprolites used in this study. Approximate distribution of relevant vegetation types at AD 1250 is shown.
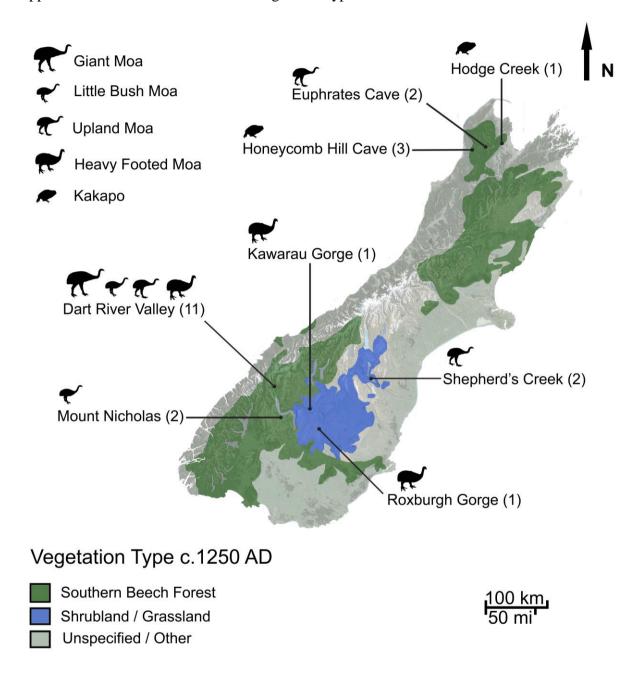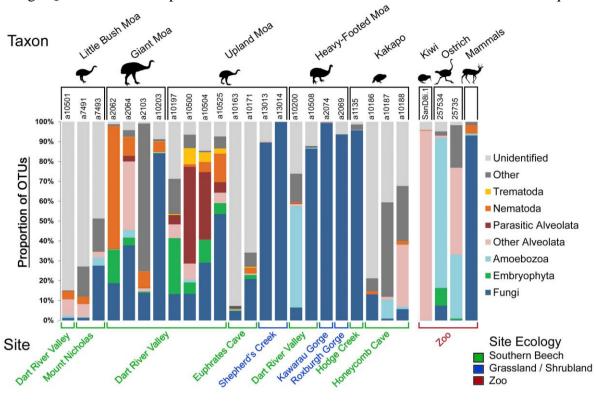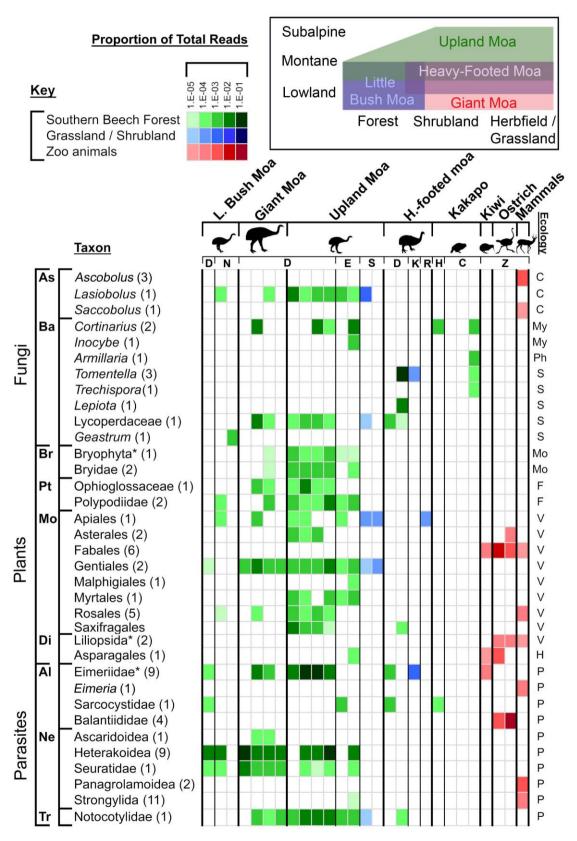
Figure 2. Proportions of reads per sample to taxonomic and ecological groups as determined through QIIME. All OTUs present in extraction blanks filtered. Note read counts non-equal.

Figure 3. Presence of selected taxa chart, identified to lowest taxonomic rank possible. Taxon deviations, right hand column: As=Ascomycota, Ba=Basidiomycota, Br=Bryophyta, Pt=Pteridiophyta (Ferns), Di=Dicots, Mo=Monocots, Al=Alveolata, Ne=Nematoda, Tr=Trematoda. Site deviations, top row: D=Dart River, N=Mt. Nicholas, E=Euphrates Cave, S=Shepherd's Creek, K = Kawarau Gorge, R=Roxburgh Gorge, H=Hodge Creek, C=Honeycomb Cave, Z=Zoo. Ecology types, right hand column: C = Coprophilous, My=Mycorrhizal, Ph=Phytopathogen (plant parasite), S=Saprobe, Mo=Moss, F=Fern, V=Variable, H=Herb, P=Parasite. * Designates taxon that includes taxon immediately following (successfully identified to lower level).

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S.2   Supplementary Information

S.2.1 Methods

S.2.1.1 Site and specimen details

Coprolites used in this study came from eight sites in New Zealand's South Island, comprising a variation of paleoecologies: one subalpine Southern beech forest (Euphrates Cave), three Southern beech forest (Dart River Valley, Honeycomb Cave, Hodge Creek), one mixture Southern beech/ conifer-broadleaf forest (Mount Nicholas), and three semi-arid shrubland / grassland (Kawarau Gorge, Roxburgh Gorge, Shepherd's Creek) (see Fig. 1, Table S1). Full site descriptions can be found in previous publications [1–16]. Species included South Island giant moa (*Dinornis robustus*, 4 from Dart River Valley), upland moa (*Megalapteryx didinus*, 4 from Dart River Valley, 2 Euphrates Cave, 2 Shepherd's Creek), *Pachyornis elephantopus* (heavy-footed moa, 2 Dart River, 1 Karawua Gorge, 1 Roxburgh Gorge), bush moa (*Anomalopteryx didiformis*, 1 Dart River, 2 Mount Nicholas), and kakapo (*Strigops habroptilus*, 1 Hodge Creek, 3 Honeycomb Cave). Full descriptions are in Table S2.

All samples were confirmed to species by aDNA analyses, most of which are in previous publications (see Table S2). The only exception was a single Mt. Nicholas specimen, which DNA identification supported either coastal moa (*Euryapteryx curtis*) or bush moa. However known biogeography of coastal moa makes an association with this species extremely unlikely, supported by the presence of confirmed bush moa coprolites from this site [5]. Radiocarbon dates were obtained for 9 of the 25 samples at the Waikato radiocarbon dating laboratory, Waikato University, New Zealand. (http://www.radiocarbondating.com/). Most dates have been previously published [5,9,16], however dates for two kakapo coprolites are new to this study (see Table S2). Overall, including coprolites not used in this study, 5/8 sites have had a range coprolite radiocarbon dates estimated, and non-dated specimens are expected to fall within these ranges (see Table S1). No dates are available of coprolites from Hodge Creek, Shepherd's Creek or Roxburgh Gorge, although they are likely to be late Holocene in age [6]. In addition to sequenced

coprolites, sequences from captive animals, comprising 23 modern mammals and 3 modern birds (1 kiwi and 2 ostrich) were included in the analyses from the study by Muegge *et al.* [17].

S.2.1.2 DNA extraction and amplification

Coprolite samples were extracted in an isolated, fully contained, ancient DNA laboratory at the Australian Centre for Ancient DNA (ACAD), using the PowerSoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA). Most extractions used were the same as those used in past studies by Wood *et al.* [3,9,11,13,14], in which information on host ID determination, radiocarbon dating, and extraction protocols can be found. In addition new extractions for this study included all four kakapo coprolites and upland moa from Shepherd's creek, each of which had host DNA amplified by primers specific either to moa or kakapo [3,9]. Extracts (including extraction blanks) were amplified by PCR (in triplicate) by the universal 18S rRNA gene-targeting, Eukaryote-specific, primers Euk1391f and EukBr based on Amaral-Zettler *et al.* [18], and can be found on the Earth Microbiome Project (EMP) webpage (http://www.earthmicrobiome.org/emp-standard-protocols/). All sets of PCR reactions were pooled and quantified, and were purified using the Mobio UltraClean PCR Clean-up kit (Mo Bio laboratories, Carlsbad, CA, USA). High-throughput sequencing (HTS) reads were generated on a single lane of the Illumina HiSeq platform at BioFrontiers Institute Next-Generation Genomics Facility at University of Colorado, Boulder.

S2.1.3 QIIME analyses

Combined reads were analysed in the QIIME software package [19], of which raw barcoded sequences were demultiplexed and quality filtered using default parameters, resulting in reads roughly ~100bp in length. Sequences were open-reference clustered into OTUs (operational taxonomic units) using an RDP classifier towards the PR^2 database (Protist 2, http://ssu-rrna.org), modified to be QIIME compatible. For a clustering threshold, we elected to utilise 97% pairwise nucleotide similarity between reads as is most often utilised by similar studies, and is widely considered to account for predicted sequencing error whilst still successfully identifying species-level diversity [20,21]. OTUs that clustered with the reference database inherited the reference taxonomy. Non-assigned hits were clustered de-

novo, and were blasted to the PR^2 database using an e-value cut-off of $1e^{-25}$. For OTUs that fell below the threshold, the RDP classifier retrained on PR^2 was used with a requirement of 50% confidence. In all instances the most abundant sequence for each cluster was selected as the representative sequence.

All OTUs present in extraction blanks were filtered from the remaining biom file to control for *in-vitro* contaminants, by identifying all OTUs present in any of the extraction blanks and filtering these from the working biom file. In addition few samples with counts of <2,500 reads, inferred to represent instances of possible sequencing or amplification failures were omitted (*a-priori* tests also generally failed to identify ecologically informative taxa from such samples). All circumstances of <5 reads per sample (maximum 0.015% of total reads and considered a sequencing error risk) were additionally removed. This was achieved by splitting all files into separate biom files for each individual sample, filtering out all reads <5 per OTU, and re-merging all into a single biom file. All OTUs that could not be assigned to a particular Eukaryote kingdom were then removed from any subsequent analyses.

This dataset was subjected to several alpha- and beta- diversity analyses in QIIME. To improve specificity and flexibility of downstream analyses, five biom files were generated representing different sample groups: all samples, all bird taxa, ancients only (all moa and kakapo), ratites only (including modern kiwi and ostrich, but not kakapo), and moa only. Each was subsequently split into groupings specified by OTU taxonomy including all samples, fungi, plants and parasites (which included all taxa identified as vertebrate parasites by BLAST and phylogenetic inferences, see below).

Each split biom file was subject to several analyses. Multiple rarefactions with minimum reads (-m) 100, maximum reads (-x) 10000, and number of repetitions (–n) 10), were run on each biom file and were used to calculate Shannon diversity and t-test differences using the script "compare_alpha.py". A phylogenetic tree of the entire dataset was calculated using a fasta file of all the reads present in each biom file, using the script 'make_phylogeny.py' (default parameters, UPGMA tree), and used as an estimation of difference matrices in downstream analyses. The script "jackknifed_beta.diversity" was used to calculate UniFrac distance matrices, using custom parameters with a rarefaction depth of 2,500 (all), 250 (fungi only) or 100 (plants or parasites only), and the number of rarefactions set to 1,000. This script then generated preliminary UPGMA UniFrac distance-matrix trees, and unweighted UniFrac distances were used to generate PCoA plots using the script "make_2d_plots.py". The script "beta_diversity_through_plots" was used to create unweighted distance matrix files (not jackknifed), used for statistical analyses

("compare_categories" adonis test, and "group_significance" g-test and Kruskal Wallis tests). All statistical tests were run independently on a diversity of categories specified in a mapping file, including coprolites vs. moderns, environment type, host genus, local site, host genus / local site, lower host taxonomy (moa, kiwi, ostrich, kakapo and mammals) and higher host taxonomy (ratites, kakapo and mammals).

S2.1.4 BLASTn and phylogenetic identifications

Despite the usefulness of curated databases such as PR2, reference data in curated databases is largely absent for large diversities of obscure taxa. Although higher-level taxonomic identifications are sufficient for most QIIME analyses, ecologically informative inferences required the deepest taxonomic identification possible. We therefore sought to improve taxon identification with alternative methods and databases. All eukaryotic 18S rRNA reads available were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) into a fasta file, which was converted into a local, custom BLAST database. To help counter genbank's lack of curation, any sequences with the tags "uncultured", "clonal", "environmental" and "unidentified" were filtered from the search terms. The fasta file generated from QIIME representative of all collapsed reads, was then blasted to the custom blast database (blastn, maximum target sequences 25, minimum percent identity match 80%). The output file was opened in MEGAN5 (http://ab.inf.uni-tuebingen.de/software/megan5/), and was collapsed into reliable taxonomic identifications using custom parameters (Min score 150, top 5%, minimum percent support 0.1%, minimum support 5).

In addition, taxa inferred to be of particular importance were subjected to phylogenetic analyses, comprising important moa parasites identified as Ascaridida nematodes (which include groups inclusive and exclusive of highly abundant Heterakoidea nematodes), Echinostomadida trematodes, or Eucoccidorida apicomplexans (either Eimeriidae or Sarcocystidae). All representative OTU sequences for each group were filtered into individual fasta files and aligned using ClustalW [22]. All NCBI 18S rRNA sequences (again using the above filtering parameters) from each taxonomic group were downloaded and assembled to a consensus sequence of each alignment in Geneious v. 7.0.5 [23]. All sequences with <95% alignment match to the OTU alignment were removed, and all remaining reads were aligned using ClustalW. The apicomplexans *Calpytospora funduli* and

Cryptosporidium parvum, the Guinea worm (*Dracunculus mediensis*), and the Common Liver Fluke (*Fasciola hepatica*) were used as outgroups for Eimeriidae, Sarcocystidae, Ascaridida and Echinostomadida respectively. Each full alignment was then subjected to phylogenetic analyses in MrBayes v. 3.2 [24–26] (10,000,000 mcmc generations, with the first 1,000,000 used as a burn-in, gamma-reversible model with a proportion of invariable sites). Trees were visualised in FigTree v. 1.4 (http://tree.bio.ed.ac.uk/software/figtree/).

S.2.2 Figures:

Table S1. Table of site details. Note that coprolite localities in New Zealand are also reviewed in detail in Wood & Wilmshurst [6]. Species codes: L = little bush Moa; G = giant moa, U = upland moa, H = heavy-footed moa, K = kakapo.

Site	Site Type	Species Known	Region	Paleovegetation	Radiocarbon Age Ranges	L	G	U	H	K	References
Dart River Valley (Daley's Flat)	Rock Shelter	Anomalopteryx (bush moa) Dinornis (giant moa) Megalapteryx (upland moa) Pachyornis (heavy-footed moa)	West Otago	Southern Beech Forest	664 ± 25 - 853 ± 25 [16]	1	4	4	2		3,13,16
Hodge Creek Cave System	Cave	Strigops (kakapo)	North West Nelson	Southern Beech Forest	725 ± 43 [2]					1	1,2
Honeycomb Cave System	Cave	Strigops (kakapo)	North West Nelson	Southern Beech Forest	172 ± 20 - 2514 ± 43 (this study) [2]					2	2,9,10
Euphrates Cave	Cave	Megalapteryx (upland moa)	North West Nelson	Subalpine Southern Beech Forest	694 ± 30 - 6368 ± 31 [11]			2			11,12
Shepherd's Creek, Waitaki Valley	Rock Shelter	Megalapteryx (upland moa)	North Otago	Dry shrubland, herbfield	Unknown			2			8
Mount Nicholas (Possum's Rockshelter)	Rock Shelter	Anomalopteryx (little bush moa) Dinornis (giant moa)	Central Otago	Southern Beech / Podocarp-Broadleaf Forest	1440 ± 30 - 1582 ± 34 [5]	2					5
Roxburgh Gorge (Rockshelter B)	Rock Shelter	Pachyornis (heavy-footed moa)	Central Otago	Dry shrubland, herbfield	Unknown				1		3,4,15
Kawarau Gorge	Rock Shelter	Pachyornis (heavy-footed moa)	Central Otago	Dry shrubland, herbfield	Unknown				1		3,4,15

Table S2. Table of all 23 coprolites used in this study.

Specimen	Species	Locality	Carbon Date	Read Count	Host DNA	Microfossils	Content DNA
A10163	*Megalapteryx didinus* (upland moa)	Euphrates Cave		174053	[11]	[3,14]	[11,14]
A10171	*Megalapteryx didinus* (upland moa)	Euphrates Cave		227405	[11]	[11,14]	[11,14]
A10186	*Strigops habroptilus* (kakapo)	Honeycomb Cave	172 ± 30 (this study)	52021	This study		
A10187	*Strigops habroptilus* (kakapo)	Honeycomb Cave	192 ± 30 (this study)	96050	This study		
A10188	*Strigops habroptilus* (kakapo)	Honeycomb Cave	1020 ± 25 [9]	47517	[9]	[9]	
A10197	*Megalapteryx didinus* (alpine moa)	Dart River Valley		193425	[13]	[13,14]	[13,14]
A10200	*Pachyornis elephantopus* (heavy-footed moa)	Dart River Valley		73537	[13]	[13,14]	[13,14]
A10203	*Dinornis robustus* (South Island giant moa)	Dart River Valley		56904	[13]	[13,14]	[13,14]
A10500 (replicate 1)	*Megalapteryx didinus* (upland moa)	Dart River Valley		90853	[13]	[13,14]	[13,14]
A10500 (replicate 2)	*Megalapteryx didinus* (upland moa)	Dart River Valley		133408	[13]	[13,14]	[13,14]
A10501 (replicate 1)	*Anomalopteryx didiformis* (little bush moa)	Dart River Valley	664 ± 25 [16]	138532	[13]	[13,14]	[13,14]
A10501 (replicate 2)	*Anomalopteryx didiformis* (little bush moa)	Dart River Valley	664 ± 25 [16]	139065	[13]	[13,14]	[13,14]
A10504	*Megalapteryx didinus* (alpine moa)	Dart River Valley		227453	[13]	[13,14]	[13,14]
A10508	*Pachyornis elephantopus* (heavy-footed moa)	Dart River Valley		137043	[13]	[13,14]	[13,14]
A10525	*Megalapteryx didinus* (alpine moa)	Dart River Valley	678 ± 25 [16]	257186	[13]	[13,14]	[13,14]
A1135	*Strigops habroptilus* (kakapo)	Hodge Creek		90386	This study		
A13013	*Megalapteryx didinus* (upland moa)	Shepherd's Creek		143787	This study		
A13014	*Megalapteryx didinus* (upland moa)	Shepherd's Creek		100579	This study		
A2062	*Dinornis robustus* (South Island giant moa)	Dart River Valley	721 ± 30 [16]	26102	[3]	[3,14]	[3,14]
A2064	*Dinornis robustus* (South Island giant moa)	Dart River Valley	841 ± 30 [16]	88940	[3]	[3,14]	[3,14]
A2069	*Pachyornis elephantopus* (heavy-footed moa)	Roxburgh Gorge		125775	[3]	[3,14]	[3,14]
A2074	*Pachyornis elephantopus* (heavy-footed moa)	Kawarau Gorge		67524	[3]	[3,14]	[3,14]
A2103	*Dinornis robustus* (South Island giant moa)	Dart River Valley		76220	[3]	[3,14]	[3,14]
A7491	*Anomalopteryx didiformis* (little bush moa)	Mount Nicholas	1440 ± 30 [5]	156679	[5,1]	[5]	
A7493	*Anomalopteryx didiformis* (little bush moa)	Mount Nicholas		139635	[5]	[5]	

Figure S1. Stacked column graph of raw (unfiltered) of total read proportions per sample, to taxonomic and ecological groups as determined through QIIME. Note read counts non-equal. Includes extraction blanks, duplicates, and all modern samples.
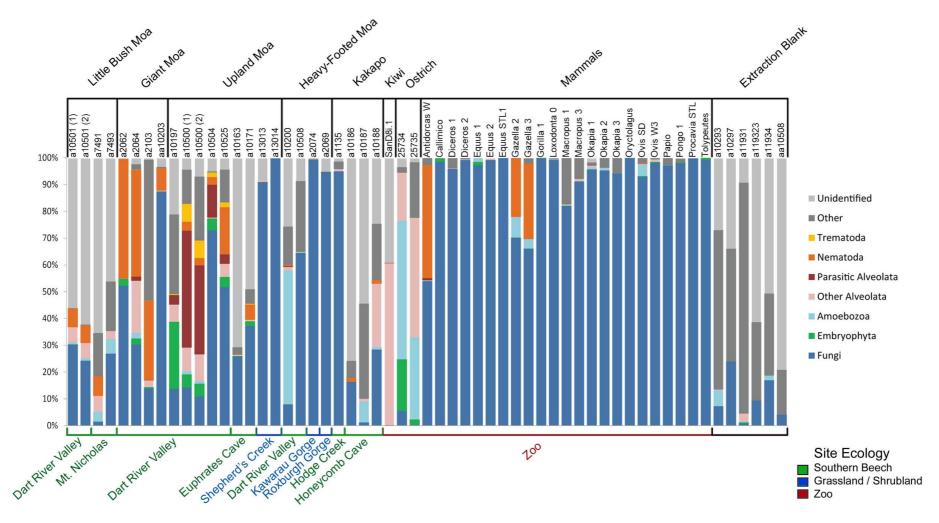
Figure S2. (Above) Stacked column graph of fungal (kingdom: Fungi) proportions post-filtering, using QIIME (PR2 database) and MEGAN (custom database) based taxonomic associations. (Below) bar graph of proportion of total reads determined as fungal by each taxonomic assignment.
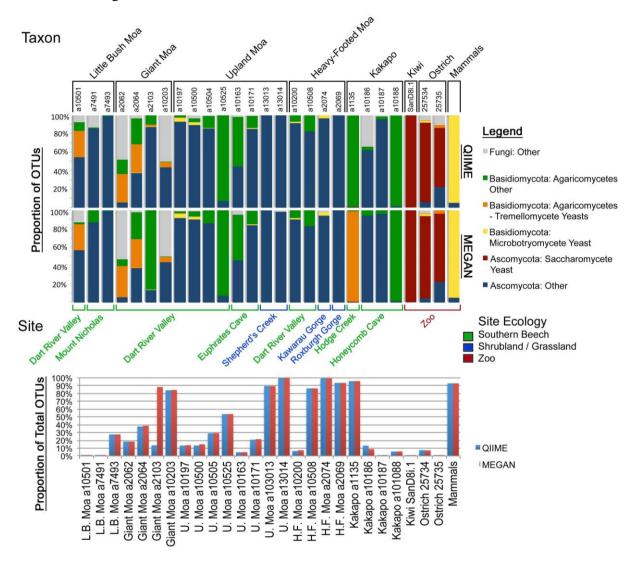
Figure S3. (Above) Stacked column graph of Embryophyta (land plant) proportions post-filtering, using QIIME (PR2 database) and MEGAN (custom database) based taxonomic associations. (Below) bar graph of proportion of total reads determined as Embryophyta by each taxonomic assignment.
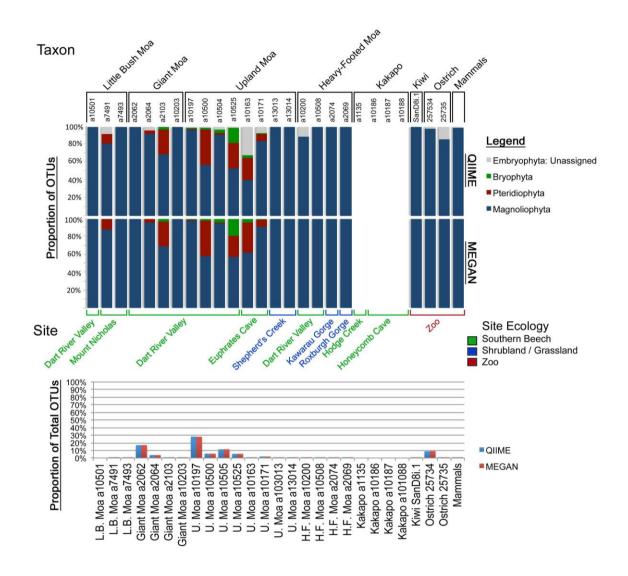
Figure S4. (Above) Stacked column graph of vertebrate parasite proportions post-filtering, using QIIME (PR2 database) and MEGAN (custom database) based taxonomic associations. Total Nematoda counts (for comparisons with Fig. 2, S2) are included. (Below) bar graph of proportion of total reads determined as parasitic / Nematoda by each taxonomic assignment.
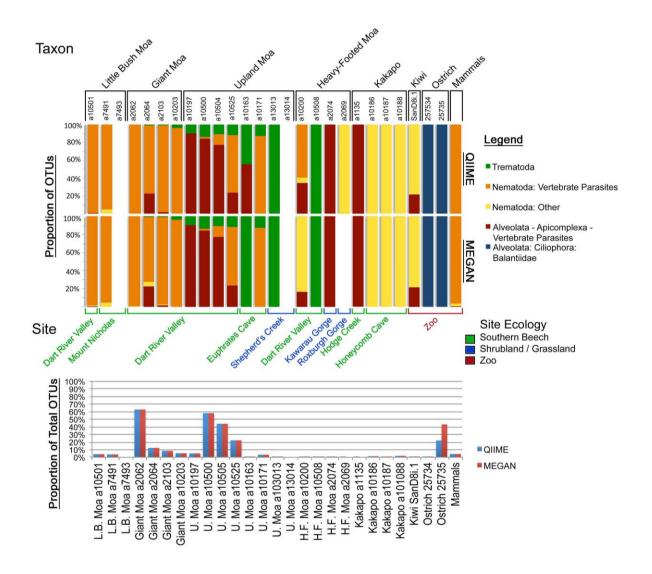
Figure S5*a*. Alpha diversity (Shannon's diversity) box and whisker plots of taxonomic groups.
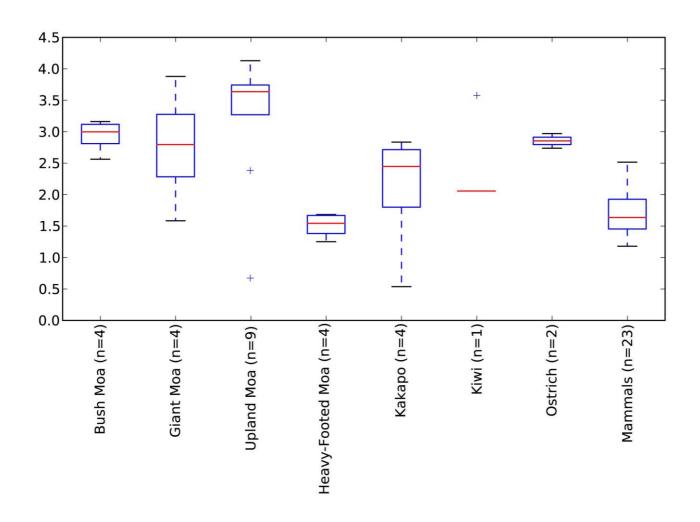
Figure S5*b*. Alpha diversity (Shannon's diversity) box and whisker plots of taxonomic groups.
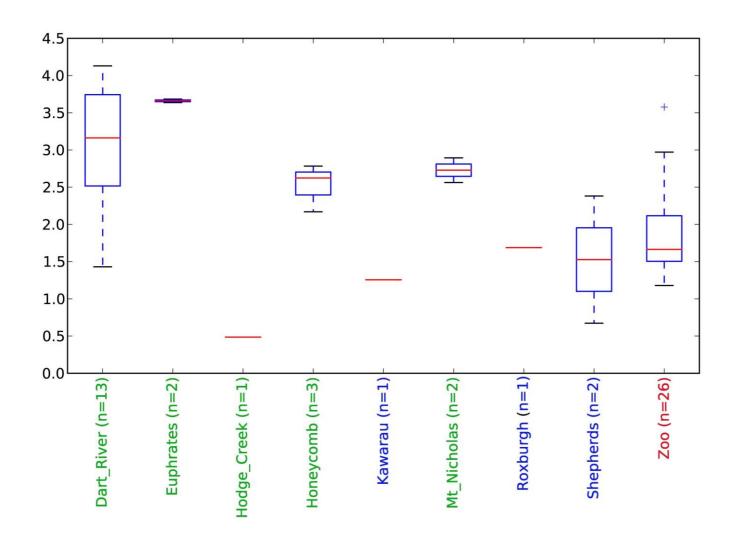
Figure S6. UPGMA tree of all samples based on UniFrac distance matrices.
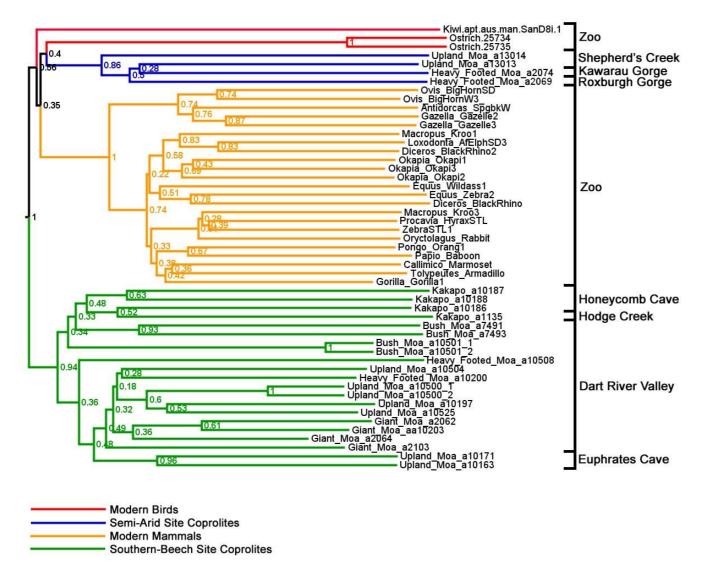
Figure S7a. PcoA plot of total reads of all samples, using weighted UniFrac distances.

Clustered to mammals (all modern mammals), to birds (including modern and ancient bird

samples).
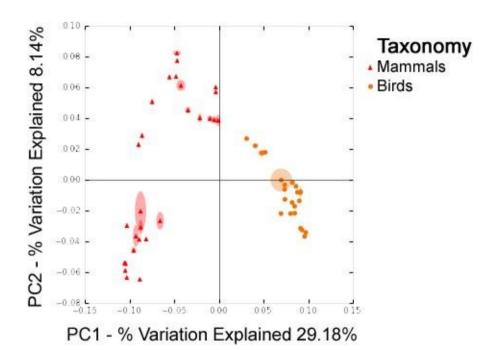
Figure S7*b*. PcoA plot of total filtered reads of bird samples only, using weighted UniFrac distances. Grouped to ecology category, host taxon, and collection site.
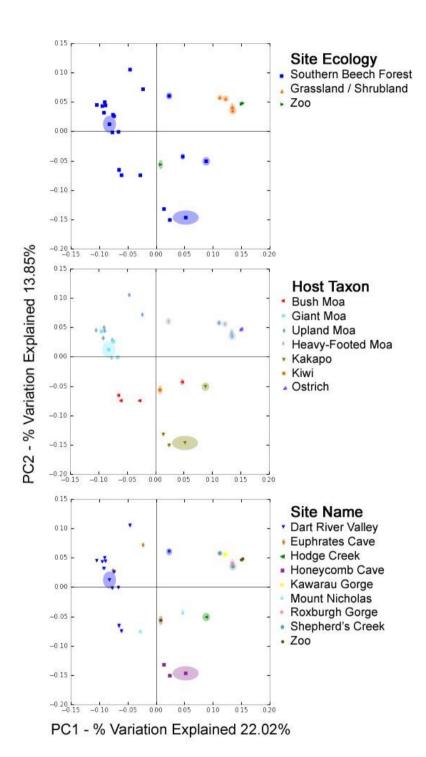
Figure S7*c*. PcoA plot of fungi reads of bird samples only, using weighted UniFrac distances. Grouped to ecology category, host taxon, and collection site.
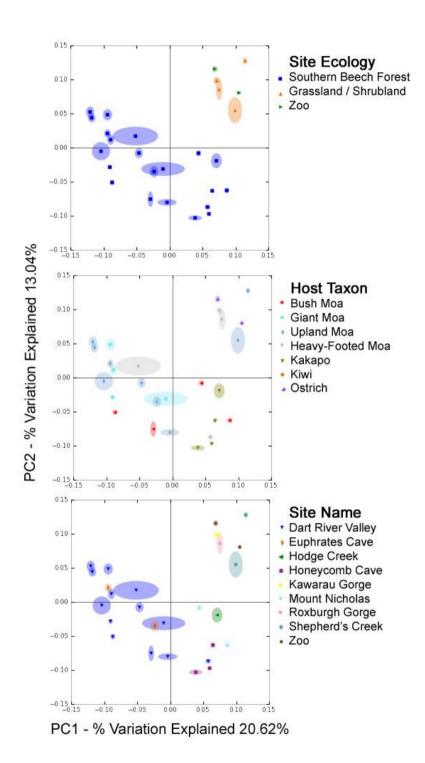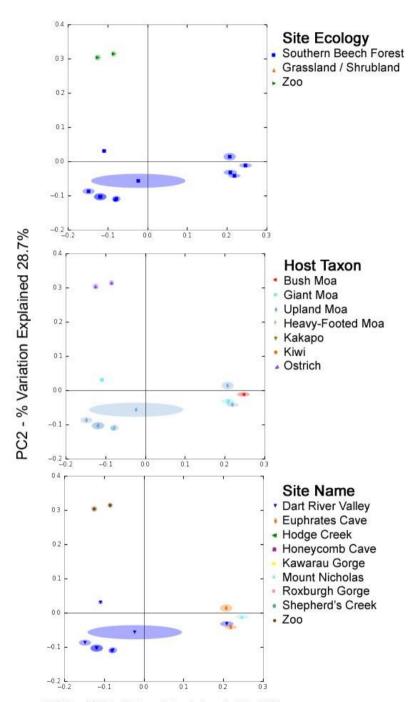
Figure S7*d*. PcoA plot of plant reads of bird samples only, using weighted UniFrac distances.

Grouped to ecology category, host taxon, and collection site.

Figure S7e. *PcoA plot of parasite reads of bird samples only, using weighted UniFrac distances. Grouped to ecology category, host taxon, and collection site.*
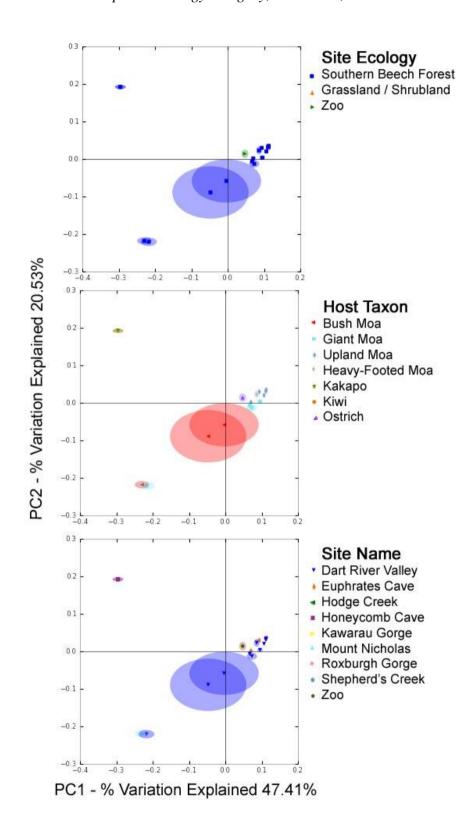
Figure S8. Bayesian phylogenetic tree of Eimeriidae (Apicomplexa: Eimeriorina: family Eimeriidae) sequences found in this study (highlighted red), and included with all available Eimeriidae sequences at this locus (repeat sequences removed). *Calyptospora* (Eimeriorina: Calyptosporidae) used as outgroup. Note all samples avian except OTU "New.Cleanup.ReferenceOTU195652" found from a modern mammalian sample.
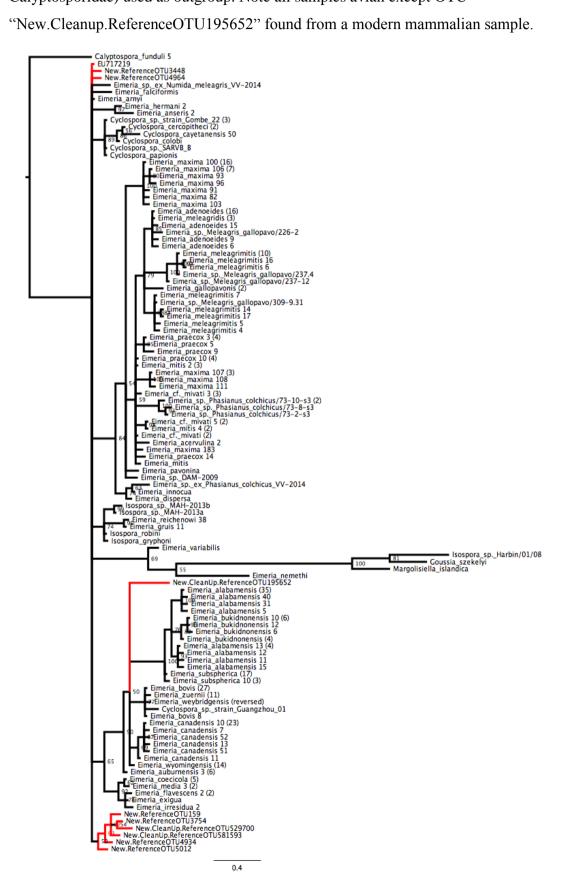
Figure S9. Bayesian phylogenetic tree the single Sarcocystidae (Apicomplexa: Eimeriorina: family Sarcocystidae) sequence found in this study (highlighted red), and included with all available Sarcocystidae sequences at this locus (repeat sequences removed). *Cryptosporidium* (Eimeriorina: Cryptosporidiidae) used as outgroup.
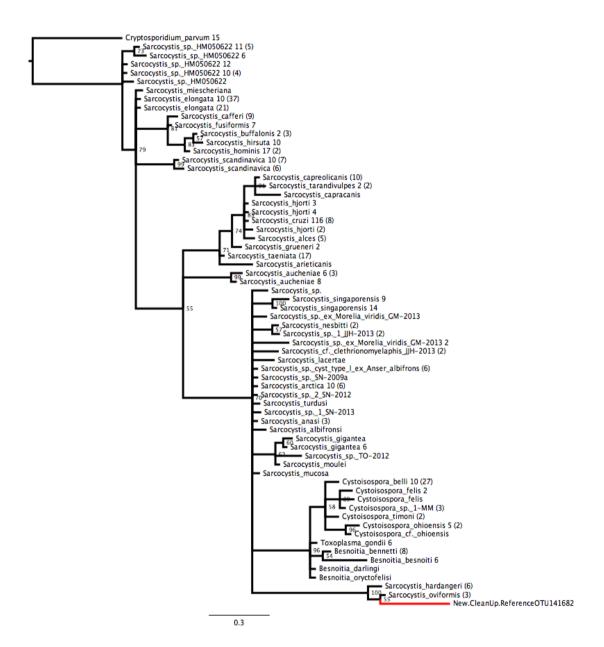
Figure S10. Bayesian phylogenetic tree of all Ascaridida nematodes (Nematoda: Spirurida:
order Ascaridida) sequences found in this study (highlighted red), and included with all
available Ascaridida sequences at this locus (repeat sequences removed). Guinea worm
Dracunculus (Spiriruidae: Camallinida) used as outgroup. Note unidentified Heterakoidea
from moa (superfamily Heterakoidea), comprise most new sequences, clustering with
Ascaridia (family Ascaridiidae) and *Heterakis* (family Heterakiidae). High branch lengths
within moa Heterakoidea support a taxon level of genus-family level or higher.
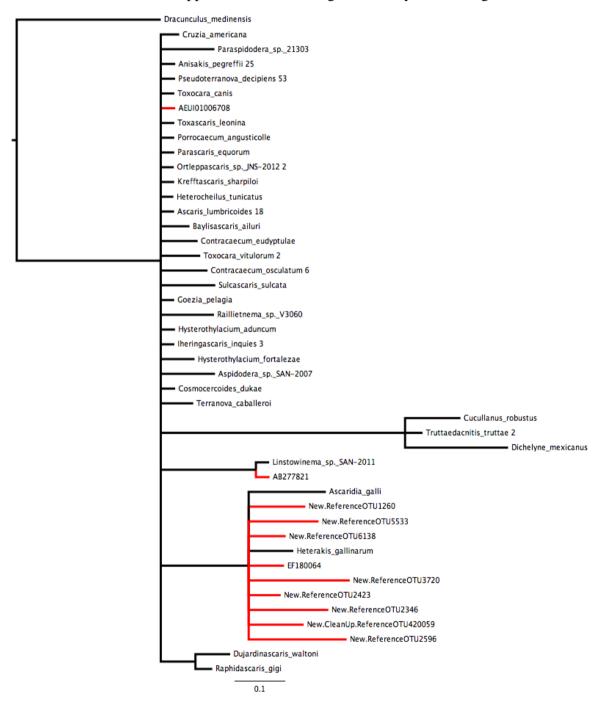
Figure S11. Figure S10: Bayesian phylogenetic tree of the single trematode (Trematoda: Echinostomida: family Notocotylidae) sequence found in this study (highlighted red) from moa, and included with all available Notocotylidae sequences at this locus (repeat sequences removed). Sheep fluke *Fasciola* (Echinostomida: Fasciolidae) used as outgroup. Note no pairwise distance found between the moa trematode, and extant *Notocotylus* and *Catatropis*.
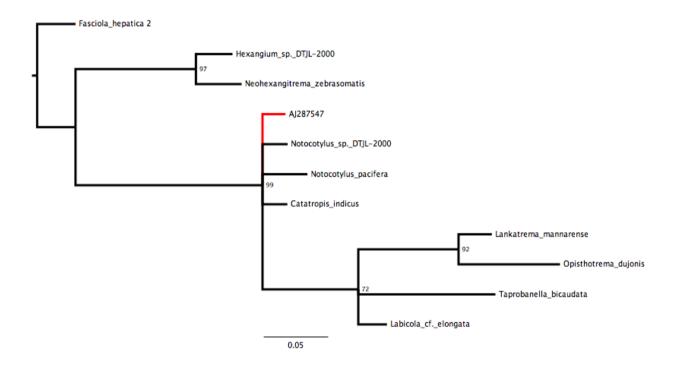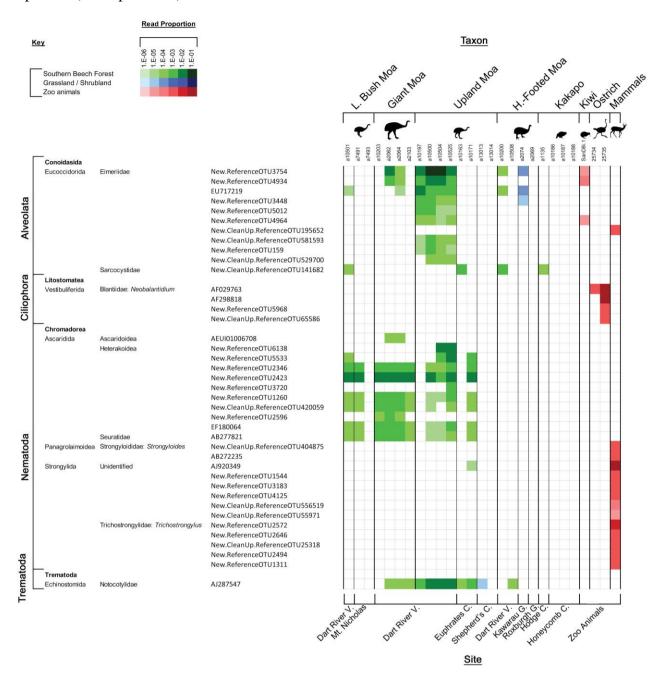
Figure S12. Chart of parasite distributions. Note only includes mammals with parasites present (3 samples total).

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CHAPTER THREE

Shotgun metagenomes of six avian coprolites from New Zealand

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Contribution to the Paper	Received initial processed data (in raw fastq format), performed bioinformatics analyses and taxon identifications. Identified major results, designed and illustrated figures, and wrote paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

   i.   the candidate's stated contribution to the publication is accurate (as detailed above);

   ii.   permission is granted for the candidate in include the publication in the thesis; and

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Name of Co-Author	Dr Kieren Mitchell
Contribution to the Paper	Performed early DNA laboratory work (including DNA amplification, libarary construction and quantification). Peformed DNA enrichment. Processed raw NGS data.
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3.1   Abstract

Obtaining detailed paleoecological information of extinct species or ancient ecosystems is limited by the availability of appropriate fossil material. Ancient DNA (aDNA) is providing increasingly high-resolution insights into past ecosystems; however most aDNA studies have utilised Sanger-sequencing and PCR methods constrained by DNA fragment length, and are especially prone to amplification bias of modern contaminants. As a result, extremely degraded specimens have been highly problematic in most previous study. Late-Holocene age coprolites (paleofaeces) have been recovered from throughout New Zealand's South Island, confirmed or believed to originate from different species of recently extirpated birds, including the megaherbivore ratite moa. These in turn provide uniquely detailed information on ecology, diet, parasites and interspecific interactions of the ancient depositing species. However, some of the largest and richest coprolites deposits found in semi-arid localities have been resistant to DNA amplification. In this study we sequence the full metagenomes of six coprolites from New Zealand using NGS shotgun data. These include two well-preserved specimens from Dart River valley (already used in successful DNA studies), and four from semi-arid localities (not previously producing amplifiable DNA). While our results return informative data for the Dart River specimens (including identification of dietary plants and fungi, parasites and methane-producing Archaea), metagenomes of the remaining four lack identifiable endogenous content, despite use of DNA-RNA hybridisation to enrich for host DNA. We conclude that problematic coprolites from these areas are too degraded to return DNA, or alternatively, unidentified organic compounds such as humic acids or maillard products are inhibiting DNA extraction. Future approaches will therefore require identification of potential inhibitors, and experimentation of a variety of DNA extraction procedures.

3.2   Introduction

New Zealand's avifauna once comprised a wide ecological and taxonomic variety of bird species, many of which are now extinct or extremely range reduced [1–3].

Among these were the large (up to 250kg) extinct, flightless, ratite moa, of which a total of nine species are now known [4]. These species varied considerably in morphology, size and diet, and were found in a variety of habitats throughout New Zealand [3,5–10]. Apart from moa, there were several other more poorly understood extinct birds, some similarly massive and flightless such as the flightless geese *Cnemiornis* spp. (15-18kg) or the adzebills *Aptornis* spp. (16-25kg), as well as numerous other smaller species [1–3]. Many became extinct in prehistory ~200 years following the ~1300 AD arrival of Maori (including all endemic land birds >5kg), or more recently following European settlement during the 18th and 19th centuries [1–3,11]. Understanding how this guild of birds interacted with other organisms and their diseases, and how they behaved, depends heavily on paleobiological data. This in turn often requires utilising specialist and advanced methods on limited materials.

Coprolites with high organic content (mid-late Holocene in age) from native bird species have been identified throughout New Zealand's South Island, and have been analysed by ancient DNA and physical fossil content [5,6,8–10,12–17]. So far coprolites have been identified by aDNA as originating from one of five moa species (little bush moa *Anomalopteryx didiformis*, heavy-footed moa *Pachyornis elephantopus*, South Island giant moa *Dinornis robustus*, coastal moa *Euryapteryx curtus*, upland moa *Megalapteryx didinus*), or the critically endangered kakapo parrot (*Strigops habroptilus*) [13]. However, no coprolites have been confirmed as originating from other bird taxa, including the remaining four moa species (eastern moa *Emeus crassus*, crested moa *Pachyornis australis*, mantell's moa *Pachyornis geranoides*, North Island Giant moa *Dinornis novaezelandiae*). Nonetheless, considerable numbers of coprolites remain unidentified, despite repeated attempts to sequence their DNA [13].

There is a strong geographical association to the success rate of DNA recovery from New Zealand bird coprolites. Coprolites recovered from relatively cooler areas (many of which are caves) in the western South Island have returned comparatively high quality DNA sequence data [5,9,10,12,13,17]. These sites, paleoecologically, were generally associated with dense, Southern beech forest. However, rich deposits found under rock-shelters from 'semi-arid' areas in west-central Otago (South Central South Island), east of the Alps have been found. Due to a rain shadow by the Alps, these areas are characterized by low rainfall with vegetation communities composed of podocarp-broadleaf trees, shrubland, herbfields and grassland [18–22]. However,

coprolite DNA recovery (when successful) from these areas tends to be relatively poor in quality, likely due to thermal effects on DNA preservation. Even some entire deposits have failed to return amplifiable DNA from any sample. Of these 'semi-arid' sites (especially "Sawers' rockshelter", Roxburgh Gorge, which currently lacks aDNA data), diverse assortments of coprolites have been found [13]. Importantly, many represent 'morphotypes' not identified to host elsewhere.

Several extinct bird species favoured the relatively open habitats found around central Otago (or similar habitats) and were rare or absent from southern-beech forest. Examples included the eastern moa, coastal moa, South Island goose (*C. calcitrans*), Haast's eagle (*Aquila moorei*) and the New Zealand quail (*Coturnix novaezelandiae*) [3]. Coprolites of these species may have been preserved and collected, but have not yet been positively identified. Furthermore, additional data from relatively well-studied species (such as South Island giant moa) would provide valuable information on how the ecology of such species varied both geographically and temporally. Finally, data from coprolites in these areas would provide vital information on the environments of these areas, especially as they have long been deforested or modified by human activity [18,21,22]. Overall, resolvable information from coprolites from 'semi-arid' areas in New Zealand's South Island would provide essential paleoecological information.

So far all attempted DNA sequencing efforts have used taxon-specific primers, either for host DNA, or for ecologically informative species represented in the coprolite matrix (such as dietary plants or parasites) [6,8–10,12,17]. Damaged DNA tends to be both low in quantity and highly fragmented, limiting the success of primers requiring specific target sites or length for successful amplification [23,24]. An alternative technique is NGS (next-generation sequencing) based 'shotgun sequencing' where DNA fragments are amplified using ligating adapters, thus allowing for a relatively unbiased range of DNA to be sequenced (the full 'metagenome') [25]. Most importantly, this method is not dependent on a minimum fragment length for successful amplification.

In this study we use a shotgun sequencing technique to amplify the metagenomes from six coprolites, four of which originate from 'semi-arid' sites in central Otago and have never yet had DNA content sequenced. This may be noteworthy as currently the only shotgun data of animal coprolites are from the European cave hyena (but these were not used for a metagenomic analysis) [26]. The

results of this study will help confirm the nature of DNA content from central-Otago coprolites, and facilitate a solution for future studies.

3.3   Methods

3.3.1   *Materials*

Six coprolites from four different localities were used in this study (Table 1, Fig 1). The first locality was the Dart River Valley (far-west Otago), which receives >1.5m mean annual rainfall [27]. Coprolites here were recovered from sediment beneath a rock overhang surrounded by mature Southern Beech forest (primarily silver beech *Lophozonia menziesii*), believed representative of the natural vegetation type of the area [10,28]. The other three sites represent semi-arid localities receiving <400mm mean annual rainfall: Roxburgh Gorge (rockshelter 'B'), Roxburgh Gorge (Sawers' rockshelter) and Kawarau Gorge. Today the modern vegetation of these areas is heavily modified, but is believed to have originally been woody shrubland (comprising small podocarps, and woody angiosperms such as *Corokia*, *Coprosma* and *Olearia*) [21,22]. Of these sites, coprolites from Roxburgh Gorge 'B' and Kawarau gorge have been successfully identified using aDNA as heavy-footed moa [6,10,12]. Coprolites from Sawers' rockshelter comprise a diversity of morphotypes (>1,000 samples), although none have had aDNA amplified [10,13,22,29].

Two coprolites from Dart River valley have been identified as upland moa (2105) and heavy-footed moa (2108), and have had coprolite taxa examined using DNA cloning and microfossil analysis in previous studies [5,10,12]. Both have been radiocarbon dated as 719 ($\pm$ 30) and 742 ($\pm$ 30) years old respectively [13]. Coprolites from Roxburgh Gorge 'B' (7526) and Kawarau Gorge (7527) have not been identified by aDNA, but may originate from heavy-footed moa (all coprolites sequenced from these sites have been assigned to this species) [6,10]. The remaining two coprolites from Sawers' rockshelter are similarly unidentified, but are not inferred to be moa. This includes a coprolite (a7550) hypothesised to originate from a waterfowl (such as the extinct Finsch's duck *Chenonetta finschi*), and a scat (7553) inferred to originate from the recently extinct (since the early 20th C.) laughing owl (*Scleoglaux albifacies*).

3.2.2 *Extraction and amplification*

Extraction methods are the same as that used in Chapter Two, two of which (both Dart River samples) were used in past studies [5]. To limit amplification bias DNA extracts for all six samples were partitioned into eight separate working reactions, later pooled for the final PCR and enrichment step (see below). Extracts were blunt-ended and repaired, and custom adapters were ligated using the protocol of Meyer and Kircher [30]. The 5' end contained a unique index, allowing both for identification of amplified DNA and downstream contaminants. Amplification of DNA was performed by three series of short PCR amplifications using specific primers complementary to the adapter sequences. Each PCR reaction contained 1 x PCR buffer, 2.5 mM MgcL2, 1mM dNTPs, 0.5 mM primer, 1.25 U AmpliTaq Gold, 2 µL DNA extract. PCR cycles followed 94 C 12 min; 12/13cycles (for 1st/2-3rd amplifications respectively,) of 94 C for 30s, 60 C for 30 s, 72 C for 40 s (plus an additional 2 s per cycle); with 72 C for 10 min. Products were purified using AMPure magnetic beads (Agencourt).

In addition, libraries were also enriched for host DNA using commercially synthesised biotinylated 80 mer RNA baits (MYcroarray, MI, USA). Baits were designed using a wide range of published whole mitochondrial genome sequences (not including control region / D-loop) of a variety of avian taxa, including paleognaths, galloanseres and neoavians. DNA-RNA hybridisation enrichment was performed using manufacturers recommended concentrations of 5.2 x SSPE, 5.2 x Denhardt's, 5mM EDTA and 0.1% SDS. This was incubated at 55 C for 36 hr with 200ng of library. Baits were then immobilised on magnetic MyOne Streptavidin Beads (Life Technologies). The baits were washed once with 1 x SCC and 0.1% SDS (15 min room temperature), and twice with 0.1 x SCC and 0.1% SDS 10 min at 50 C), then resuspended in 0.1 M NaOH pH 13.0 which destroyed the RNA baits and released captured DNA. The resulting DNA solution was purified using a Qiagen Minelute spin-column, and subjected to yet another round of PCR (12 cycles, using above protocol).

Both 200ng of enriched and non-enriched library were followed by a final round of PCR (7 cycles, using above protocol), using fusion primers to ligate full-length Illumina sequencing adapters to each library for sequencing. Libraries were

diluted to 2M (quantified using qPCR), and run on a single lane of an Illumina MiSeq using 2 x 150 (paired-end) sequencing procedures.

3.2.3   Analysis and identification

Sequenced reads were demultiplexed using the 5' adapter barcode using 'sabre' (https://github.com/najoshi/sabre) (default parameters: no mismatches allowed). In addition, five base pairs were removed from the 3' reverse read end using fastx_trimmer (FASTX-toolkit v0.0.13; http:hannonlab.cshl.edu/fastx_toolkit) to ensure forward and reverse reads were of equal length for merging. Adapter sequences were removed, and paired reads merged using Adapter Removal v1.5.4 [31]. Low quality bases were trimmed using (<Phred20 --minquality 4) and merged reads shorter than 25 bp were discarded (--midlength 25).

Merged fastq files (non-enriched libraries only) were then subjected to BLASTn searches on a local database comprising the entire NCBI nucleotide (nt) database (http://www.ncbi.nlm.nih.gov/genbank/) downloaded as of September 2014. Blast xml files were loaded into MEGAN5 V 5.10.3 (http://ab.inf.uni-tuebingen.de/software/megan5/) for filtering using custom parameters (minimum (bit) score 150, max-expected hits 0.01, top percent 10%). Taxon assignments from MEGAN5 were then used to determine overall composition of the containing data, and high-resolution identifications of specific containing taxa. As the primary focus was to determine the nature of any DNA sequenced from all samples, read counts per taxon were not taken into account in this analysis and putative contaminant taxa were not filtered. Short reads were also not filtered, although bit score parameters were believed sufficient to prevent spurious taxon identifications due to short read lengths (e.g. reads <50 bp).

3.3.4   Host DNA identification

Aside from BLAST matches (to non-enriched data only), both enriched and non-enriched library reads were assembled to a range of reference bird mitochondrial genomes using geneious mapper v 7.0.5 [32] (using inbuilt "medium" sensitivity

parameters). These include complete mitochondrial genomes for three moa species, as well as the morepork owl (*Ninox novaeseelandiae*) and common shelduck (*Tadorna tadorna*); as well as assembled mitochondrial genomes for the extinct South Island Adzebill (*Aptornis otidiformis*) and South Island goose (*Cnemiornis calcitrans*) (unpublished data).

3.4   Results

3.4.1   *Sequencing depth and read proportions*

All sequenced (post-collapsing) libraries returned 66,180-237,036 reads each with read lengths ranging from 25-277bp (see Table 1, Fig. 2). Read length distributions varied considerably between libraries (Fig. 2), with both Dart River valley samples demonstrating a bias towards shorter read lengths (peak 50-75 bp) than other samples, potentially representing a higher endogenous (and thus fragmented aDNA content). The sample Sawers' 7550 'Duck' showed a similar distribution, although less pronounced. Remaining samples showed bias towards longer read lengths; with the sample Sawers' 7553 'Owl' demonstrating an almost completely uniform read distribution (e.g. read counts at 25bp were similar to those at 277bp).

The vast majority of reads were not identified; either returning no hits ('no hits'), or alternatively failing to match to any specific taxon ('not assigned') (See Fig. 3). In total only 2.02-4.26% or reads were assigned to a taxonomic group in MEGAN5, demonstrating a considerable decrease in efficiency compared to metabarcoding methods (refer to Chapter Two). Of groups assigned, most reads were inferred to be prokaryotic although proportions varied. Furthermore, prokaryotes for most samples were unanimously bacteria - generally common aerobes such as the Actinobacteria *Mycobacterium* and *Streptomyces,* and the proteobacterium *Cellvibrio* (also widespread in all samples). However, in both Dart River valley samples, archaea were prevalent and even dominant in sample 2108 (heavy-footed moa), entirely comprised of Methanobacteriaceae (*Methanobrevibacter* and *Methanosphaera*). Eukaryotic reads were mostly Metazoa, although plant reads overwhelmingly (and

uniquely) dominated in Dart River samples (See Fig. 4). Fungi formed a third important group, with few other eukaryotes being identified.

3.4.2 Host DNA content

Blast searches and assemblies to reference moa mitochondrial genomes, found that only a single sequence from the upland moa coprolite (2105) represented moa DNA. This was a strand of cytochrome B, weakly assigned by MEGAN as originating from the eastern moa *Emeus crassus* (there is not currently a complete mitochondrial genome available for upland moa) (See Table 2). Enrichment improved the sequenced proportion of host DNA. A total of 60 and 44 reads from the Dart-River upland and heavy-footed moa samples respectively, successfully assembled to reference moa mitochondrial genomes. Of the semi-arid samples only two short reads (44bp) from the duck sample (sawers' 7550) were assembled, which aligned to all reference genomes used. BLAST searches found identity matches (<94%) to a wide range of vertebrates including mammals, demonstrating that these sequences are unlikely to represent captured host DNA. As a result enrichment did not appear to improve capture of endogenous host DNA from the four semi-arid samples.

3.4.3 Eukaryotic taxon assignments

Gut-bacteria from birds remain poorly studied [33], and bacterial aDNA study is especially vulnerable to contamination [24]. As a result, we elected to focus on eukaryotic sequences. Of the eukaryotic reads assigned, most with robust assignments (i.e. to plausible taxa, or hits to a range of related species) were either referenced to organelle (mitochondrial or chloroplast) sequences, or to common barcoding nuclear loci such as 18S and ITS (internal transcribed spacer), likely due to a wider availability of sequences from these loci. Many remaining sequences hypothesised to originate from obscure nuclear loci were generally assigned spurious assignments. For example, large numbers of reads in most samples (especially in semi-arid samples) were matched to a single sequence of the marine echinoderm *Lytechinus variegatus*

(5S ribosomal RNA), with a strong support (e.g. 180/181 pairwise match, e-value 5.1e-87), which were discarded.

Final taxon assignments demonstrated considerable differences between semi-arid and Dart River samples (see Table 2). Semi-arid samples were generally entirely comprised of few protozoans, mould-type fungi, and typical contaminating mammals (e.g. domestic dog, pig, cattle or human). Few plant reads were generally assigned to the Poaceae (grasses) or Rosaceae. Although native species within these families are found in Central Otago, it is also likely they originate from environmentally common taxa (for example cultivated genera such as Roses, the stone-fruit genus *Prunus*, or strawberries). A sequence of the blood-borne apicomplexan parasite *Babesia* was identified from the Kawarau Gorge 7526 'Moa' sample, although may represent a contaminant. Overall, semi-arid samples contained no identified taxa considered to be endogenous.

Dart River samples (2105 upland moa, and 2108 heavy-footed moa) however contained a wide diversity of eukaryotes, closely correlating with findings from past studies (including Chapter Two). This predominantly comprised a variety of plant reads (Table 2). Compared to 18s metabarcoding data (Chapter Two), many could be identified to genera. Identified taxa included a variety of ferns (*Asplenium* and *Ophioglossum*), aquatic plants (*Myriophyllum*), shrubs (*Coriaria, Muehlenbeckia, Gaultheria, Gonocarpus*), herbs (*Ranunculus*, *Rumex*, *Senecio*, *Viola*), and sedges (*Cyperus*). Differences between the samples were present, potentially representing dietary differences between the two species. For example only heavy-footed moa contained sedge DNA (both contained general grasses however, which could be contaminant). This sample also contained macrofungal DNA, assigned to Geastraceae (earthstars) and Gomphales (matches representing the closely related Gomphaceae, Lentariaceae or Clavariadelphaceae [34]).

Finally, parasites were identified from the Dart River coprolites. These included a trematode sequence identified specifically as an unidentified *Notocotylus*, found in the upland moa coprolite. Furthermore, a *Leishmania* (Trypanosomatidae) sequence was identified from the heavy-footed moa coprolite. However the prevalence of leishmaniasis in birds is poorly studied, and not known to naturally occur in New Zealand, so it is unclear whether this is a valid result (i.e. contaminating or a misidentification). Importantly however, no sequences were assigned to members of the Eimeriidae or to any parasitic nematodes, known to be abundant in Dart River

moa coprolite from past studies (including Chapter Two). As a result, it is likely that the relatively low number of identifiable reads represented insufficient read depth to obtain the full coprolite diversity.

3.5   Discussion

3.5.1   Endogenous DNA content

Shotgun sequencing in this study was demonstrated as an effective measure of the overall metagenome, obtaining detailed information on Dart River Valley samples that firmly correlate with past studies [5,12]. However, no discernible endogenous DNA could be recovered from any of the four coprolites from semi-arid sites. Read lengths also supported that sequenced DNA from semi-arid samples generally had modern origins (as opposed to being endogenous, but unidentifiable due to damage and/or fragmentation). All identified taxa from semi-arid samples also tended to comprise typical contaminating types (such as humans, domestic species, and ubiquitous common fungi and bacteria) [23,24]. Finally, and importantly, secondary enrichment for host DNA was also ineffective for the semi-arid samples (although greatly increased the amount of host DNA in Dart River Valley samples). Overall, DNA extracted from these samples using conventional approaches likely contains little (if any) amplifiable, endogenous genetic material.

3.5.2   Ecological inferences

As a counter-measure, the semi-arid coprolites therefore served as a partial control for endogenous content in the Dart River coprolites. For example Basidiomycota fungi and plants were extremely rare or absent from the semi-arid samples. Furthermore, this may also suggest that Archaea uniquely found in Dart River samples may also be endogenous. These Archaea were identified as forms of Methanobacteriaceae (*Methanobrevibacter* and *Methanosphaera*), which are methane-releasing (methanogens) common in animal guts, and play an important role

in digesting fermentation products of polysaccharides (polymeric carbohydrates) [35,36]. Both *Methanobrevibacter* and *Methanosphaera* have previously been identified in birds, including chickens and the folivorous hoatzin [35,37]. However, they are apparently absent in the kakapo parrot, the largest surviving flightless herbivorous bird in New Zealand [38,39]. Further investigation may be needed to identify whether these microbes are endogenous, whether they demonstrate hindgut fermentation in moa, and their overall prevalence in other ratite species.

Eukaryotic taxa correlated strongly with past studies on these coprolites [5,12,29], including findings of an unidentified *Notocotylus* being abundant in upland moa. However, very few new examples of dietary taxa were found. Examples included some new fungal taxa not identified in the metabarcoding study (several putative species of Gomphales). These may be dietary as these fungi ("coral fungi"), are often brightly coloured, widespread in New Zealand and edible; although are saprobic (and thus potentially coprophilous) [40,41]. Plant species generally represented taxa found in past DNA and pollen studies (*Coriaria*, *Gaultheria*, *Gonocarpus*, *Muehlenbeckia*, *Myriophyllum, Ranunculus*, *Viola*), representing a variety of herbs and woody species [3,5–10]. Other new findings included DNA of specific ferns (improving on 18s data), including *Asplenium* and *Ophioglossum* (from heavy-footed and upland moa respectively). Furthermore, a wide variety of low, small lying herbs and fungi were found only in upland moa (e.g. *Ophioglossum*, *Viola*, *Senecio*), supporting past inferences that this species foraged much closer to the ground than the larger heavy-footed moa. Overall, while there were intriguing insights, the sample size was too small to develop robust conclusions.

3.5.3 *Comparison between Metabarcoding / Shotgun datasets*

Compared to the 18S metabarcoding study of moa coprolites, shotgun methods demonstrated a considerable drop in efficiency. Only a very low proportion of reads could be identified (<5%). As a result, much greater read depths would be needed to capture the abundances and diversity obtained using metabarcoding. For example, despite similar read depths between the shotgun and metabarcoding studies (~100-200k reads), shotgun methods failed to capture identifiable sequences of some

of the most ecologically significant and abundant taxa as identified using metabarcoding (such as apicomplexan and nematode parasites).

Nonetheless, lack of sequencing bias to a specific locus allowed for a broader diversity of taxa to be identified. Firstly, a) other well-represented loci could be utilised, for example plastid DNA or internal transcribed spacer (ITS). For example, plants could be identified to genus level, not currently possible using 18S alone. Secondly, b) DNA fragments could be sequenced in relative completion. For example, a longer amplicon of 18S was sequenced from the moa trematode. Finally, c) sequencing targets are not restricted to rare, short (as required by aDNA), variable loci bound by conserved regions for primer binding (such as 18S short subunit). This is especially useful for loci such as ITS (used as a barcode for fungi), where variable regions are >200bp and thus short fragments are difficult to amplify across a diversity of species [42]. Despite these advantages however, shotgun sequencing did not appear to outperform metabarcoding or conventional PCR, concerning especially damaged coprolite samples.

Database improvements and use of a variety of barcoding loci will increasingly nullify shotgun's advantage in point a). However, the latter two points represent important uses for shotgun data. As a result, while metabarcoding represents a far better method in terms of cost and efficiency, shotgun data does represent some useful advantages compared to metabarcoding. Improvements in techniques such as enrichment (for example to plant DNA only) may greatly improve efficiency towards target species, although this remains relatively untested and will be both costly and time-consuming. At this current stage, metabarcoding studies should be sufficient for most future projects on materials such as coprolites. However, supplementary shotgun sequencing will be useful in providing both initial 'snapshot' analyses allowing for targeted metabarcoding, as well as sequencing of taxa or loci difficult to obtain using barcoded primers.

3.5.4   DNA preservation in coprolites and future directions

Ultimately, this study suggests that 'semi-arid' coprolites failing to return amplifiable DNA using conventional PCR efforts are unlikely to contain identifiable endogenous DNA. Overall, why coprolites from eastern 'semi-arid' localities

apparently do not preserve DNA compared to western 'wet' localities needs to be considered. Radiocarbon dates have demonstrated that coprolite ages differ little in age between the regions, thus DNA recovery cannot be due to temporal differences alone [10]. Semi-arid environments around central Otago are both relatively exposed and the region experiences hot temperatures during summer months [27]. Furthermore, central Otago experiences the greatest seasonal temperature extremes in New Zealand. Therefore heat damage, and a fluctuating temperature regime should greatly increase DNA damage [23,24,43]. Overall, it is conceivable that the cooler, stable microclimates found in 'wet' coprolite sites may be vital for any long-term DNA preservation for degradable organic materials such as coprolites.

However, it is also plausible local chemistry or molecular reactions may be affecting DNA extractions. For example DNA extraction and sequencing from environmental samples are often affected by the presence of humic acids, which can both bind to DNA and inhibit PCR reactions [44-46]. Humics represent a variety of compounds generated by biological decomposition, and may be more prevalent in semi-arid samples due to local soil chemistry or breakdown of the coprolites. Furthermore, the effects on heat could produce Maillard reactions ('browning'), binding sugars (such as plant cellulose) and amino acids into polymeric melanoidin products, previously detected in sloth coprolites [47]. These in turn may be preventing DNA release during extraction steps. Extraction methodologies such as incorporation of a Maillard reversant such as PTB (N-phenacylthiazolium bromide) may dramatically alter DNA recovery [47,48].

Overall, this study confirms that current extracts do not contain any readily obtainable endogenous DNA content. This may frustrate the use of these coprolites in future study, as although microfossil content can be analysed, this information is of limited use if at least the depositing taxa cannot be identified. Future steps to reattempt DNA recovery would be to alter methodological approaches in early phases of laboratory work. Assessment of soil and coprolite chemistry should be assessed and compared with rates of DNA recovery, and a wide variety of extraction and DNA sequencing procedures should be attempted. The large coprolite deposits from near Central Otago represent an enormous and still relatively untapped source of paleoecological information on ancient New Zealand. Refining a method allowing for widespread successful DNA amplification of these samples would be an exciting and important discovery.

3.6   Figures

Table 1. Chart of samples used in this study (ACAD, Australian Centre for Ancient DNA). * Represents estimated coprolite age (less than 4,000 years).

ACAD #	Locality	Region	Species	DNA Data	Envrionment	Age (years)
2105	Dart River Valley	West Otago	Upland moa	Yes	Wet / Cool	719 ± 30
2108	Dart River Valley	West Otago	Heavy-footed moa	Yes	Wet / Cool	742 ± 30
7526	Kawarau Gorge	Central Otago	Heavy-footed moa?	No	Semi-Arid	<4,000*
7527	Roxburgh Gorge, Rockshelter B	Central Otago	Heavy-footed moa?	No	Semi-Arid	<4,000*
7550	Roxburgh Gorge, Sawer's Rockshelter	Central Otago	Duck?	No	Semi-Arid	<4,000*
7553	Roxburgh Gorge, Sawer's Rockshelter	Central Otago	Owl?	No	Semi-Arid	<4,000*

Table 2. Chart of taxa identifiable to family level, or selected taxa identified to order level or higher. Blue squares designate likely endogenous taxa; Red squares designate likely contaminant taxa. Coprolite designations as follows: 1) Dart River 2105 Upland Moa, 2) Dart River 2108 Heavy-footed moa, 3) Kawarau Gorge 7526 'Moa', 4) Roxburgh B 7527 'Moa', 5) Sawer's 7550 'Duck', 6) Sawer's 7553 'Owl'. * taxon identified as Emeiidae due to lack of appropriate host reference sequence (Megalapterygidae).

Taxon	Order	Family / Genus	Description	Native?	1	2	3	4	5	6
Flabellinea	Thecamoebida	Thecamoebidae	Free-living amoebas	Y					B	
Apicomplexa	Piroplasmidae	Babesiidae: Babesia	Blood parasite	N				R		
Apicomplexa	Unidentified	Unidentified	Invertebrate parasite?	Y					R	
Oomycota	Saprolegniales	*Aphanomyces*	Water Mold	Y	R					
Magnoliophyta	Apiales	Araliaceae	Ivy family	Y		B				
Magnoliophyta	Asterales	Asteraceae	Daisy family	Y	B					
Magnoliophyta	Asterales	Asteraceae: *Senecio*	Ragworts and Groundsels	Y	B					
Magnoliophyta	Caryophyllales	Caryophyllaceae	Carnation family	Y	B					
Magnoliophyta	Caryophyllales	Polygonaceae	Knotweed family	Y	B					
Magnoliophyta	Caryophyllales	Polygonaceae: *Fagopyrum*	Buckwheats	N		R				
Magnoliophyta	Caryophyllales	Polygonaceae: *Muehlenbeckia*	Maidenhairs	Y	B					
Magnoliophyta	Caryophyllales	Polygonaceae: *Rumex*	Docks and Sorrels	Y	B					
Magnoliophyta	Caryophyllales	Unidentified	Herbs	Y	B					
Magnoliophyta	Curcurbitales	Coriariaceae: *Coriaria*	Shrubs / small trees	Y	B					
Magnoliophyta	Ericales	Ericaceae	Heather family	Y	B					
Magnoliophyta	Ericales	Ericaceae: *Gaultheria*	Shrubs	Y	B					
Magnoliophyta	Malphigiales	Euphorbiaceae	Spurge family	Y	B					
Magnoliophyta	Malphigiales	Violaceae: *Viola*	Pansy genus	Y	B					
Magnoliophyta	Myrtales	Onagraceae	Willowherb	Y	B					
Magnoliophyta	Poales	Cyperaceae: *Cyperus*	Sedges	Y	B					
Magnoliophyta	Poales	Poaceae	Grasses	Y	R	R			R	
Magnoliophyta	Ranunculales	Ranunculaceae *Ranunculus*	Buttercups	Y	B					
Magnoliophyta	Rosales	Rosaceae	Rose family	Y	R	R				
Magnoliophyta	Sapindales	Rutaceae: *Citrus*	Citrus fruit	N		R				
Magnoliophyta	Saxifragales	Halograceae: *Gonocarpus*	Raspworts	Y	B					
Magnoliophyta	Saxifragales	Halograceae: *Myriophyllum*	Watermilphoil	Y	B					
Pteridiophyta	Ophioglossales	*Ophioglossum*	Adder's tongue ferns	Y	B					
Pteridiophyta	Polypodiales	Asplenaceae: *Asplenium*	Spleenwort Ferns	Y	B					
Pteridiophyta	Pteriodopsida	Unidentified	Leptosporangiate Ferns	Y	B					
Euglenozoa	Trypanosomatida	Trypanosomatidae: *Leishmania*	Leishmaniasis Parasites	Y ?	B					
Ascomycota	Eurotiales	Aspergillaceae	Mold fungi	Y			R		R	
Ascomycota	Oxygenales	Arthrodermataceae	Skin fungi	Y					R	
Ascomycota	Sordariales	Chaetomiaceae: *Chaetomium*	Mold fungi	Y		R			R	
Ascomycota	Sordariales	Hypocreomycetidae	Mold fungi	Y				R		
Basidiomycota	Geastrales	Geastraceae	Earthstars	Y	B					
Basidiomycota	Gomphales	Unidentified	Coral Fungi	Y	B					
Basidiomycota	Polyporales	Coriolaceae: *Poria*	White rot fungus	Y					R	
Zygomycota	Mortirellales	Mortierellaceae: Mortierella	Soil Fungi	Y		R				
Arthropoda	Collembola	Entomobryoidea	Springtails	Y	R					
Aves	Dinornithiformes	Emeidae*	Moa	Y	B					
Mammalia	Artiodactyla	Bovidae: Bovinae	Cattle and relatives	N			R			
Mammalia	Artiodactyla	Suiidae: *Sus scrofa*	Pig	N			R			
Mammalia	Carnivora	Canidae: *Canis familiaris*	Domestic dog	N			R			
Mammalia	Primates	Hominidae: *Homo sapiens*	Human	Y	R		R		R	
Trematoda	Echinostomida	*Notocotylidae: Notocotylus*	Parasitic Flukes	Y	B					

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Figure 1. Map of sampling locations used for this study. A) Dart River Valley (West Otago, wet southern beech forest), B) Kawarau Gorge (Central Otago, semi-arid), C) Roxburgh Gorge – including rockshelter B and Sawer's rockshelter (Central Otago, semi-arid).

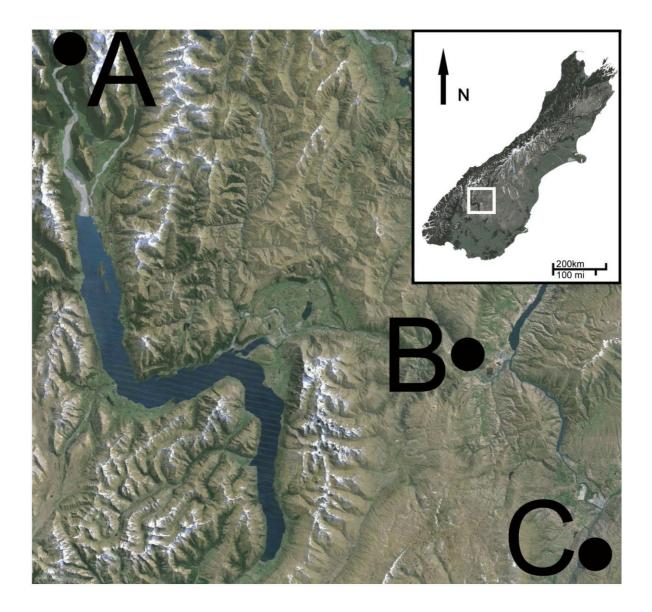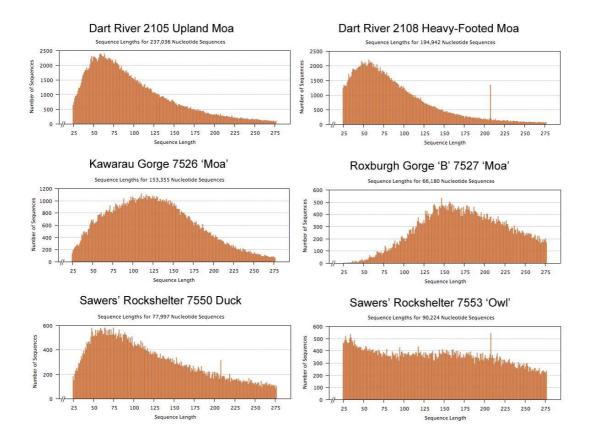**Figure 2**. Proportions of merged read lengths for each coprolite library.

Figure 3. Proportions of taxon assignments identified by BLASTn searches and filtered through MEGAN5. A) represents all assignments, B) only reads with BLAST hits, C) only reads assigned to a specific taxonomic group.
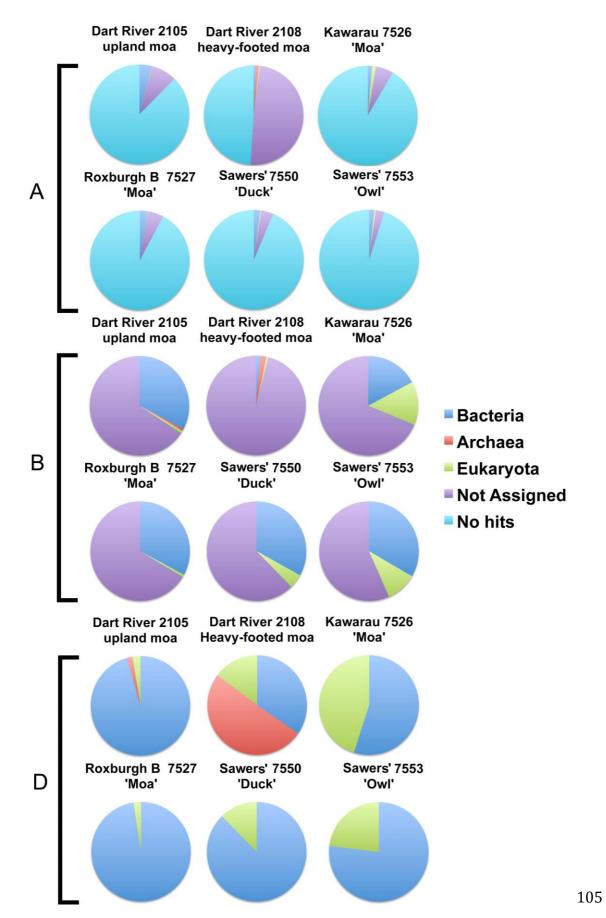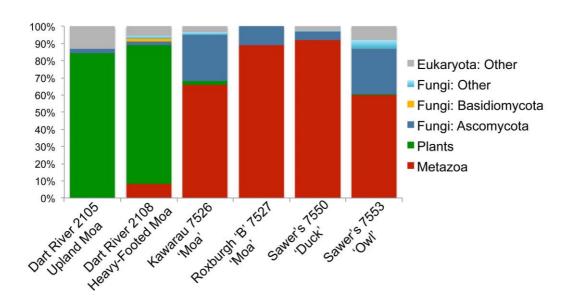
Figure 4. Proportions of major taxonomic groups of eukaroytes identified by BLASTn searches and filtered through MEGAN5.

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CHAPTER FOUR

Mitochondrial genomes of New Zealand's extinct, enigmatic adzebills (Aves: Aptornithidae †) resolve taxonomic placement within the superfamily Ralloidea (Gruiformes), and identifies biogeographic link between New Zealand and the Afrotropics

Statement of Authorship

Title of Paper	Mitochondrial genomes of New Zealand's extinct enigmatic adzebills (Aptornithidae) resolve taxonomic placement within the Ralloidea (Gruiformes)
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Publication Style
Publication Details	In preparation for submission to a peer reviewed journal (currently planned for Proceedings of the Royal Society B)

Principal Author

Name of Principal Author (Candidate)	Alexander Boast
Contribution to the Paper	Performed early DNA work including DNA extraction and amplification, and library construction and quantification. Assisted with RNA-DNA hybridisation enrichment. Assembled mitochondrial genomes. Peformed phylogenetic analyses and molecular clock estimates. Identified major results, designed and illustrated figures, and wrote paper.
Overall percentage (%)	
Signature	Date 11/06/2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

  i.   the candidate's stated contribution to the publication is accurate (as detailed above);
  ii.   permission is granted for the candidate in include the publication in the thesis; and
  iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Alan Cooper
Contribution to the Paper	Assisted with paper and study design, and supervised research. Provided samples.
Signature	Date 09/06/2015

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Contribution to the Paper	Performed and supervised early DNA work including DNA extraction and amplification, and library construction and quantification. Performed RNA-DNA hybridisation enrichment. Processed raw NGS data. Provided edits.
Signature	Date 9.6.15

Please cut and paste additional co-author panels here as required.

4.1 Abstract

The recently extinct adzebills (Aptornithidae, *Aptornis* spp.) were massive flightless birds endemic to New Zealand, which have long eluded precise taxonomic assignment. Using NGS sequencing and RNA-DNA hybridisation enrichment, we successfully amplify and assemble two near-complete ancient mitochondrial genomes of both the North Island (*A. otidiformis*) and South Island (*A. defossor*) adzebill. Our molecular phylogeny confirms that adzebills are part of the Ralloidea (Gruiformes), sister taxon to a 'heliornithoid' clade with a predominantly Afro-Madagascan distribution (including finfoots, flufftails and the rail *Canirallus*). Molecular clock analyses reveal a split time of approximately 35.4 ± 6.1 Ma for divergence of the adzebill lineage, predating the Oligocene marine inundation of New Zealand. We suggest that the biogeographic link between New Zealand and Africa most likely supports that the heliornithoid clade was once more widespread, now largely replaced by a later expansion of crown Rallidae (31.3 ± 5.1 Ma) during the early Oligocene. Inferred expansion date estimates for both major ralloid clades may indicate utilisation of vacant niche space following extinction and environmental changes at the Eocene-Oligocene boundary at ~33.9 Ma. We hypothesise the adzebill lineage persisted in New Zealand by exploiting an omnivore/insectivore niche involving powered physical excavation. This is supported by possible convergent evolution of similar, unrelated flightless Rallidae on young remote islands (*Diaphorapteryx, Aphanapteryx, Erythromachus*). Finally, we suggest that the relatively recent divergence between the North and South Island adzebill species (1.5 ± 0.8 Ma) was driven by the closing of the 'Manawatu Strait' (<2 Ma) between New Zealand's North and South Islands.

4.2 Introduction

The Adzebills (Aptornithidae, *Aptornis* spp.) were massive (up to 25kg), specialised, flightless, birds endemic to New Zealand, becoming extinct roughly 500 years ago [1–3] (Fig. 1). These birds have long perplexed taxonomic, biogeographic and ecological placement since described by Sir Richard Owen in the 19th C., and still

remain among the most enigmatic of all recent bird lineages. Unlike other recently extinct bird families with similarly uncertain affinities (e.g. the dromornithid 'thunder birds' of Pleistocene Australia, or the 'giant megapode' *Sylviornis* of New Caledonia), subfossil remains of *Aptornis* contain preserved DNA [4–6]. However, previous ancient DNA studies of *Aptornis* have been limited to the amplification of short Sanger-sequenced fragments, which have so far been insufficient for building a robust phylogeny.

New Zealand's lack of terrestrial mammals, as well as age and long isolation since separation from Gondwana (~85-52 Ma), has allowed the persistence and macroevolution of unusual and phylogenetically distinct avian lineages [7–10]. For example, New Zealand's endemic Acanthisittidae and Strigopoidea represent the oldest lineages of all living songbirds (Passeriformes) and parrots (Psittaciformes), respectively: two of the most speciose, successful and widespread all bird orders [11,12]. Other striking examples are the endemic ratite kiwi (Apterygiformes) and moa (Dinornithiformes), identified as sister taxa to the geographically and morphologically distant elephant birds of Madagascar and the flighted tinamous of South America, respectively [13,14]. Resolving the evolution of these ancient New Zealand lineages provides insight into the dispersal and evolution of much more widespread related avian groups during the early Cenozoic. However, they are also important in understanding New Zealand's biological history, having been radically altered by an extensive marine inundation of the island group during the 'Oligocene drowning' (~34-23 Ma) [15]. As a result, endemic and enigmatic taxa such as *Aptornis* are of prime research importance for evolutionary biogeography.

Aptornis includes two recently extinct species, *A. otidiformis* and *A. defossor,* endemic to the North and South Islands of New Zealand, respectively. Both adzebills were described by Owen, (*A. otidiformis* in 1844, and *A. defossor* in 1871) [16–19], although their bones were long confused with similarly sized moa and the giant geese *Cnemiornis* spp. [1,20]. Fossil fragments of an older adzebill (*Aptornis proasciarostratus*) 16-19 Ma in age have been described from the St. Bathans deposits and, apart from a smaller size and more gracile build, are almost indistinguishable from modern forms [20]. Both recently extinct adzebill species were robust, almost wingless, superficially rail-like birds almost 1m in height, possessing a massive, heavily reinforced skull and bill (Figure 4) [1]. The Holocene distribution of both species was restricted to the drier eastern lowlands of both islands, and both

species were apparently absent from high altitudes and southern-beech forest [1]. Adzebills also appear to be absent from semi-arid grassland and shrubland areas in the south-central South Island, though fossil evidence suggests they were more widespread during glacial forest retreats during the Pleistocene. It seems probable that the restricted distribution of adzebills played a role in their extinction.

Evidence from bone isotopes suggests that adzebills consumed animal food, and it is hypothesised that they subsisted on a diet of invertebrates, reptiles, ground-dwelling birds (including nesting seabirds) and carrion [1]. Its enormous bill would undoubtedly have been used in the excavation or immobilization of food or prey. Owen proposed that "[feeding] involved frequent strong and deep thrusts into the ground and the quest was for animal, not vegetable matters… larvae and earthworms were probably part of the food of *Aptornis*" [21]. However, as with similar birds such as rails *Aptornis* may have also opportunistically consumed available fruit, roots, seeds and fungi. Overall, the ecology of *Aptornis* remains equivocal. Both species became extinct ~1500AD roughly 200 years following the arrival of humans ~1300AD (along with other birds including the New Zealand moa) [2,3], and it is known from archaeological deposits they were targeted by human hunting [1,22,23].

The derived and specialised osteology of adzebills have long frustrated efforts to classify *Aptornis*, which have been further confused by hypotheses of Gondwanan biogeography. For example, a relationship between *Aptornis* and the similarly enigmatic, flightless and massive Dromornithidae of Australia and *Sylviornis* of New Caledonia has been proposed [24], although all evidence supports *Aptornis* as a neoavian, with both of the former being members of Galloanseres (fowl) [24–27]. More recently, *Aptornis* has been included within the 'Gruiformes': an order traditionally comprising a wide diversity of cursorial, plesiomorphic bird families. A relationship between *Aptornis* and the 'gruiform' kagu of New Caledonia (*Rhynochetus jubatus*) has been debated since Fürbringer's assessment in 1888 [28–31]. A possible relationship between a kagu/*Aptornis* clade and another 'gruiform', the sunbittern of the neotropics (*Eurypyga helias*), has also been suggested [28,29]. However, molecular methods have since rejected the monophyly of traditional 'Gruiformes', finding most members to be largely unrelated. Both kagu and sunbittern comprise their own order (Eurypygiformes) as do bustards (Otidiformes), mesites (Mesitornithiformes) and seriamas (Cariamiformes) [32–34].

Currently accepted Gruiformes *sensu stricto* now solely comprise the Ralloidea (the rails, Rallidae; finfoots, Heliornithidae; and flufftails, Sarothruridae), and Gruoidea (the cranes, Gruidae; trumpeters, Psophiidae; and limpkins, Aramidae) [5,35,36]. A close relationship between *Aptornis* and core Gruiformes (independent of kagu/sunbittern) has been proposed as early as Parker [37] who demonstrated similarities with the South American *Psophia*. Similarly Cracraft proposed a clade containing *Psophia* and *Aptornis* (although included the unrelated kagu, sunbittern and seriamas). Other assessments, in particular that of Lowe [38] found *Aptornis* to instead fall near the base of the 'gruiform' clade. Only since aDNA sequencing of a 12S rRNA fragment of *A. defossor* by Houde *et al.* has *Aptornis* finally been confirmed a gruiform unrelated to kagu/sunbittern [5,39]. However, Houde *et al.* was unable to unequivocally resolve the phylogeny, associating *Aptornis* with either to the Rallidae or the Heliornithidae. Furthermore, the encompassing 'Ralloidea' clade also contained *Psophia*, confirmed as falling within the Gruoidea by later studies. Therefore, the specific relationships of *Aptornis* must still be considered unresolved.

Overall, *Aptornis* represents one of the most phylogenetically and morphologically distinct members of the Gruiformes and New Zealand's endemic fauna. However, even after almost 200 years of study, the origins, evolution or ecology of the adzebills are still poorly understood. Aptornithidae represent the last of New Zealand's endemic bird families lacking confident taxonomic assignment at the ordinal level, which is significant considering several others (moa, kiwi, acanthisittid wrens, strigipoid parrots) are now pivotal in discussions of bird evolution during the early Cenozoic. Next generation sequencing (NGS) and RNA-DNA hybridisation enrichment has recently allowed for vast improvements in ancient DNA recovery [13,40–43]. Using such methods, a phylogeny of *Aptornis* may now finally be resolvable. A complete phylogeny should provide essential insights into the early evolution and biogeography of crown Gruiformes, as well as elucidate the origins and evolutionary history of this enigmatic taxon.

4.3   Methods

4.3.1   Specimen Sampling and DNA extraction

For *Aptornis* DNA sequencing we used two bone samples sourced from femurs, each from the extinct North Island and South Island Adzebill species (*A. otidiformis* and *A. defossor* respectively). The *A. otidiformis* sample was recovered from a cavern near Coornor, Wairarapa, south-eastern North Island, and the *A. defossor* sample from the honeycomb hill cave system, north-western South Island. Both were found amongst deposits of other extinct New Zealand bird species, and were differentiated from similar-sized and co-occurring moa species using characteristic osteological features. Neither sample has been radiocarbon dated, although both are certain to be >500 years in age based on the approximate extinction time of *Aptornis*. As the honeycomb hill deposit is not considered to fall within known *A. defossor* distributions during the Holocene [1], this sample may be Pleistocene (>12,000) in age, which may also be supported by Pleistocene-aged carbon dates of moa from associated deposits [44].

DNA extraction and library preparation of both *Aptornis* samples were performed in a purpose-built, physically isolated, ancient DNA laboratory at the Australian Centre for Ancient DNA (ACAD), University of Adelaide. To control for contaminants, the exterior surfaces (~1mm) of both bone samples were removed using a dremel tool, and exposed surfaces were cleaned with bleach and exposed to UV irradiation for 15min. Samples were powdered using mikrodismembrator (Sartorius). 0.2-0.3g of bone powder from each sample was lysed by rotational incubation at 37°C overnight in 4ml of 0.5M EDTA pH 8.0 followed by a second round of incubation at 55 °C with 60 µL of proteinase-K. The resulting solution was bound, washed and eluted to a DNA extract using the silica-based method of Rohland and Hofreiter [45].

In addition we also extracted DNA from muscle tissue of the extant grey-winged trumpeter, *Psophia crepitans* (Psophiidae), sampled from a private frozen tissue collection (in turn sourced from deceased captive birds at the Smithsonian National Zoological Park). DNA was isolated using a modified salting-down extraction, where ~0.3g tissue was mixed with 300 µL cell lysis solution, 3µL

proteinase K and incubated at 55 °C. The resulting solution was purified to DNA using a salting-down method [46]. The DNA extract (*Psophia* only) was sheared to ~300bp using an ultrisonicator designed for library preparation (Covaris). Ancient extracts were not sheared due to the already fragmented nature of the endogenous DNA.

4.3.2   DNA sequencing and amplification

Extracts were blunt-end repaired, and custom adapters were ligated using the library preparation protocol of Meyer and Kircher [47]. Each adapter (5' and 3') contained a unique 7 mer index, allowing both for identification of amplified DNA and removal of downstream contaminants. Following library preparation, each sample was amplified using PCR. Each library was partitioned into eight separate PCR reactions in order to reduce amplification bias. Each reaction contained 1 x PCR buffer, 2.5 mM MgCl2, 1mM dNTPs, 0.5 mM primer, 1.25 U AmpliTaq Gold, 2 µL DNA extract. Reactions were subjected to the following thermocycling regime: 94 °C 12 min; 13 of 94 °C for 30s, 60 °C for 30 s, 72 °C for 40 s (plus an additional 2 s per cycle); and a final extension of 72 °C for 10 min. Individual PCR products for each library were pooled following amplification, and purified using AMPure magnetic beads (Agencourt). H20

All libraries were enriched for host mitochondrial DNA using commercially synthesised biotinylated 80 mer RNA baits (MYcroarray, MI, USA). Baits were designed using a wide range of published whole mitochondrial genome sequences (not including control region / D-loop) of a variety of avian taxa, including paleognaths, galloanserans and neoavians, as used in several past aDNA studies [13,48,49]. DNA-RNA hybridisation enrichment was performed using manufacturer's recommended concentrations of 5.2 x SSPE, 5.2 x Denhardt's, 5mM EDTA and 0.1% SDS. This solution was incubated for 44 hr (3 hr at 60 °C, 12 hr at 55 °C, 12 hr at 5-°C, 17 hr at 55 °C) with 200ng of library. Following incubation, baits were immobilised on magnetic MyOne Streptavidin Beads (Life Technologies). The baits were washed once with 1 x SCC and 0.1% SDS (15 min at room temperature), and twice with 0.1 x SCC and 0.1% SDS (10 min at 50 °C), then resuspended in 0.1 M NaOH pH 13.0 which destroyed the RNA baits and released captured DNA. The

resulting enriched DNA solution was purified using a Qiagen Minelute spin-column, and subjected to another round of PCR (12 cycles, eight reactions, using above recipe).

Enriched libraries were subjected to a final round of PCR (seven cycles, five reactions, using above recipe), using fusion primers to add full-length Illumina sequencing adapters for sequencing. Libraries were diluted to 2M (quantified using KAPA qPCR) and run on an Illumina MiSeq using 2 x 150 (paired-end) sequencing chemistry.

4.3.3   Genome assembly

Sequenced reads were demultiplexed using the 7 mer 5' and 3' barcode sequences using 'sabre' (https://github.com/najoshi/sabre) (default parameters: no mismatches allowed). Adapter sequences were removed, and paired reads merged using Adapter Removal v1.5.4 [50]. Low quality bases were trimmed (--minquality 4) and merged reads shorter than 25 bp were discarded (--minlength 25).

Collapsed reads were imported into Geneious v 7.0.5 (http://www.geneious.com/) and were assembled to related reference taxa, using an assembled mitochondrion of the weka *Gallirallus australis* (Rallidae, K425525 Genbank accession) for both *Aptornis* libraries, and the black-crowned crane *Balearica pavonina* (Gruidae, NC_020570 Genbank accession) for the *Psophia* library. Each reference sequence had control region removed for assembly. We used exhaustive mapping iterations (until an assembled read plateau was reached) using inbuilt 'medium-low' sensitivity parameters. Due to the greater sequencing depth of the modern *Psophia* library, and control for contaminants and ancient DNA damage of the *Aptornis* libraries, we utilised an additional step for the ancient DNA data only. From each initial *Aptornis* assembly, a consensus sequence (majority threshold), was used as a reference for a stringent round of mapping using BWA v0.7.8 [51] ('aln' –l 1024, -n 0.1, -o 2). Mapping reads with a Phred score >30 were selected using SAMtools v.14 [52] view command (-q 30), and read duplicates were discarded using the 'FilterUniqueSAMCons.py' python script [53]. Final consensus sequences for all three final assemblies (checked by eye) were generated in Geneious (75% threshold, min 3 read coverage, ambiguous nucleotides with IUPAC ambiguities).

Merged and trimmed reads for each of the three libraries comprised sequence counts of 224,419 (*A. defossor)*, 274,845 (*A. otidiformis*), and 1,247,336 (*Psophia*), of which 5,586 (2.5%), 7,034 (2.6%) and 527,315 (42.28%) reads mapped to the reference sequence respectively. Mapping statistics varied between each library (note *Aptornis* assemblies had duplicates removed during BWA step), for *A. defossor* (mean read length = 76.7 ± 31.2, sequencing depth = 18.3x ± 11.6 max 57), *A. otidiformis* (mean read length = 76.7 ± 31.2, sequencing depth = 5.9x ± 4 max 24), and *Psophia* (mean read length = 111.1 ± 28.2, sequencing depth = 1,925x ± 2,804 max 19,323). All consensus sequences comprised near complete mitochondrial genomes, with 97.7%, 97% and 100% genome coverage for *A. defossor, A. otidiformis*, and *Psophia* respectively (not including ambiguous nucleotides, or d-loop which was not enriched for). Regions missing from the ancient genomes comprised approximately 64bp of COX1, 154 bp of ND4L/ND4, 176bp of cytB, 16bp of ND6 and 56 bp of tRNA-Glu from *A. defossor*; and a continuous 346 bp fragment comprising the terminal end of ND6 and all of tRNA-Glu from *A. otidiformis.* Both assembled *Aptornis* genomes closely matched an available 673 bp 12S rRNA fragment of *A. defossor* sequenced by Houde *et al.* [5] (666/673 pairwise match for the *A. defossor* sequence with all mismatches representing ambiguous nucleotides, and 670/673 pairwise match for the *A. otidiformis* sequence). We also identified close matches between our *Psophia* library and available sequences of all three extant *Psophia* species (best hit 1142/1143 pairwise match with *P. crepitans* cytB sequence DQ485900).

4.3.4   *Phylogenetic analyses and taxon sampling*

A priori tests using a wide range of assembled mitochondrial genomes of various bird species (including an unpublished assembly of the sunbittern as well as an available kagu mitochondrion), unanimously confirmed *Aptornis* as nesting within the gruiformes. This also confirms results from previous studies [5,39], and refutes the long-proposed relationship with *Aptornis* and the Eurypygiformes (sunbittern and the kagu, *Rhynchocetus jubatus*). As a result we elected to utilize a dataset comprised solely of Gruiformes (Table 1).

We first designed an initial dataset of complete mitochondrial genomes available for individual gruiform genera only (unless stated otherwise we did not use

more than one species per genus), as downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). For representative outgroups to root the tree we used the Southern rockhopper penguin (*Eudyptes chrysocome*) representative of the Sphenisciformes, and the black-browed albatross (*Thalassarche melanophrys*) representative of the Procellariiformes. We used these two orders (now accepted as sister taxa) for fossil calibration purposes (see below) and their relative close relationship to the Gruiformes (following the closest gruiform relatives, the Charadriiformes and Opisthocomiformes) [34]. All mitochondrial genomes had control region / d-loop removed, and were aligned using MAFFT v. 7.017 [54,55] (automatic / default settings) as implemented in Geneious. Alignments were checked by eye, and short-length, non-coding intergenic spacers were manually removed.

This dataset covered most gruiform families, including both Gruidae subfamilies (Gruinae 2/3 genera, and Balearicinae 1/1 genera), Psophiidae (1/1 genera), Heliornithidae (1/3 genera), and Rallidae (9/ max. 48 genera). However, we were unable to include flufftails (Sarothruridae, *Sarothrura* spp.), and the limpkin (Aramidae, *Aramus guarauna*). We were also unable to include the rail genus *Canirallus* identified as unrelated to the remaining Rallidae, and sister to the Sarothruridae by Garcia-R *et al.* [56].

To improve taxon sampling, we developed a secondary dataset utilising only eight mitochondrial markers: 16S RNA, tRNA-Val, 12S RNA, Cytochrome Oxidase subunit 1 (COX1), ATP synthase subunit 6 (ATP6), tRNA-Gly, NADH dehydrogenase subunit 3 (ND3) and Cytochrome B (CytB). We obtained all mitochondrial data for gruiform genera not already covered by complete mitochondrial genomes from GenBank (Table 1). Sequences were then assembled to a representative sequence of the weka *Gallirallus australis* mitochondrial genome reduced only to the loci above, using geneious mapper (High-sensitivity settings). These assemblies were checked by eye for correct gene alignment. While single species were used when possible, congeneric taxa were merged into single assemblies if this improved coverage, comprising three species each of *Anurolimnas* and *Pardirallus* (Table 1). Consensus sequences of each assembly (75% threshold), were then aligned to a reduced version (to the above loci) of the initial alignment, non-inclusive of the two outgroup taxa (*E. chrysocome* and *T. melanophrys*), rooted using the topology estimate by the initial 'complete' dataset. This assembly was aligned

using MAFFT (using E-INS-i algorithm, maximum gap open penalty), following the above procedures.

This second dataset considerably improved taxon coverage, and included all gruiform genera and families. The exceptions were several Rallidae (~26/ max. 48 genera were covered) although this family was difficult to sample given polyphyly and instability of several genera [56]. Our Rallidae dataset, however, included 2/3 species of *Canirallus* (*C. beankaensis* and *C. kioloides*), both included due to the taxonomic importance of this genus. We were also able to include most members of the Rallidae identified as isolated or basal lineages by morphological analysis (other than *Canirallus*) by Livezey [29]. This includes the Nkulengu rail (*Himantornis haematopus*), which has frequently been considered the most primitive of the Rallidae [29,57,58]. Other distinctive genera covered included *Eulabeornis*, *Aramides* and *Habroptila*. However we were unable to include *Gymnocrex* or *Rougetius*. Most taxa were covered at least by CytB, although others (for example *Amaurolimnas* or *Micropygia*) were only covered by alternative loci such as COX1.

To identify appropriate partition schemes and substitution models we used PartitionFinder v 1.1.1 [59], with alignments subdivided into individual mitochondrial loci (including protein-coding genes, rRNA genes, and tRNAs). PartitionFinder favoured eight and two partitions for the 'complete' and 'incomplete' alignments respectively, and provided a range of substitution model schemes (Table 2). Phylogenetic trees were estimated using Bayesian estimates as implemented through MrBayes v 3.2.5, using the partition schemes and substitution models suggested by PartitionFinder. These used a total of 1×10^6 ('complete') and 1×10^7 generations ('incomplete'), with the first 25% of sampled trees used as a burn-in to estimate tree topology.

4.3.5   *Fossil calibrations and molecular clock analyses*

Molecular clock estimates (on the initial, 'complete' dataset only) were performed using BEAST v 1.7.5. We employed a total of four fossil-based calibrations for molecular clock estimates (Table 3). For the split between the Sphenisciformes and Procellariiformes we used the fossil penguin *Waimanu manneringi* [60]. *Waimanu* fossils from the New Zealand Wairarapa Greensand have

been dated 60.5-61.6 Ma, providing a conservative minimum age for the penguin lineage.

A diversity of different bird fossils have been assigned to Gruiformes, however most are highly fragmentary and their affinities remain contentious. For example, of these fossil 'gruiformes', the only fossil limpkin (Aramidae) is an early Oligocene tarsometatarsus from the South Dakota Brule formation [61,62] and the oldest likely finfoot (Heliornithidae) is a Miocene (12 Ma), distal humerus end from the North Carolina Lee Creek formation [63]. The only fossil with affinities to the South American trumpeters (Psophiidae) is a small, undescribed coracoid from the Quercy-Fissure fillings (mid Eocene – early Oligocene) in France [64]. Furthermore, the conservative morphology of some gruiforms limits precise taxonomic placement of fossil taxa. For example, the oldest definitive rail (early Oligocene) *Belgirallus* [65,66], cannot be used as a calibration for a Ralloidea split, as both 'basal rails' (including *Canirallus*), and 'true' rails (Rallidae *senso stricto*) currently lack any accepted differentiating characters, and in turn it may be assumed that extinct stem Ralloidea were morphologically similar. Overall, most internal nodes could not be calibrated.

To calibrate the split between the Gruoidea and the Ralloidea we used the abundant and exceptionally well-preserved messelornithid *Messelornis cristata* from the Messel shale deposits (>47Ma) (of which *M. cristata* comprise roughly half all bird fossils) [67,68]. Messelornithids are generally accepted as a sister taxon to, or nested within, crown Ralloidea [69–71]. However other, older 'messelornithids' (e.g. *M. nearctica* from the Green River formation at 53.5-48.5 Ma) are considerably more fragmentary and their taxonomic placement is less certain [71,72]. Similarly, a number of putative Paleocene-Eocene 'gruiformes' considered Gruoidea relatives (e.g. Parvigruidae, Geranoididae, Eogruidae) have uncertain affinities [71]. Fossils often assigned to the Gruidae have been assigned as far as the mid-Eocene (e.g. *Paleogrus* spp.), however again are sparse and some also have possible affinities with Anseriformes [71,73]. We followed the relatively conservative calibration of 12 Ma as used by Krajewski *et al.* [73] for the divergence between the Gruidae subfamilies Gruinae and Balearicinae, using the fossil gruines *Grus afghana* and *G. miocenicus* [74]. Finally, the fossil Aptornithid *A. proasciarostratus* from the New Zealand St. Bathans deposit (16-19Ma) provided a clear minimum bound for age of the *Aptornis* lineage [20].

For calibration schemes for the initial 'complete' dataset, for the *E. chrysocome* (Procellariiformes) and *T. melanophrys* split we used a lognormal distribution with an offset of 60.5 Ma (mean 1, standard deviations 1). Due to likely underestimates of other calibrations (e.g. the already highly derived morphology of *A. proasciarostraus*) and lack of clear upper-bound constraints, we used uniform distributions for remaining calibrations: 47-65 Ma for the Gruoidea-Ralloidea, 12-60 Ma for the Balearicinae-Gruinae split, and 16-60 Ma for the *Aptornis* lineage.

4.4   Results

4.4.1   *Tree topology and biogeographic assessment*

Our analyses unequivocally support that *Aptornis* is a member of Gruiformes, falling within a clade comprising Heliornithidae, Sarothruridae, and the 'rail' *Canirallus*, with 100% posterior distribution (Fig. 2,3), which we collectively refer to as 'heliornithoids' (*Heliornis* has nomenclatural priority). *Aptornis* was moderately supported as the basal taxon (66%) in this group. Alternative topologies instead found *Aptornis* as sister to *Sarothrura-Canirallus* with the Heliornithidae as basal, which may be an artefact due to the observed fast evolutionary rates in the latter group (Fig. 3). Both *Aptornis* species are confirmed closely related sister taxa, with branch distances supporting separate species status. With the exception of the Afrotropical genus *Canirallus,* a monophyletic clade containing all 'true' rails (Rallidae) is supported, sister to the 'heliornithoid' clade. Biogeographic inferences based on the wholly Afrotropical distribution of the Sarothruridae, *Canirallus* and the basal Heliornithidae (*Podica senegalensis*), suggests a possible African/Madagascar origin for heliornithoids except the New Zealand *Aptornis*. This disjunct distribution is likely best explained by an early expansion of heliornithoids either outcompeted or displaced by a later radiation of crown Rallidae.

Our Rallidae topology correlates closely to the phylogeny by García-R *et al.* [56,75] with many of the major clades being supported (with the exception of *Megacrex*, *Himantornis* and *Micropygia* which were not included in their study). Differences observed (e.g. the placement of *Rallus* and *Rallina*; or disagreement between the

relationship of the "*Fulica*" and *"Aramides"* clades) were minor and met with low posterior support values. Our chief concern was to test the monophyly of all available Rallidae sequences non-inclusive of *Canirallus*, which is supported. Phylogenetic inference does not indicate the biogeographic origin for 'true' rails, although most diversity is found within the Asiatropics. Reciprocal monophyly of the separated Ralloidea and Gruoidea clades is supported, with Gruoidea possibly having a neotropical origin based on the biogeography of the basal Psophiidae and Aramidae.

4.4.2   Divergence times

Although molecular clock estimates (Fig. 2, Table 4) were performed only for the 'complete' dataset, this tree included most nodes of interest. The split between *Aptornis* and *Heliornis* (representing the monophyletic basal afrotropical ralloid clade) is determined to be early Eocene – Late Oligocene (35.4 ± 6.1 Ma). The North Island *A. otidiformis* and South Island *A. defossor* are estimated to have diverged in the mid-late Pleistocene (1.5 ± 0.8 Ma), further justifying separate species status. Extant Rallidae are determined as having a slightly younger age (31.3 ± 5.1) in the Oligocene, disagreeing with early Eocene origins by García-R *et al.* [56,75]. This also supports a hypothesis of a later 'true' rail radiation, occurring after the radiation of the *Aptornis* lineage. In addition, the most recent ancestor of all extant ralloids (43.2 ± 5.6 Ma) is mid Eocene which is younger than a number of fossil ralloidea (such as messelornithids), suggesting these fossils may be stem rather than crown Ralloidea [69–71]. Primary divergences of extant Gruiformes (55.8 ± 5.1 Ma) and Gruoidea (49.1 ± 6.3) are estimated to have occurred during the early Eocene. Finally, for the Gruidae, we infer approximate splits of the Balearicinae and Gruinae (26.6 ± 8 Ma) to occur in the Oligocene and split of *Grus-Anthropoides* (<10 Ma) to be Miocene. Most of these dates are congruent with those found by Krajewski *et al.* [73] and Fain *et al.* [35] (which found only slightly older estimates).

4.5   Discussion

4.5.1   *Age, origins and Phylogeny of* Aptornis

Our phylogenetic dataset finds unanimous support that the Aptornithidae represent an early diverging branch of the Ralloidea evolutionary tree, and gives new and unexpected insights into evolution of this cosmopolitan bird group. We infer the disjunct association with Afrotropical taxa, similar to the kiwi-elephant bird relationship found by Mitchell *et al.* [13], is very unlikely to demonstrate long-distance dispersal or continental vicariance. Instead we propose that heliornithoids (*Aptornis* and other non-rallid ralloids) represent the remnant of an early expansion of ralloid birds occurring during the late Eocene. This expansion has since been displaced or outcompeted by crown Rallidae, in turn inferred as having slightly younger early Oligocene origins. The timing of these events correlate closely with the appearance of recognisably modern rails (e.g. *Belgirallus*) in the early Oligocene [65,66]. Furthermore, the timing of both apparent radiations may also represent an evolutionary bottleneck and/or utilisation of vacant niche space following extinction, climatic cooling and vegetation changes associated with the Eocene-Oligocene boundary at ~33.9 Ma [76–82]. We argue that *Aptornis* and the Heliornithidae (which have successfully radiated through the worldwide tropics) have persisted due to extreme specialisation, whereas the afrotropical taxa (*Sarothrura, Canirallus*) have successfully competed with true rails based on the comparative lack of endemic African rail genera or genera-clades.

As we infer that closely related taxa to *Aptornis* are long extinct, it is difficult to determine a time of origin within New Zealand. Even so, given the dispersal capabilities of extant rails [83], it is plausible that early ralloids were rapidly present in New Zealand. In addition, our phylogeny determines the common ancestor of Aptornithidae would have been recognised as a modern Rallidae *senso lato* (considering the relationship to *Canirallus*). This therefore supports that *Aptornis* represents an extraordinary case of macroevolution including almost total wing-loss, and a 55-90 × weight increase from a hypothetical volant ancestor (based on maximum weight estimates of *Canirallus* at 280g) [84]. Finally, the presence of almost morphologically modern aptornithids Miocene (16-19 Ma) in age, demonstrates that most of these changes had already occurred by this time [20]. In addition, these same

fossil deposits also contain abundant rail bones [85] similar in size and morphology to extant volant Rallidae. Whether these are relatives to *Aptornis*, or represent later colonisers, cannot be concluded without further evidence, although their small stature supports some degree of niche monopolisation by *Aptornis.* Based on these observations, we suggest that *Aptornis* has been present in New Zealand since the Eocene/Oligocene.

We can clearly include *Aptornis* as one of a guild of birds with a long presence in New Zealand, which also comprise the endemic ratite kiwi (Apterygiformes) and moa (Dinornithiformes), the New Zealand 'wrens' (Acanthisittidae) and New Zealand parrots (Strigopoidea). All these groups, including *Aptornis*, have been hypothesised as having vicariant gondwanan origins. However, even upper limits of our estimates (41.5 Ma) post-date the latest possible separation of New Zealand from Gondwana at >52 Ma [8]. It is unlikely that this could be an age underestimate, as this would require node ages of crown neoavians and gruiformes to be shifted to the mid-late Cretaceous, unsupported by most molecular estimates and fossil data [33,34]. Similarly, research now consistently supports flighted origins for all other ancient New Zealand bird lineages during the mid-Cenozoic [13,14]. Overall, we support a flighted, dispersal origin of aptornithids to New Zealand, prior to peak marine inundation of New Zealand at ~26 Ma [10,86–88] (Late Oligocene).

4.5.2   *Ralloidea topology and taxonomy*

Our topology confirms [56] that current Rallidae are paraphyletic to the Sarothruridae, Heliornithidae and Aptornithidae if placement of *Canirallus* is determined to be correct. These *Canirallus* sequences are likely to be accurate, originating from multi-species dataset based solely on the genus by Goodman *et al.* [89]. However, due to the deep divergences and age estimates found within the Ralloidea, we consider that separate family-level statuses of the major ralloid clades (including Aptornithidae) are justified. Therefore, paraphyly of current Rallidae with respect to *Canirallus* would be best resolved by transferring *Canirallus* to the Sarothruridae or erecting the Canirallidae (as opposed to delegating Aptornithidae, Heliornithidae and Sarothruridae as subfamilies of the Rallidae).

Given the general discordance between morphology and molecular based tree topologies of the Ralloidea, it is plausible other unexpected rail taxa may be found to fall within the heliornithoids [29,56,75,90]. Unsampled rallidae that may have heliornthoid affinities comprise the genera *Gymnocrex* (asiatropics) and *Rougetius* (afrotropics) assigned to fall close to the base of the rail tree by cladistic methods [29,90]. The great remainder of unsequenced genera (*Aphanapteryx*, *Aphanocrex*, *Atlantisia*, *Capellirallus*, *Erythromachus*, *Mundia*, *Nesotrochis*, *Vitirallus*, *Hovacrex*) all represent oceanic island forms (many extinct), likely to have recent dispersal origins [83,91]. The final remaining unsequenced genera show close affinities to other well-sampled taxa, to which they are sometimes included (*Tribonyx – Gallinula*; *Aenigmatolimnas/Crecopsis – Crex*; *Cyanolimnas – Pardirallus/Neocrex*). Nonetheless our assessment of ralloid taxonomy and evolution may be altered with additional taxon coverage.

4.5.3   *Divergence between North and South Island* Aptornis

Our inclusion of both *Aptornis* species in our molecular clock analysis permitted a divergence estimate between the two species at 1.5 Ma ± 0.8 Ma. The mean estimate at 1.5 Ma is closely congruent with the closing of the 'Manawatu strait' at 2-1.5 Ma, which had long separated the geological precursors of New Zealand's North and South Islands since ca. 30 Ma [44] (Fig. 4). The development of the modern, ephemeral Cook Strait (closing during sea level drops in Pleistocene glaciations), is a more modern feature at ~0.45 Ma [44,92]. Age estimates between North Island and South Island giant moa (*Dinornis novaezealandiae* and *D. robustus* respectively) by Bunce *et al.* [44] are highly congruent with our estimates at 1.45 ± 0.8 Ma. Similar estimates (specific dates not provided in-text) were also found between the North Island *Pachyornis geranoides* (Mantell's moa) and South Island *P. elephantopus* (heavy-footed moa). Similar to Bunce's estimates with moa, *Aptornis* likely evolved in the larger Southern New Zealand fragment, only dispersing into the North Island during the Pleistocene isthmus. This is supported by the presence of *A. proasciarostratus* in the Miocene (16-19 Ma) St. Bathans fauna in Otago, South Island. The early connection between both islands, however, may have been tenuous and ephemeral, resulting in almost immediate allopatry. Thus geographic speciation

between both North and South populations may have occurred prior to the complete development of the Cook Strait. Alternatively, terrestrial ecological or geographic barriers between the two populations may have been sufficient to limit or prevent gene flow. This may be supported by the apparent limitation of *Aptornis* to a narrow habitat range as determined by fossil data [1].

4.5.4   *Macroevolution and ecological niche of* Aptornis

Aptornis represents one of the most morphologically 'aberrant' [20] and ecologically mysterious of all New Zealand's recent bird fauna. Holocene-aged fossils of *Aptornis* are generally restricted to drier, eastern areas of both islands and are entirely absent from high altitudes or areas comprised by southern-beech forest. This appears to demonstrate a habitat preference of *Aptornis* to specific forms of well-drained, lowland podocarp-broadleaf forest or shrubland [1]. However, discerning the specific nature of these vegetation types has been complicated by extensive prehistoric habitat modification of areas recently occupied by adzebills [93–96]. Furthermore, wider distributions of both species during Pleistocene forest retreats also infer a preference to relatively open habitats. Yet, adzebills appear to have been entirely absent from semi-arid areas of the South Island originally comprised by grassland and shrubland [97–99]. These details signify a highly specialised niche, the nature of which is still equivocal. It may therefore be possible that viable adzebill populations occurred only where seasonally abundant sources of protein (e.g. infrequent masting of specific tree species, or breeding seabirds) permitted successful reproduction, as is well documented for the extant kakapo parrot [100,101].

Adzebills have long been considered either predatory or omnivorous [1]. The enormous head and bill (Fig. 1) were also reinforced with specialised and advanced osteological features, which in turn were attached to evidently extensive musculature for hatchet-like movements of the head [1]. Together these likely designate adaptions for excavation of dead wood or deep soil. Both extant *Sarothrura* and *Canirallus* generally subsist on a diet of insects, also occasionally taking small invertebrates and seeds [102]. Similarly, preserved gut contents of the Eocene ralloid *Messelornis cristata* containing fish and seeds demonstrate a diverse diet of early ralloids [103,104]. These indicate that adzebills, or at least their ancestors, had broad opportunist diets with a

predisposition for animal prey. Furthermore, adzebills were likely sympatric with early moa or kiwi which may have already occupied specialised megaherbivore or probing insectivore niches, respectively, millions of years earlier [13].

We hypothesise that *Aptornis* successfully exploited and rapidly adapted to a niche preventing displacement by later true rails. The St. Bathans deposits containing both Miocene Aptornithids and rails may indicate a long presence of both lineages within New Zealand [85] (assuming these 'rails' do not fall within the heliornithoid clade). However, most extant or recently extinct endemic New Zealand rail taxa have much more recent origins [83,91,105]. For example, most of New Zealand's recent rails were comprised of a morphologically diverse guild of related taxa appearing to originate from independent colonisations of *Gallirallus*, of which the only extant member is the weka (*G. australis*) [1,3,83,91,105]. As with much of likely heliornithoid diversity, or the mid-Eocene aged ralloid messelornithids, it appears that ralloidea may be unusually prone to waves of extinction and replacement/displacement by related lineages. *Aptornis* therefore may have persisted either by occupying a more stable niche. Alternatively, *Aptornis* was among the first ralloids to colonise New Zealand, and was effectively able to compete with later arrivals due to its specialisation. Elsewhere, the *Aptornis* niche space was likely to be filled by terrestrial mammals, which with the exception of a possible Miocene age shrew-sized species [106] were largely absent from New Zealand.

Possible convergent evolution between *Aptornis* and true rails may be found within the New Zealand archipelago. Of the afore-mentioned '*Gallirallus*' rails to colonise New Zealand, the largest by a wide margin was the ~2kg Hawkin's rail (*Diaphorapteryx hawkinsi*), endemic to the geologically young (<2 Ma) Chatham Islands ~680km to the east of mainland New Zealand. It was also both allopatric to, and in some ways superficially similar to, *Aptornis*. It also appeared to have no clear related analogues in the mainland rail fauna (the often-cited analogue of the weka *G. australis* was likely instead represented on the Chatham group by the extinct Dieffenbach's rail *G. dieffenbachii*) [91]. Despite the young age of the taxon (<2Ma), *D. hawkinsi* was already ~8-15 × the weight of volant *Gallirallus* such as *G. phillipenensis* [1]. In addition, entirely unrelated extinct, flightless rails on Réunion (*Erythromachus*), and Mauritus (*Aphanapteryx*), were so similar to *D. hawkinsi* they have previously been considered congeneric [107,108].

That no mainland New Zealand Rallidae evolved a similar morphology may be a result of direct competition with the long-established *Aptornis*. Furthermore, these rail species may therefore represent early-stage examples of convergent evolution with *Aptornis* and provide clues into its ecology and evolution. Possible live accounts of these particular rail taxa support opportunistic diets, and in the case of *D. hawkinsi* a propensity for fernroot of which it continuously excavated for by "rooting like a pig" [109]. Isotopic evidence of *D. hawkinsi* bones fall between those of herbivorous and predatory birds (from the same deposits) supporting an omnivorous diet [48]. Strikingly, isotope analyses of *Aptornis* bones have found measurements (^{15}N/^{13}C ratios) almost identical to *D. hawkinsi* [1], although this may be an artefact of the differing source deposits. It is plausible that *Aptornis* may have foraged for more vegetable matter than has been previously accepted, and its specialised bill was similarly used for excavating plant roots. Furthermore, inferring a hypothetical prey diet of *Aptornis* is constrained by a probable, though poorly understood, extinction and/or wide scale destruction of New Zealand's invertebrate and reptile fauna following arrival of the Pacific rat [1,110–115].

Overall, although our phylogeny provides clues into the niche of *Aptornis*, discerning the paleobiology of these extraordinary birds in the New Zealand ecosystem will require a multidisciplinary dataset. This will need to include a comprehensive survey of Holocene / Pleistocene fossil occurrences in both islands, identifying specific paleoenvironments where *Aptornis* occurred, reconstructing past prey animal communities (including seabirds, invertebrates, and herpetofauna), and a developing a more comprehensive isotopic analysis of *Aptornis* bones to correctly determine its trophic level. Nonetheless our phylogeny provides considerable new resolution into the evolution of this highly unusual bird lineage. We can now confirm that *Aptornis* clearly represents one of the older members of New Zealand's fauna, and represents one of the most extraordinary examples of macroevolution on these remote islands.

4.6 Figures

Table 1: List of all taxa and sequences used in this study. Each separate cell represents a single sequence generated into a consensus for phylogenetic analyses, note use of three species for single *Anurolimnas* and *Pardirallus* sequences (see methods for details).

Superfamily	Family	Genus	Species	Common name	Complete Mitochondrion	16S	12S/rRNA-Val	COX1	ATP6	tRNA-Gly/ND3	CytB
Outroup	Diomedeidae	*Thalassarche*	*melanophyrs*	Black-browed albatross	NC_007172						
Outroup	Spheniscidae	*Eudyptes*	*chrysocome*	Southern rockhopper penguin	NC_008138						
Gruoidea	Aramidae	*Aramus*	*guarauna*	Limpkin		DQ485854	AGU76104 DQ485816	DQ433321			DQ485899
Gruoidea	Gruidae	*Anthropoides*	*paradiseus*	Blue crane	NC_020573						
Gruoidea	Gruidae	*Balearica*	*pavonina*	Black-crowned crane	NC_020570						
Gruoidea	Gruidae	*Grus*	*vipio*	White-napped crane	NC_021368						
Gruoidea	Psophiidae	*Psophia*	*crepitans*	Grey-winged trumpeter	This study						
Ralloidea	Aptornithidae	*Aptornis*	*defosor*	South Island adzebill†	This study						
Ralloidea	Aptornithidae	*Aptornis*	*otidiformis*	North Island adzebill†	This study						
Ralloidea	Heliornithidae	*Heliopais*	*personatus*	Masked finfoot			DQ485820				DQ485903
Ralloidea	Heliornithidae	*Heliornis*	*fulica*	Sungrebe / American finfoot	NC_025499						
Ralloidea	Heliornithidae	*Podica*	*senegalensis*	African finfoot			DQ485821 PSU76021	HQ998023			DQ485904
Ralloidea	Rallidae	*Amaurolimnas*	*concolor*	Uniform crake				JQ173979 JQ173980			
Ralloidea	Rallidae	*Amaurornis*	*phoenicurus*	White-breasted waterhen	NC_24593						
Ralloidea	Rallidae	*Anurolimnas*	*castaneiceps*	Chestnut-headed crake			ACU77156				
			fasciatus	Black-banded crake		KC614006		KC614046			KC614090

			viridis	Russet-crowned crake		KC614010		JQ1740052 JQ174053 KC614049 KM896240			KC614094
Ralloidea	Rallidae	*Aramides*	*ypecaha*	Giant wood rail		K613984		FJ27146 FJ27147 FJ27148 JQ174065			K614067
Ralloidea	Rallidae	*Aramidopsis*	*plateni*	Snoring rail			JQ360466				JQ347988
Ralloidea	Rallidae	*Coturnicops*	*exquisitus*	Swinhoe's rail	NC_12143						
Ralloidea	Rallidae	*Crex*	*Crex*	Corn crake		KC613986		GQ481654 GU571355 GU571845 GU571846			KC614069
Ralloidea	Rallidae	*Diaphorapterix*	*hawkinsi*	Hawkin's rail†							KC614124
Ralloidea	Rallidae	*Dryolimnas*	*cuvieri*	White-throated rail		KC613987		KC614039			KC614070
Ralloidea	Rallidae	*Eulabeornis*	*castaneoventris*	Chestnur rail	NC_025501						
Ralloidea	Rallidae	*Fulica*	*atra*	Eurasian coot	NC_25500						
Ralloidea	Rallidae	*Gallicrex*	*cinerea*	Watercock		KC613997	AY447958	ABB43517 JQ342129 JQ342130 KC349319			JQ342151 JQ342152 KC439273 KC614080
Ralloidea	Rallidae	*Gallinula*	*chloropus*	Common moorhen	NC_15236						
Ralloidea	Rallidae	*Gallirallus*	*australis*	Weka	KF425525						
Ralloidea	Rallidae	*Habroptila*	*wallacii*	Invisible rail			JQ360501				JQ347984
Ralloidea	Rallidae	*Himantornis*	*haematopus*	Nkulengu rail				KC614126			

Ralloidea	Rallidae	*Laterallus*	*melanophais*	Rufous-sided crake		DQ485859	DQ485823 LMU76018				DQ485906
Ralloidea	Rallidae	*Lewinia*	*muelleri*	Auckland Island Rail	NC_025502						
Ralloidea	Rallidae	*Megacrex*	*inepta*	New guinea flightless rail			JQ360504 MIU77153				JQ347987 MIU77173
Ralloidea	Rallidae	*Micropygia*	*schomburgkii*	Ocellated crake				JQ175374 JQ175375 JQ175376			
Ralloidea	Rallidae	*Neocrex*	*erythrops*	Paint-billed crake		KC614011		KC614050			KC614095
Ralloidea	Rallidae	*Nesoclopeus*	*woodfordi*	Woodford's rail		KC614012	JQ360506 JQ360505				JQ348018 JQ348017 KC614096
Ralloidea	Rallidae	*Pardirallus*	*maculatus*	Spotted rail				JQ175674			KC614114
			nigricans	Blackish rail		KC614020		KC614054			KC614104
			sanguinolentus	Plumbeous rail		KC614025		FJ027940 JQ175675 JQ175676			KC614113
Ralloidea	Rallidae	*Porphyrio*	*porphyrio*	Purple swamphen / pukeko	NC_25508						
Ralloidea	Rallidae	*Porzana*	*carolina*	Sora crake		DQ485862	DQ485826	AY666466 DQ433143 DQ433993 HM033711			DQ485909 AY509685
Ralloidea	Rallidae	*Rallina*	*eurizonoides*	Slaty-legged crake	NC_12142						
Ralloidea	Rallidae	*Rallus*	*longirosris*	Mangrove rail		DQ485861	DQ485825 JQ360507 RLU6016	DQ433164			DQ485908 JQ348019

Ralloidea	Rallidae*	*Canirallus*	*beankaensis*	Tsingy wood rail					HQ403665 HQ403667	HQ403657 HQ403663	HQ403669 HQ403671
Ralloidea	Rallidae*	*Canirallus*	*kioloides*	Madagascan wood rail					HQ403664	HQ403653 HQ403654 HQ403655 HQ403656 HQ403662	HQ403668 HQ403670
Ralloidea	Sarothruridae	*Sarothrura*	*rufa*	Red-chested flufftail		KC614033		HQ997939			JC614121

Table 2. Partitions and model schemes used for phylogenetic analyses as determined by PartitionFinder v1.1.1.

Locus	Complete Alignment		Incomplete Alignment	
	Partition	Model	Partition	Model
tRNA-Phe	1	GTR+I+G		
12s-rRNA	1	GTR+I+G	1	GTR+I+G
tRNA-Val	4	TVM+G	1	GTR+I+G
16S-rRNA	1	GTR+I+G	1	GTR+I+G
tRNA-Leu1	2	K80+I		
ND1	3	GTR+I+G		
tRNA-Ile	2	K80+I		
tRNA-Gln	2	K80+I		
tRNA-Met	4	TVM+G		
ND2	3	GTR+I+G		
tRNA-Trp	4	TVM+G		
tRNA-Ala	4	TVM+G		
tRNA-Asn	1	GTR+I+G		
tRNA-Cys	1	GTR+I+G		
tRNA-Tyr	4	TVM+G		
COX1	5	GTR+I+G	2	GTR+I+G
tRNA-Ser1	2	K80+I		
tRNA-Asp	4	TVM+G		
COX2	5	GTR+I+G		
tRNA-Lys	1	GTR+I+G		
ATP8	6	HKY+I+G		
ATP6	3	GTR+I+G	2	GTR+I+G
COX3	5	GTR+I+G		
tRNA-Gly	4	TVM+G	1	GTR+I+G
ND3	3	GTR+I+G	2	GTR+I+G
tRNA-Arg	4	TVM+G		
ND4L	3	GTR+I+G		
ND4	3	GTR+I+G		
tRNA-His	4	TVM+G		
tRNA-Ser2	5	GTR+I+G		
tRNA-Leu2	4	TVM+G		
ND5	3	GTR+I+G		
CYTB	3	GTR+I+G	2	GTR+I+G
tRNA-Thr	4	TVM+G		
tRNA-Pro	4	TVM+G		
ND6	6	HKY+I+G		
tRNA-Glu	4	TVM+G		

Table 3. Calibration priors used for molecular clock estimates.

Taxon Split	Fossil taxon	Taxon age (Ma)	Prior
Procellariiformes | Sphenisciformes	*Waimanu tuatahi*	60.5-61.6	Lognormal (Mean 1, S.D. 1, Offset 60.5)
Gruoidea | Ralloidea	*Messelornis cristata*	>47	Uniform (47-65)
Aptornis| Heliornis	*Aptornis proasciarostratus*	16-19	Uniform (16-60)
Gruinae | Balearicinae	*Grus afghana* & *Grus miocenus*	>12	Uniform (12-60)

Table 4. Molecular age estimates (in Ma) as inferred from molecular clock analyses (* represent nodes with calibrated priors).

Node	Clade	Mean	Median	95% Standard Deviation
Root	Passerea (non-inclusive of Otidae)	66.261	65.2925	5.1255
Procellariiformes | Sphenisciformes*	Austrodyptornithes	63.86	62.811	3.3043
Gruoidea | Ralloidea*	Gruiformes	55.837	55.5828	5.1298
Psophia | Gruidae	Gruoidea	49.136	49.1226	6.2691
Gruinae | Balearicinae*	Gruidae	26.647	26.0529	7.9728
Aptornis | Heliornis *	"heliornithoids"	35.382	35.4331	6.1224
(*Aptornis + Heliornis*) | Rallidae	Ralloidea	43.1985	43.1187	5.6338
Rallidae	Crown Rails (- *Canirallus*)	31.3051	30.9468	5.099
Aptornis defossor - A. otidiformis	Modern *Aptornis* (N / S Is.)	1.504	1.3254	0.8094

Figure 1. Near-complete articulated skeleton of *Aptornis defossor* (South Island adzebill). Note this *Aptornis* skeleton is missing detachable lachrymals, which enclosed the front half of the orbit. Image source: Wikimedia commons (public domain).

Figure 1. Beast maximum clade-credibility consensus tree of the 'complete' datatset utilising only taxa with complete mitochondrial genomes. Scale represents time as in millions of years (Ma) before present. Families/Subfamilies and superfamilies designated on right. Error bars represent 95% standard deviation estimates from mean node age estimates. Node numbers represent bayesian % posterior support values
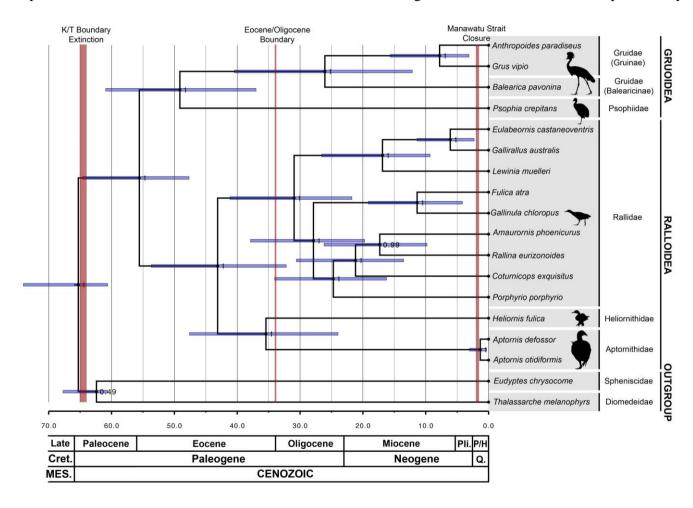
Figure 2. Bayesian phylogenetic analysis of the 'incomplete' dataset using a total of 8 mitochondrial loci. Node values show % posterior probability support. Coloured bars represent biogeography of each genus (inclusive of congeneric unsampled species).
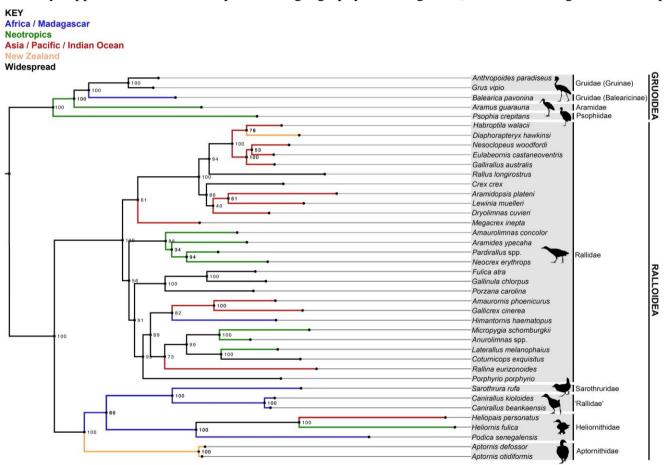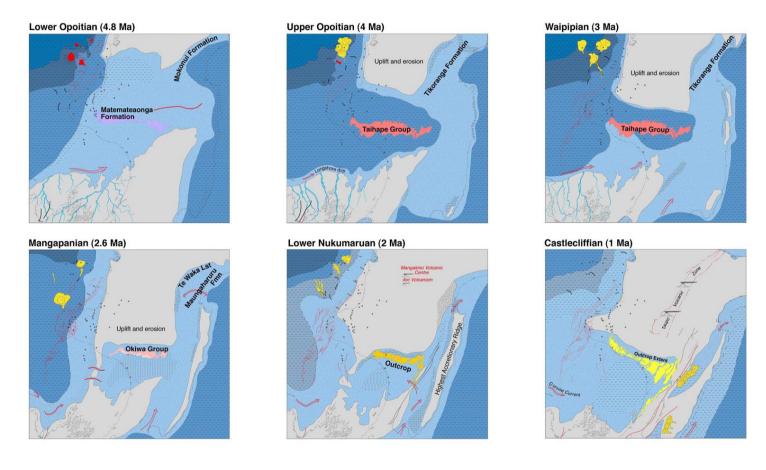
Figure 3. Changing geographic landscape of central New Zealand during the Miocene-Pleistocene, and closure of the 'Manawatu strait' permitting faunal exchange between the North-South Island fragments for the first time since 30 Ma. Fossil and molecular evidence supports that both moa and *Aptornis* evolved on the lower Southern fragment later dispersing north. Note the modern Cook Strait did not appear until ~0.45. Ma. Modified from Bunce *et al.* [44]

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CHAPTER FIVE

General Discussion

5.1   General Summary, significance, and limitations of this thesis

This thesis includes some of the few studies to have used next-generation sequencing (NGS) to research the recently extinct bird fauna of New Zealand. Overall, the scope of this work is broad, and themes covered include co-extinction, lost species interactions, prehistoric animal behaviour and diet, parasitology, evolutionary biology, biogeography and phylogenetics. However, all concern the extinct avifauna of New Zealand, focusing on the interconnected areas of ecology and evolutionary biology – neither of which can be understood in isolation without regarding the other. The avifauna of New Zealand comprised the majority of the large vertebrate fauna, therefore are key in understanding the greater New Zealand ecosystem and its long and complex history. However, this extraordinary community has been so drastically impacted by human activity, that ancient DNA (aDNA) data is necessary to understand it in relative completion.

Of special significance were the metabarcoding and metagenomic studies of avian coprolites. These methods have been applied to very few animal coprolites in past studies, comprising shotgun sequencing of a cave hyena coprolite (which focused on host and mammalian prey DNA), and plant-specific metabarcoding of coprolites from Pleistocene megafauna [1,2]. Not only were we able to determine the effectiveness of these methods to elucidate ancient species interactions, we were able to obtain entirely new and original results not found in years of prior aDNA study on moa coprolites. This included the first direct observation that New Zealand's extinct moa on occasion fed on fungi – including taxa mycorrhizal to some of New Zealand's most important forest-forming tree species (southern beech). Possible co-extinction was observed between moa and a whole clade of parasitic taxa. Finally, several subtle indicators for moa behaviour were also inferred from the data.

The shotgun data analysis also revealed some new key results. For example, DNA of methanogenic bacteria was identified from moa coprolites and supports some degree of gut fermentation in these extinct animals, which is not a universal feature among herbivorous birds [3–5]. I was also able to determine that some of the richest (yet most problematic from an ancient DNA perspective) coprolite deposits from semi-arid areas in the south-central South Island are unlikely to provide any genetic data.

This unfortunate result may demonstrate that alternative sequencing or extraction methods of these samples may need to be trialled.

Finally, the last research chapter of this thesis (Chapter Four) resolved the phylogenetics of one of New Zealand's most mysterious members of the bird fauna (*Aptornis*). It was revealed that this mysterious bird taxon fell within an unusual clade of several small bird families, which today have a largely African distribution. This striking biogeographical discordance has been observed between a few other New Zealand and Afrotropical bird taxa [6,7], and likely represents extinction of biogeographical intermediates. This in turn may demonstrate the unusual capacity of New Zealand to retain relictual evolutionary lineages [8]. Furthermore, this taxon is demonstrated as having a considerable age (~35 Ma), representing one of the oldest members of the endemic avifauna and perhaps supporting that this lineage persisted through marine inundation of New Zealand during the 'Oligocene drowning' event (~34-23 Ma)

However, there were a considerable number of challenges and limitations from these projects. Many of these were largely logistic. For example, the metabarcoding coprolite study (Chapter Two) was constrained to a small number of samples spread between a wide number of sites and species. Therefore interpreting species/environment-specific results could not be made with complete confidence. The shotgun sequencing study (Chapter Three) was limited due to low sequencing depth, and was again confounded by limited samples. In addition, this shotgun study did not sequence extraction blanks to control for laboratory contaminants. This was as the major objective was to determine whether DNA persisted in several of the samples (principally by identifying whether moa DNA was present). However this study design limited the level of new paleoecological data that could be inferred. Finally, the *Aptornis* study (Chapter Four) was restricted by available genetic data, as relatively few new sequences were developed for this study.

The remainder of this discussion examines specific aspects of this thesis in more detail, and proposes future research directions. These are divided firstly to a section regarding the coprolite studies, and a second for the *Aptornis* study. A final third section proposes a number of indirectly related projects on New Zealand's biota (with a particular focus on aDNA and the avifauna), all of which aim to resolve problems discussed throughout this thesis.

5.2 Ancient DNA of late Quaternary animal coprolites from New Zealand

5.2.1 Metabarcoding and metagenomic studies

As presented throughout Chapters Two and Three, NGS methods to obtain DNA from coprolites presented several considerable advantages over Sanger-sequencing and cloning methods. However, there were several important issues that greatly impeded resolution. For the metabarcoding study (Chapter Two) the study was entirely restricted to a single locus (18S rRNA). While useful for obtaining a broad diversity of all eukaryotes, use of this single locus and 'universal' primers (amplifiable across most eukaryotes) presented at least two major limitations.

Firstly, the broad specificity meant that huge numbers of read sequences originated from environmentally abundant, and often ecologically uninformative taxa. For example, massive read counts were identified as a small number of environmentally abundant ascomycete fungi taxa. Whether these represented endogenous DNA sequences or modern contaminants could not be determined. Furthermore, their great abundance may have even prevented informative species being sequenced. This resulted in a large redundancy of data, and a reduced level of efficiency.

Secondly, identification of this data depended heavily on the availability and quality of 18S rRNA reference data. Although a relatively wide taxonomic diversity of 18S rRNA sequences are available in several databases allowing for identification of sequenced data, this locus is nonetheless under sampled for several major taxa compared to other loci. For example, 'DNA barcoding' projects have elected to use the loci ITS (internal transcribed spacer) for fungi, *rbc*L or *trn*L for green plants, or COX1 (cytochrome oxidase I) for Metazoa [9–14].

Both these two limitations can be easily overcome through use of taxon specific primers, utilising only well-referenced loci. Therefore, although 18S rRNA may provide an informative broad census of DNA content, targeted studies (including future studies on moa coprolites) would greatly benefit from utilising a variety of primers and loci.

We also utilised a metagenomic, shotgun approach. The issues and advantages with this particular method are discussed in Chapter Three. In general, shotgun methods result in a massive reduction in efficiency, as this method generally represents a random sample of the whole DNA content or 'metagenome'. Most reads result from obscure loci and therefore cannot be identified, and there is no control for sequencing of abundant contaminating taxa such as bacteria. Nonetheless, shotgun methods provide some considerable advantages, in particular a lack of restriction to a particular fragment length (thus allowing both short and long reads alike to be amplified and sequenced). RNA-DNA hybridisation utilising custom baits specific to particular taxa or loci may greatly improve efficiency, but may not currently be cost-effective. Furthermore, this approach may be the only way to amplify extremely variable loci across a broad diversity of taxa. For example, the fast evolving fungal 'barcoding' gene ITS is widely amplifiable due to the presence of conserved loci at either end of the variable region [9,10]. However at >200bp, this fragment length is generally too long to be amplifiable for aDNA using conventional PCR.

5.2.2 *Prehistoric avian dispersal of New Zealand's mycorrhizal fungi*

An important result in this thesis was the identification of likely dietary fungi from moa coprolites. In the metabarcoding study these comprised species of the mycorrhizal genera *Cortinarius* and *Inocybe*, as well as the phytopathogenic *Armillaria*. However, it is plausible these represent environmentally contaminating taxa. Alternative mechanisms for which DNA may arrive in the coprolite are post-depositional – including contamination by aerobic spores, or by invasion of the coprolites by the fungal hyphae. Alternatively they may be pre-depositional, in that moa were passively consuming fungi either through the ingestion of spores, or they were ingesting soil-borne hyphae with uprooted herbs or swallowed gizzard stones (gastroliths).

However, these particular species, being mycorrhizal or phytopathogenic, are entirely co-dependent with plant roots, and are extremely unlikely to invade any coprolites. All moa also consumed gizzard stones, therefore these fungi have been widespread among moa if this was the source of spore ingestion [15]. Furthermore, aerobic spores of these ubiquitous forest fungi would likely settle throughout the

environment. Therefore, we infer that if these fungal DNA sequences were largely environmental then they would be found in most coprolites at low abundance. However, instead their presence was restricted to species that are known to feed on forest herbs (upland moa, giant moa) or the kakapo, a confirmed mycovore [16]. These fungi were found only in isolated examples, suggesting a wholly non-random distribution. Finally, they also occurred in high read counts, which would suggest that if consumed by these particular birds, then they were being consumed in a greater abundance than would be suggested by a feeding artefact (e.g. ingestion through soil). However, the possible ingestion of soil hyphae still remains the most likely alternative explanation, which could be rejected or confirmed by identification of fungal spores in the coprolite matrix.

Two of the inferred dietary fungi are mycorrhizal to some of the most important forest tree species in New Zealand (southern beech) [17]. Therefore, it is possible that moa may have played a role in dispersal of these fungi, affecting the health and dispersal/regeneration capacity of New Zealand's forests. New Zealand also has a diversity of 'sequestrate' (fruiting mushroom bodies, being entirely enclosed and preventing unassisted spore release) fungal species, most of which are mycorrhizal [18–20]. In addition, many of these are brightly coloured – in particular shades of red, or less commonly blues and purples. These appear superficially similar to plant 'berries' and almost certainly represent adaptations for bird dispersal. However, most of New Zealand's likely mycovorous birds are extinct (e.g. moa, or most flightless rails) or endangered (such as the kakapo parrot). These sequestrate species are a diverse non-monophyletic group of several species, a large number of which fall in the genus *Cortinarius* (which was found in some moa coprolites) [20]. However, we were unable to identify these to specific species, and therefore whether these features represent adaptations for bird dispersal remains unconfirmed. Future metabarcoding studies using well-referenced fungal loci (in particular ITS) on forest-dwelling moa and on the kakapo, will be needed confirm the presence of any particular species of these fungi in the coprolites.

5.2.3 Parasite-host co-extinction

The extinction of parasites may be among the largest unidentified sources of biodiversity loss worldwide [21]. Parasites represent ubiquitous yet poorly understood components of all ecosystems and are believed have important effects on trophic systems, mitigation of competition and host behaviour [21,22]. In addition, due to the common involvement of secondary or even tertiary hosts, removal of a parasitic taxon may directly impact a number of host taxa. Finally, research on parasitic species can provide important insights into host behaviour, diet and even evolution.

Ancient DNA from moa coprolites (both in our study and that of Wood *et al.* [23]) identified a diversity of parasites, many only being identified to family level or above. Furthermore, most were genetically divergent from any reference species already sequenced. Of particular interest was a diversity of related species of nematodes identified as members of the superfamily Heterakoidea. In New Zealand, morphologically distinctive and hypothetically 'Gondwanan' members of the Heterakoidea have been identified from the tuatara (*Hatterianema*), and the kiwi (*Kiwinema*), together comprising the proposed 'Kiwinematidae' [24,25]. A final, possible member of this family has also been identified from African mole rats [26]. However, genetic information for any members of this group is unavailable, and we were also unable to locate any specimens. Therefore, DNA from modern kiwi or tuatara scats may be required. If a relationship between these parasites is discovered, this would provide a fascinating insight into the evolution of their particularly ancient host species. Similar research on other extant ratites (such as emu or ostrich) may also help to understand the phylogenetic relationships of moa parasites, for example members of the Eimeriidae.

The relationship between closely related species of parasites we identified was unclear. For example, we identified evidence for as many as nine species of heterakoid nematodes from moa, however we were unable to obtain more than a short fragment of DNA from each taxon. This provided insufficient resolution to even confirm monophyly of these species. Depending on DNA quality, longer fragments may be sequenced utilising deeper shotgun sequencing (no nematodes were obtained from our low-depth shotgun analysis), or parasite specific primers. If monophyly and host-specificity to moa of these taxa can be confirmed, this would provide a rare observed example of an entire clade of hosts and parasites becoming co-extinct.

A final and much more technically complex analysis would be to obtain a genome of a host-specific moa parasite. Due to the comparatively short genomes of parasitic taxa (for example, nematodes) this would be considerably less costly an labour intensive than sequencing and assembling an entire moa genome [27]. Subsequently, the population demography of the parasite genome could then be tracked using analyses such as Pairwise Sequentially Markovian Coalescent (PSMC) modelling [28], and used as a proxy for that of the moa hosts. A parasite genome could be sequenced relatively easily by isolating individual parasite eggs from coprolite matrix for use in high-depth NGS sequencing. However, the host specificity or host-range of individual parasite species would require confirmation. For example, host specificity was either unsupported or unconfirmed for the parasites sequenced in this thesis, although specificity to moa as a group seems likely. If the parasite was moa specific (rather than specific to one moa species), this analysis would instead serve as a proxy for fluctuations in the populations for moa as a whole. This would present a unique research opportunity, as a single demographic analysis of all moa is impossible using a single moa species alone.

5.2.4 *Paleoecological and parasitic changes of the kakapo parrot*

Aside from moa, considerable numbers of coprolites from several deposits in the South Island are believed to originate from the extant but critically endangered kakapo parrot (*Strigops habroptilus*) [29–31]. Four kakapo coprolites were included in Chapter Two, however only limited information could be obtained from these samples (which may be further resolved using more informative or taxon-specific metabarcoding loci). The kakapo declined from a population of hundreds or tens of thousands to <60 in a few centuries [16,32]. Therefore, examination of kakapo coprolites in detail will provide valuable data into understanding several of the more subtle impacts of species decline. This may include identifying important roles kakapo had as dispersers (including dietary plants and fungi) or pollinators in New Zealand's ancient ecosystem. This would also reveal whether endemic kakapo parasites became extinct due to a host-population decline, or whether new parasites were introduced from human arrival. Furthermore, this data would also make a major contribution to understanding the ecology of an intensely conservation-dependent species.

5.2.5   *Further aDNA coprolite studies*

Late Quaternary aged animal coprolites from a diversity of species have been found throughout New Zealand [31]. Among these are coprolites from introduced mammals including the Pacific rat, or Polynesian dog (kuri). DNA from Pacific rat coprolites may reveal which native species were being consumed, and clarify the extent of early rat-mediated extinctions in New Zealand. Dog coprolites instead may serve as a proxy for the species consumed by the human communities with which they occurred [33–35]. Finally, huge varieties of coprolites of unknown host provenance have been found in semi-arid areas of the south-central South Island, which should be a priority for future studies (examined specifically in Chapter Three).

5.3   The adzebills: an evolutionary and ecological enigma

5.3.1   *Phylogenetic analyses and additional taxon sampling*

Although our dataset resolved the phylogenetic relationships of *Aptornis*, as outlined earlier, this dataset was in many ways incomplete. Much of the confidence of our analysis rested on the use of complete mitochondrial genomes, in particular for analyses such as molecular dating. However, several key taxa (in particular the remaining non-aptornithid 'heliornithoids' (non-Rallidae ralloids) comprising *Podica*, *Heliopais*, *Canirallus* and *Sarothrura*)) could not be included in this analysis, due to a lack of available mitochondrial genomes. We were able to include all these taxa in larger dataset, using more taxa at the cost of reduced resolution. However, as we elected not to use molecular dating on this dataset (as a result of low confidence in the incomplete data), the evolution of the heliornithoids, including *Aptornis*, could not be interpreted in full. In addition, a small number of rail genera were missing from the entire analysis. Given the relatively unexpected phylogeny of the 'rail' *Canirallus* within the heliornithoid clade, it is plausible other accepted Rallidae may have similar relationships. The phylogeny of the Rallidae itself also remains incompletely resolved, especially regarding the diversity of recently extinct island taxa (also see

section 5.4.2). Therefore the early biogeography of this family could not be resolved, and limiting our ability to infer much regarding the evolutionary history of this group.

5.3.2   *Population genetics and paleoecology*

The ecology of *Aptornis* was not investigated, although the phylogenetic relationship within the Ralloidea provided ground for some hypotheses. However a multidisciplinary study would provide important details into paleobiology and behaviour of this bizarre bird taxon. Population genetics, (although labour intensive using ancient bone material) may elucidate population structure and dispersal capacity. If the apparent specific habitat requirements of *Aptornis* are correct (general restriction to lowland, well-drained forest, woodlands and shrubland not dominated by southern beech), then it would be expected that *Aptornis* had high population structuring, and could not disperse across several habitat types. For example, populations in Southland may have been genetically isolated from those in Otago and Canterbury, due to a narrow, continuous belt Southern beech forest that once occurred between these regions [36] (now largely deforested).

No coprolites are currently assigned to *Aptornis*, therefore diet of either species cannot be observed directly. However, isotopic analyses have been conducted on co-occuring *Aptornis,* herbivorous moa and an extinct species of insectivorous owlet-nightjar [15], which revealed that *Aptornis* occupied the highest trophic level of the three species. More such analyses using a number of deposits and other species, especially known predators (such as birds of prey), or even other rails (such as the weka *G. australis*), would be considerably more informative. Ultimately however, inferring the niche of *Aptornis* may also rest heavily on identifying the reasons for its habitat restrictions. This would therefore require identifying the specific paleoenvironments where *Aptornis* occurred, whilst correcting for vegetation changes during the Pleistocene [15]. Furthermore, identifying the niche of *Aptornis* may also require a more complete understanding of the distribution, abundance and diversity of possible prey, such as reptiles, invertebrates and seabirds (see sections 5.4.3-4).

5.4   Evolution and paleoecology in New Zealand: outstanding questions

Overall, this thesis investigated a number of questions on the paleoecology and evolution of New Zealand's biota, with a specific focus on the extinct avifauna. Although each chapter concerned specific taxa and objectives, many of our results and hypotheses were connected with a variety of other ecological or evolutionary problems. A number of additional future projects regarding subjects that have been discussed at different points throughout this thesis are outlined briefly (although each of these relate more specifically to Chapter Four). Due to extensive extinction of New Zealand's animal species, many of these projects will require at least some input of ancient DNA data.

5.4.1   Biogeography of New Zealand in the Late Paleogene-Neogene

For ~28 Ma, New Zealand was divided into the early precursors of the current North and South Islands. These islands first separated around ~30 Ma, and only re-joined around ~2 Ma with the closing of the 'Manawatu Strait'. In Chapter Four (phylogenetics of the adzebills), it was discussed how some members of New Zealand's flightless avian biota (including both *Aptornis* and moa) evolved on the larger southern fragment, and only dispersed into the northern fragment during the Pleistocene (<2.5 Ma). Furthermore, ancient fauna of the southern fragment is well represented by the 16-19 Ma St Bathans fossil fauna, which includes ancient forms of kiwi, moa, *Aptornis,* nestorine parrots, acanthisittid wrens and other taxa. However, presumably a diversity of species evolved in the northern fragment in complete isolation from that of the South. This 'northern' fauna remains a total enigma. Whether any of these species persisted until present will require a considerably more extensive review and molecular dating analyses of the endemic biota to be conducted.

5.4.2   Discordance with fossils and present phylogenetics: evolution of New Zealand's rail and waterfowl faunas

The St Bathans fossil fauna as outlined above comprised a diversity of avian species. Among these were an assemblage of rails (Rallidae or Ralloidea) and waterfowl (Anatidae) [37,38]. However, almost all modern endemic members of these families appear to have extremely recent origins, and have likely been in New Zealand no longer than the Pleistocene [39]. This suggests that a considerable faunal turnover has occurred. Understanding the evolution of Rallidae and Anatidae in New Zealand may in part be resolved by DNA data, yet both families have suffered considerable extinction in the past ~700 years (therefore aDNA methods will be required) [15,40]. Furthermore, these recent colonisers can provide fascinating insights into macroevolutionary processes. For example the extinct Finsch's duck (*Chenonetta jubata*) demonstrates a measurable historic reduction in wing size continuing to present [41]. Another project, and one that directly relates to Chapter Four, would be to more thoroughly examine the evolution of New Zealand's rails. Among these are five species believed to represent separate colonisations of the *Gallirallus* clade (which would extend to six if the extinct Macquarie rail, *G. macquariensis* is included) [40], which have yet to be studied in depth [42–44]. These species together demonstrate a fascinating example of evolutionary niche partitioning and macroevolution.

5.4.3   New Zealand's seabird communities

As presented in the introduction, New Zealand's breeding seabirds may have occurred in great numbers, and would have had a massive ecological impact in both marine and terrestrial environments [15,45]. For example, seabirds would have offloaded enormous amounts of marine-derived nutrients onto the land, and had a direct impact on vegetation structure, land topography and invertebrate communities [15]. They would have also provided an important source of seasonal prey (such as for *Aptornis*) [45]. However, there are few paleoecological analyses to test the wider impacts of the seabird community. DNA may provide direct evidence of seabird population decline, most feasibly through a combined population genetic analysis of subfossil bones and modern birds. Furthermore, environmental DNA of areas hypothesised to have

represented ancient colonies may provide some insight. Finally, non-DNA research on common environmental indicators such as tree rings may identify whether a periodic offload of marine nutrients was occurring, if it suddenly ceased.

5.4.4 *New Zealand's lost invertebrate and herpetological faunas*

This thesis focused almost entirely on the New Zealand avifauna. However, New Zealand's birds represent only the larger members of a once complex ecosystem, which also included a diversity of lizards, tuatara, frogs, snails, insects and spiders. However, an oft-quoted 'plague' of the pacific rat doubtless extensively destroyed much of the smaller fauna soon after the arrival of human colonists [15,46]. Remains of New Zealand's herpetofauna are common in subfossil deposits (such as in caves) yet are often too small or lacking in osteological characteristics to allow for any taxonomic assignment [15]. Therefore 'bone-grab' ancient DNA analyses of such material [47] would no doubt provide fascinating and entirely new information on this aspect of the New Zealand ecosystem.

Even more poorly known is New Zealand's invertebrate fauna, which even today still includes several extraordinary, and highly endangered taxa. Among these are the cricket-like giant weta (*Deinacrida* spp.), some of the heaviest insects in the world (71g, 3 × heavier than a house mouse), and large carnivorous snails in the Rhydidae [48,49]. It is likely these now rare taxa originally played a much greater functional role in New Zealand's ecosystems. The preservation record of invertebrates is poor, yet some Holocene-aged extinctions of large New Zealand insects have nonetheless been identified [50]. Identifying the total diversity and ecology of this particular lost community may only be resolvable by use of environmental, ancient DNA analysis.

5.5   Concluding remarks

New Zealand is unique in a number of important regards. Firstly, it is the only large continental-aged landmass, isolated enough to have generally lacked indigenous

mammals. With the exception of a mysterious extinct, shrew-sized terrestrial mammal from fossils 16-19 Ma in age [51], or a few species of bat which arrived by flight, there is no evidence that mammals ever comprised a significant portion of the native fauna. Therefore, this niche space was filled by invertebrates, reptiles, and most importantly, native birds. No other landmass of its size or age is known to have had a similar fauna. Secondly, it is also the only large continental-aged landmass to have been settled by humans within the last 1,000 years. This feature is also complemented by a climate and landscape (e.g. caves / high-altitudes) also amenable to biological preservation, Therefore, the pre-human ecosystem and its evolution can be understood in far greater completion than other continental landmasses, or even recently settled islands.

The long isolation of New Zealand and its unusual species assemblage no doubt contributed to the development of a complex and deeply interconnected ecosystem which we still barely understand. The recent extinction of so many of its endemic species has greatly increased the challenge of elucidating its origins and how it functioned. Doing so requires a multidisciplinary approach. Among the newest of these disciplines is ancient DNA, which provides some of the highest-resolution forms of data currently available. Recently, next-generation sequencing became widely available and as a result we are now on the cusp of a new age of exploration. Further application of the approaches used in this thesis will lead to a greater understanding of New Zealand's reduced and remarkable fauna. New Zealand's unique ecosystem may never truly recover, and the loss of so much of its fauna will always represent a great tragedy. However, ancient DNA will help us to finally see it as it once was.

5.6   References

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