# Ancient DNA studies of the New Zealand kiwi and wattlebirds: evolution, conservation and culture.

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# Abstract

Ancient DNA was used to provide a temporal perspective for examining a number of evolutionary, conservation and cultural questions involving members of the New Zealand avifauna. Ancient mitochondrial DNA (mtDNA) sequences were used to examine the past levels and patterns of genetic diversity in the five species of New Zealand kiwi (Apterygidae). Brown kiwi, particularly in the South Island, exhibited high levels of genetic structuring with nearly every population exhibiting private mitochondrial haplotypes. The extinction of a large number of brown kiwi populations has, therefore, led to the loss of a large amount of genetic variation in these species. The past ranges of great spotted kiwi and the three brown kiwi species, whose bones are morphologically indistinguishable, were determined. This information can aid conservation programmes aiming to re-introduce kiwi to regions where they are now extinct.

In contrast to the high level of genetic structuring in South Island brown kiwi, the majority of little spotted kiwi samples from the South Island shared a common haplotype. The difference in phylogeography between brown kiwi and little spotted kiwi is hypothesised to relate to differences in their dispersal behaviour and/or their population histories. The addition of ancient samples of little spotted kiwi from the North Island indicated a complex relationship with great spotted kiwi.

Nuclear microsatellite DNA markers were isolated from North Island brown kiwi and tested for cross amplification in the other kiwi species. Five loci were polymorphic in all kiwi species. Preliminary analyses of genotyping results indicated that the kiwi species were distinguished by assignment tests and that subdivision may occur within several of the species.

An extensive reference database of modern and ancient mtDNA sequences was used to determine species and provenance of a number of unlabelled museum subfossil bones and skins. This method was also used to examine provenance of brown kiwi feathers from Maori artefacts (cloaks and baskets).

Ancient DNA methodology was also used in a molecular examination of the relationships of a second endemic avian family, the New Zealand wattlebirds (Callaeatidae). Analyses of nuclear gene sequences, *c-mos* and RAG-1, revealed kokako, saddleback and huia comprised a strongly supported monophyletic group. A divergence time estimate for the New Zealand wattlebirds indicated that they are more likely to have arrived by transoceanic dispersal than have a Gondwanan origin. Sequences from three mtDNA genes, 12S, ND2 and cytochrome b, were also analysed but could not resolve the relationships between the three genera.

Microsatellite DNA from the extinct New Zealand huia exhibited considerable genetic variation, exceeding that found in extant North Island saddleback, from which the loci were isolated. Assignment tests indicated no genetic structuring within huia, although interpretation was complicated by a lack of provenance details for many of the skins.

The results presented here suggest that ancient DNA can not only provide information about the relationships of extinct taxa but also demonstrates the importance of placing the present day genetic diversity found in endangered taxa within the context of past patterns and levels of genetic variation.

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# CHAPTER ONE

## General Introduction

This thesis focuses on the application of ancient DNA methods to a number of evolutionary, conservation and cultural issues involving two endemic New Zealand avian families: kiwi (Apterygidae) and the New Zealand wattlebirds (Callaeatidae). This introductory chapter provides background information on the topics of ancient DNA, kiwi and the New Zealand wattlebirds.

*Ancient DNA: a window to the past*

Ancient DNA provides a direct historical perspective for studies of molecular genetics and evolution by enabling the isolation of DNA from plant or animal remains. The first ancient DNA study involved the cloning of DNA from the 150-year-old skin of an extinct quagga (Higuichi et al., 1984). Following this initial study, considerable advances in molecular biology have been made and DNA has since been successfully retrieved from a wide range of sources including bone, hair, teeth, coprolites, feathers, seeds and sediments. The amount and type of DNA sequence retrieved has also increased and whole mitochondrial DNA (mtDNA) genome (e.g., Cooper et al., 2001; Haddrath and Baker, 2001) and nuclear DNA sequences (e.g., Shepherd, 2001; Huynen et al., 2003) have now been obtained. However, the retrieval of authentic DNA from ancient biological tissues is restricted by the *post mortem* decay of DNA. DNA spontaneously degrades over time, mainly through strand breaks, hydrolysis and oxidation (Lindahl, 1993). The degree of degradation has been demonstrated to vary with both the tissue type and preservation environment, with bones preserved in cool, dry conditions lacking micro-organisms most favourable for ancient DNA survival (Burger et al., 1999). The small quantities and degraded nature of surviving DNA, and therefore, the high risk of contamination from modern DNA, presents technical difficulties when working with ancient DNA. Suggestions to overcome these problems include the physical isolation of ancient DNA extractions and PCR set-up, the use of negative controls for extraction and PCR, independent replication in a separate laboratory, amplifying short overlapping DNA fragments, performing multiple,

independent PCRs and cloning and sequencing of several clones (Pääbo, 1989; Handt et al., 1994; Cooper and Poiner, 2000; Pääbo et al., 2004).

*The applications of ancient DNA*

Ancient DNA has most commonly been used to examine the systematics of extinct organisms (Wayne et al., 1999), particularly using mitochondrial DNA sequences, but recent studies have encompassed a far wider range of research topics. For example, ancient DNA has allowed the detection of changes in the genetic variation of populations over time, in both the distribution (e.g., Leonard et al., 2000; Shepherd, 2001; Goldstein and DeSalle, 2003; Hofreitner et al., 2004) and levels of diversity (e.g., Bouzet et al., 1998; Shapiro et al., 2004). One important point evident from these studies is that interpreting patterns and levels of genetic variation solely from modern samples may be misleading (Pääbo, 2000; Matocq and Villablanca, 2001). The retrieval of DNA from large numbers of subfossil bones, combined with radiocarbon dating, has permitted the estimation of rates of evolution for the mitochondrial control region of Adélie penguins (Lambert et al., 2002) and bison (Shapiro et al, 2004). DNA has been recovered from coprolites (e.g., Hofreitner et al., 2000) and sediments from permafrost and caves (Willerslev et al., 2003), allowing past ecosystems to be investigated. Ancient DNA has permitted insight into human history, particularly aspects of migration (e.g., Matisoo-Smith and Robins, 2004), trading (Arndt et al., 2003) and the domestication of plants and animals (e.g., Leonard et al., 2002; Jaenicke-Després et al., 2003; Larson et al., 2005).

*Previous ancient DNA studies in New Zealand*

New Zealand has an extensive, well-characterised Holocene subfossil record (Worthy and Holdaway, 2002) and is therefore an ideal location for studies using ancient DNA. Many of the remains of extinct species and populations are relatively young owing to the late settlement of humans in New Zealand (approximately 800 years ago; McGlone and Wilmshurst, 1999; Holdaway and Jacomb, 2000); therefore, the DNA is likely to be reasonably well preserved. However, the full potential of ancient DNA research in New Zealand is only just beginning to be explored.

The majority of studies of New Zealand taxa that have employed ancient DNA methodology have, as in early ancient DNA studies overseas, examined the systematics

of extinct organisms: e.g., moa (Cooper et al, 1992; Cooper et al., 2001; Haddrath and Baker, 2001; Huynen at al., 2003; Bunce et al., 2003; Lambert et al., 2005; Baker et al., 2005), adzebill (Houde et al., 1997), piopio (Christidis et al., 1996), North Island takahe (Trewick, 1997), huia (Tebbutt and Simons, 2002) and Haast's eagle (Bunce et al., 2005). More recently in New Zealand there has been a shift towards using ancient DNA for conservation and population-focused work, particularly for marine organisms. For example, studies have examined the loss of genetic diversity in the Hector's dolphin (Pichler and Baker, 2000) and the New Zealand snapper (Hauser et al., 2002). However, both of these studies involved a relatively short time frame, with samples less than 150 years old. In contrast, a recent study isolated ancient DNA sequences from sediments dated at 600 years BP from a New Zealand cave (Willerslev et al., 2003). DNA sequences were detected from animals now extinct or not currently present in the vicinity of the cave. Obtaining DNA from sediments offers the advantage of examining 'palaeoenvironments' while preventing damage to subfossil bone specimens. However, potential problems of using sediment as a DNA source include difficulties with dating owing to movement of DNA since deposition and obtaining sufficient sequence since short fragments cannot be combined as they may have derived from different individuals or taxa (Pääbo et al., 2004).

*The New Zealand avifauna*

New Zealand separated from Gondwana 82-85 mya (Cooper and Millener, 1993) and has been largely isolated from other landmasses ever since. Consequently the New Zealand biota is thought to comprise both ancient taxa present from when New Zealand separated (e.g., tuatara, moa and frogs) and taxa that have managed to successfully colonise via long distance dispersal (e.g., bats and many plant species) (Cooper and Millener, 1993; Daugherty et al., 1993). The New Zealand fauna is characterised by a high level of endemism, flightlessness, gigantism and niche shifts (Daugherty et al., 1993). Birds are a particularly well-represented component of the fauna and many are thought to have evolved to fill niches commonly occupied overseas by mammals (Daugherty et al., 1993). Prior to the arrival of humans and their commensals, 245 bird species are believed to have had breeding populations in New Zealand (Holdaway et al., 2001). Nearly a third of these have since become extinct (Holdaway et al., 2001) and almost half of the remaining land bird species have been restricted in distribution such that they are considered threatened or endangered (Wilson et al., 2004).

The two avian families investigated in this thesis are both endemic to New Zealand and are thought to have had a long history in New Zealand. Populations of species in both families have been reduced in number and range since human arrival.

*Kiwi*

Kiwi belong to the endemic New Zealand family Apterygidae which is a member of the ratite birds. Other ratites include tinamous, rhea, ostrich, cassowary, emu, and the extinct moa and elephant bird. The monophyly of the ratites is well established, in that they are all more closely related to one another than to any other birds. However, the relationships amongst the ratites are still debated, particularly the placement of the kiwi (Worthy and Holdaway, 2002 and references therein).

*Kiwi taxonomy*

There are two principal morphological groups of kiwi: brown and spotted kiwi. At least ten species of kiwi were described in the genus *Apteryx* in the nineteenth century (Herbert and Daugherty 2002), but modern kiwi taxonomy stems from Oliver (1930) and Matthews (1931). Oliver described four species: two brown kiwi species, *A. mantelli* (North Island brown kiwi) and *A. australis* (tokoeka); and two spotted kiwi species, *A. haastii* (great spotted kiwi) and *A. owenii* (little spotted kiwi). Matthews (1931) subsequently modified this classification by grouping brown kiwi into a single species, *A. australis*, with three subspecies: *A. a. mantelli* (North Island), *A. a. australis* (South Island) and *A. a. lawryi* (Stewart Island). He also moved the little spotted kiwi into a new genus, *Stictapteryx,* with three subspecies: *S. owenii owenii* and *S. o. occidentalis* in the South Island, and *S. o. iredalei* from the North Island (Matthews, 1931; 1935). Prior to the first genetic studies in the 1990s, the taxonomy of Matthews was largely followed, although little spotted kiwi was usually included in *Apteryx*, and its subspecies were not recognised.

Initial genetic studies of mtDNA and allozymes revealed an extremely high level of genetic structuring in brown kiwi (Baker et al., 1995; Herbert and Daugherty, 2002). In addition, these authors proposed that the pattern of allozyme and mitochondrial sequence variation indicated two morphologically cryptic species within brown kiwi: *Apteryx mantelli* in the North Island and a small population at Okarito in the South

Island, and *A. australis* in the south of the South Island and Stewart Island. Consequently, these genetic data were in conflict with the morphologically-based taxonomy of Oliver (1930) and Matthews (1931) where the main north-south division occurred at Cook Strait. Herbert and Daugherty (2002) also suggested that the Haast population of brown kiwi had significant genetic differences from other southern brown kiwi and possibly represented another kiwi species.

More recently, Burbidge et al. (2003) suggested that the geographically isolated brown kiwi population at Okarito be granted specific status. They reasoned that the genetic divergence of Okarito kiwi from North Island brown kiwi, as determined from mitochondrial DNA sequences, exceeded that separating the reproductively isolated great and little spotted kiwi. This species has since been named *A. rowi* (Tennyson et al., 2003) and given the common name rowi.

In summary, five species of kiwi are presently recognised; two spotted kiwi species (*A. haastii* and *A. owenii*) and three brown kiwi species (*A. mantelli*, *A. rowi* and *A. australis*). Furthermore, two subspecies of tokoeka are recognised (*A. a. australis*, south-west South Island and *A. a. lawryi,* Stewart Island). The current distributions of these species are shown in Figure 1.

*The history of kiwi in New Zealand*

Subfossil evidence suggests that kiwi, as a group, were once distributed throughout New Zealand. However, only little spotted kiwi can be positively identified by bone morphology because they are significantly smaller than both brown and great spotted kiwi (Worthy and Holdaway, 2002). Little spotted kiwi remains have been found throughout the North and South Islands. The other kiwi species cannot be distinguished by either bone shape or size (Worthy, 1997). Therefore, their precise past distributions are unknown.

It is assumed that kiwi numbers began to decline with the arrival of humans and their commensals. Maori hunted kiwi for meat, skins and feathers with dogs and traps (Peat, 1990). The arrival of Europeans and the species they introduced (cats, dogs, possums and mustelids) resulted in a further decline of kiwi numbers (Heather and Robertson, 2000). Europeans cleared large areas of forest for pasture, thus reducing kiwi habitat.

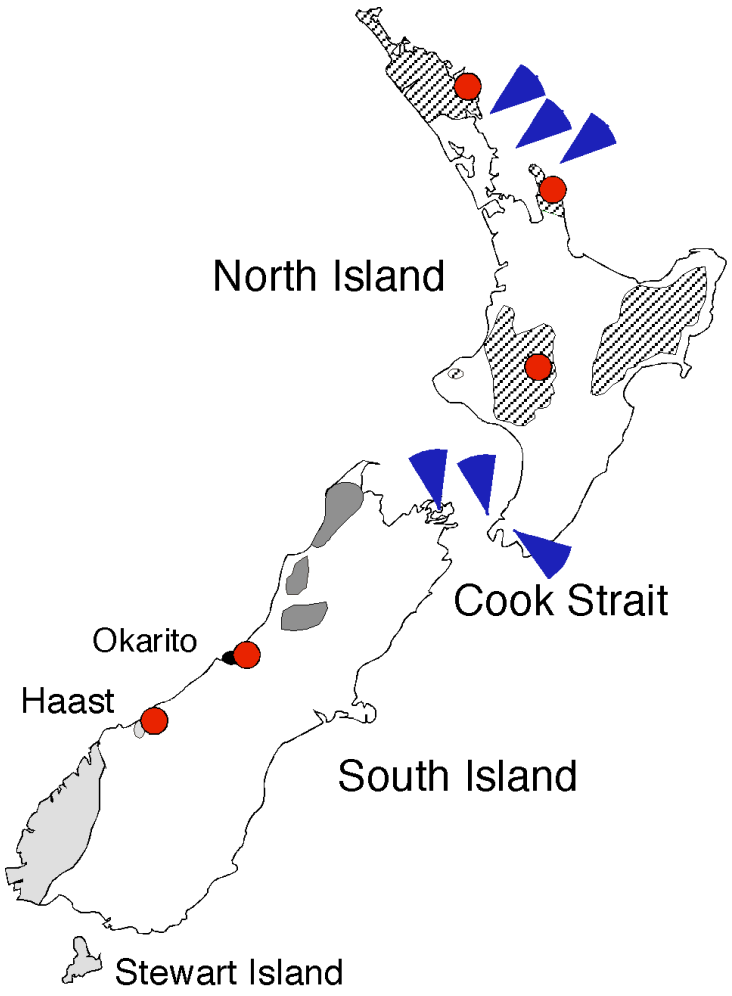


**Figure 1.** Current distributions of the five kiwi species: North Island brown kiwi, *Apteryx mantelli* (striped), rowi, *A. rowi* (black), tokoeka, *A. australis* (light grey), great spotted kiwi, *A. haastii* (dark grey) and little spotted kiwi, *A. owenii* (blue arrows). The five intensively managed mainland kiwi sanctuaries are indicated by red circles.

Kiwi were also extensively hunted by Europeans for their skins which were popular in overseas museums. In 1896 all the species of kiwi were declared protected. However, kiwi numbers continued to decrease because of ongoing land clearance and introduced predators. Unmanaged mainland populations of all species of kiwi are estimated to be currently decreasing by about 6% per year and all kiwi taxa are presently considered threatened (Robertson, 2003).

The 'Kiwi Recovery Programme' was established by the Department of Conservation to "maintain and, where possible, enhance the current abundance, distribution and genetic diversity of kiwi" (Robertson, 2003). Current conservation actions include establishing populations on predator-free offshore islands, maintaining selected populations in intensively managed mainland sanctuaries (Figure 1) and captive breeding. A further goal is to identify genetically distinct kiwi populations and ascertain their taxonomic status (Robertson, 2003).

*The New Zealand wattlebirds*

The New Zealand wattlebirds are an endemic family, Callaeatidae, of three genera, all with limited powers of flight. The New Zealand wattlebird family comprises saddleback (*Philesturnus carunculatus*), kokako (*Callaeas cinereus*), and huia (*Heteralocha acutirostris*). Both saddleback and kokako have distinct forms on the North and South Islands, variously considered species or subspecies. Saddleback, which is common in subfossil deposits on the mainland (Worthy and Holdaway, 2002), became restricted to offshore islands after human arrival. The distribution of kokako has also declined and is believed to be extinct in the South Island. Kokako remain in small fragmented populations on the North Island mainland and have been introduced to a number of predator-free offshore islands. In contrast, huia were only present in the North Island and are believed to be extinct with the last confirmed sighting in 1907 (Heather and Robertson, 2000). The huia has attracted much interest in theoretical ecology because the female and male of this species differed greatly in beak morphology. Darwin (1871) used the bill dimorphism of huia as an example of an adaptation that reduces competition between the sexes for food.

*Thesis outline*

This thesis comprises seven research chapters, in addition to a general introduction and discussion. The research chapters are written as 'stand-alone' scientific papers. Chapter Two describes the application of ancient DNA to conservation by analysing ancient DNA sequences from kiwi. Specifically it examines past species ranges of brown and great spotted kiwi, species boundaries within brown kiwi and the loss of genetic diversity over time in brown kiwi. Chapter Three is a comparison of the phylogeography of little spotted kiwi with brown kiwi. Chapters Four and Five describe the use of ancient DNA to investigate provenance of unlabelled kiwi bones,

skins, feather cloaks and baskets (kete) from museums. The isolation of nuclear microsatellite DNA markers from North Island brown kiwi is described in Chapter Six and preliminary population genetic analyses for all kiwi species is included. These markers were initially isolated with the aim of amplifying them from ancient samples. Chapter Seven is an investigation of the relationships of the New Zealand wattlebirds. It also includes an examination of the date of divergence of the New Zealand wattlebirds from the passerines. In Chapter Eight, the patterns and levels of genetic diversity in the extinct New Zealand huia are examined through cross-amplification of microsatellite DNA markers. Finally, Chapter Nine provides a summary of the main findings of this thesis and proposes possible future research.

Appendix A includes details of modern and ancient kiwi samples used in this study and is included to facilitate linkage between chapters. Appendix B includes two manuscripts published during this PhD research that, although not directly related to the topics presented here, use similar methodology. The first, 'Serial population bottlenecks and genetic variation: translocated populations of the New Zealand saddleback (*Philesturnus carunculatus rufusater*)', was published in *Conservation Genetics*. This paper was the collaborative effort of a number of researchers over a considerable number of years. My contribution to this manuscript was to score some of the microsatellite DNA data, and to analyse most of the allozyme, microsatellite, and minisatellite data. I was also involved in writing the manuscript and co-ordinating input between the co-authors. The second manuscript, '*Asplenium* ×*lucrosum* nothosp. nov.: a sterile hybrid widely and erroneously cultivated as *Asplenium bulbiferum*', was published in *Plant Systematics and Evolution*. In this study I was involved in the collection of samples and the extraction and isolation of DNA from the herbarium samples.

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# CHAPTER TWO

## Ancient DNA and conservation: lessons from the kiwi of New Zealand.[1]

**Abstract**

Conservation genetics typically seeks to map the distribution of genetic variation across space and to use these genetic parameters to infer likely short-term evolutionary consequences for rare and endangered species. Recent developments in ancient DNA now provide the opportunity to extend genetic variation studies backwards in time, and therefore provide a framework context in which to document temporal genetic changes. Ancient DNA research can also help to determine past levels of genetic diversity, identify species' boundaries and reveal former ranges amongst morphologically-cryptic taxa. Ancient DNA sequence data for the New Zealand kiwi (*Apteryx* spp.) are presented to illustrate how such studies can aid conservation efforts. New Zealand brown kiwi have lost over a third of their past genetic variation. Ancient DNA methodology is also used to determine past species distributions for brown kiwi and great spotted kiwi whose bones are morphologically indistinguishable. Furthermore, ways in which species' boundaries within brown kiwi may be investigated are suggested.

### Introduction

Anthropogenic factors, such as the introduction of exotic species, harvesting, hunting and pollution, have resulted in the decline of many plants and animals around the world (Lande, 1999). Consequently, many once widespread species only survive now in remnant populations. Both genetic and morphological studies of these survivors may be misled without knowledge of how the current genetic variation compares to past patterns and levels of diversity. Ancient DNA methodology provides a tool to examine past genetic variation, which may have important implications for conservation biology.

[1] Shepherd, LD and Lambert, DM. To be submitted to *Molecular Ecology*.

There are several key conservation issues that ancient DNA is able to directly address. Firstly, ancient DNA allows a direct examination of the levels of past variation, plus a measure of any loss of diversity in reduced and/or small contemporary populations. Some authors have suggested that the loss of genetic diversity is of importance for conservation because of a proposed relationship between loss of genetic diversity and long-term population viability, e.g., Frankham et al. (2002).

Secondly, ancient DNA may also aid the mapping of past patterns of genetic variation and facilitate accurate species delimitation. The identification of species' boundaries in endangered taxa is often difficult because they commonly have fragmented distributions and therefore do not occur in sympatry. It is important to establish the specific status of each population to allow for the development of appropriate management strategies and priorities. However, the determination of species status in allopatric taxa is problematic because they cannot be directly assessed for evidence of divergence in mate recognition (Paterson, 1985).

Two approaches have been used to address this problem. Some have opted for the use of Evolutionarily Significant Units (Ryder, 1986; Moritz, 1994). This essentially represents an attempt to avoid the species problem in conservation, by the creation of a new category that can be delineated using a set of prescribed rules.

Another approach has been to use molecular markers such as microsatellite DNA loci, allozymes and/or mitochondrial DNA sequences to calculate a genetic distance between pairwise combinations of populations. This distance is then compared with that calculated between congeneric species whose taxonomic status is not in question. If the distance between the reference species is equivalent to that between the two populations being tested, then the latter are considered to be different species (Frankham et al., 2002). Classic examples include tuatara (Daugherty et al., 1990), orangutan (Xu and Arnason, 1996) and coelacanth (Holder et al., 1999). However, if a large genetic distance distinguishes two recently isolated populations then these might represent remnants of a once more continuous distribution of a single, genetically diverse, species. Under this scenario the loss of geographically intermediate populations, possibly combined with the change in frequency (even fixation) of alternate alleles, will result in these fragmented populations being highly divergent. Hence this pattern is a

result of population loss, rather than speciation. Indeed, the fixation of a nucleotide character state through recent human-mediated extinction and fragmentation has recently been demonstrated in the northeastern beach tiger beetle (*Cicindela dorsalis*) (Goldstein and DeSalle, 2003). An alternative possibility is that two species with discrete differences did exist in the past, despite an otherwise continuous range, but that only remnant allopatric populations of these species survive today. Analysing ancient DNA from extinct geographically intermediate populations allows these alternate hypotheses to be tested.

Ancient DNA can also potentially provide valuable information regarding the prior ranges of morphologically similar taxa that have been recently restricted in distribution. The success of re-introduction programs is likely to be increased by selecting sites within the historical range of the species in question (Frankham et al., 2002). Therefore, information regarding previous distributions can subsequently be used to select suitable locations for re-introduction. For example, DNA extracted from wombat museum skins established that an extinct population consisted of the northern species of hairy-nosed wombat rather than the currently geographically closer southern species (Taylor et al., 1994). Additionally, accurate past distributions are essential for studies investigating the dynamics of species' declines. It has been suggested that most species that have undergone range contractions persist in the periphery rather than the core of their former range (Channell and Lomolino, 2000). However, accurate historical distributions are essential to test such ideas (Brooks, 2000).

In this study ancient DNA was used to examine past ranges, species' boundaries and loss of genetic diversity in the kiwi (*Apteryx*), a highly distinctive, flightless, endemic New Zealand genus of ratites (Table 1). Kiwi distributions have been reduced through predation and habitat loss to the extent that the conservation of kiwi is now actively managed (Robertson, 2003). Kiwi are ideal species to study past pre-human diversity because population decline and fragmentation occurred relatively recently and there is an excellent subfossil record.

Currently two morphological groups of kiwi are recognised: the spotted and the brown kiwi. The spotted kiwi group comprises two species: the great spotted kiwi (*Apteryx haastii)* and the little spotted kiwi (*A. owenii*). The number of brown kiwi species, as

**Table 1.** Species of kiwi with their present distributions and conservation status.

| Species | Current Distribution[1] | Current population estimate[1] | Conservation status[2] |
|---|---|---|---|
| North Island brown kiwi (*Apteryx mantelli*)  |  | 25 000 | Seriously declining |
| Rowi (*Apteryx rowi*)  |  | 250 | Nationally critical |
| Tokoeka (*Apteryx australis*)  |  | 300 at Haast and 30-33 000 in Fiordland and Stewart Is. | Gradually declining |
| Little spotted kiwi (*Apteryx owenii*)  |  | 1 200 on Kapiti Is. and a total of 200 on other islands and mainland sanctuaries. | Range restricted |
| Great spotted kiwi (*Apteryx haastii*)  |  | 17 000 | Gradually declining |

[1]Bank of New Zealand Kiwi Recovery™ Trust. http://www.kiwirecovery.org.nz

[2]Hitchmough (2002)

determined by morphology, has been the topic of considerable debate with up to six species previously described. However, recent molecular research has led to the description of three allopatric brown kiwi species: North Island brown kiwi (*A. mantelli*), Okarito brown kiwi or rowi (*A. rowi*) and tokoeka (*A. australis*) (Baker et al., 1995; Burbidge et al., 2003; Tennyson et al., 2003). Additionally, two subspecies of tokoeka are currently recognised: *A. australis australis* and *A. a. lawryi*. The three

species were previously classified as one (*A. australis*) but the large genetic distances between them, which are similar to the distance between the two species of spotted kiwi, has been used to argue for specific status (Baker et al., 1995; Burbidge et al., 2003). Nuclear DNA (allozyme data) also showed high levels of differentiation between the three brown kiwi species (Baker et al., 1995; Herbert et al., 2002). The presence of a fourth, extinct, species of brown kiwi (the 'Eastern' brown kiwi) has been suggested from subfossil bones throughout the eastern South Island (Worthy, 1997; 1998a; b).

Subfossil kiwi remains are distributed across New Zealand indicating that kiwi were once more widespread (Worthy and Holdaway, 2002). Little spotted kiwi (*A. owenii*) is the only taxon that can be positively identified by bone morphology, because individuals of this species are significantly smaller than both brown kiwi (*A. mantelli, A. rowi* and *A. australis*) and great spotted spotted kiwi (*A. haastii*) (Worthy and Holdaway, 2002). The other kiwi species cannot be distinguished by either bone shape or size (Worthy, 1997). Therefore, large kiwi bones from the South Island, where both great spotted and brown kiwi currently occur, have been regarded as indeterminable (Worthy and Holdaway, 2002). Large kiwi bones from the North Island are usually classified as brown kiwi because great spotted kiwi have not been historically recorded from the North Island. However, the possibility that some of these North Island bones may be great spotted kiwi cannot be excluded on morphology alone (Millener, 1981). Clearly a new approach is needed.

Ancient DNA methodology was here applied to conservation issues using the endangered New Zealand kiwi as a case study. Specifically, the past distributions and levels of genetic variation in brown kiwi and great spotted kiwi were explored. Whether ancient DNA can further illuminate the specific status of the allopatric brown kiwi populations presently regarded as distinct species was also examined.

## Methods

### *Samples*

We obtained 45 ancient 'large' kiwi specimens (i.e., morphologically they could belong to either brown kiwi or great spotted kiwi) from the Museum of New Zealand Te Papa Tongarewa and the Canterbury Museum (Table 2, Appendix 1). These specimens were

**Table 2.** Ancient kiwi samples from which full-length DNA sequence was obtained, their localities, museum numbers and GenBank accession numbers. Samples marked with an * were independently re-extracted at the University of Auckland. Museum abbreviations: CM - Canterbury Museum, MNZ – Museum of New Zealand Te Papa Tongarewa.

| Sample identifier | Haplotype group | Museum Number | Museum | Locality | Sample Type | GenBank number (cytochrome b) | GenBank number (control region) |
|---|---|---|---|---|---|---|---|
| 32 | *Apteryx mantelli* | DM10276 | MNZ | Waverley | Toe pad | AY713367 | AY713332 |
| 33 | *Apteryx mantelli* | DM9375 | MNZ | Ohakune | Toe pad | AY713368 | AY713335 |
| 34 | *Apteryx mantelli* | DM13551 | MNZ | Waitotara Valley, Wanganui | Toe pad | AY713369 | AY713336 |
| 36 | *Apteryx rowi* | S.24355.1 | MNZ | Perini Creek, Buller Gorge | Bone | AY713348 | AY713337 |
| 37 | *Apteryx rowi* | S.24355.2 | MNZ | Perini Creek, Buller Gorge | Bone | AY713349 | AY713339 |
| 38 | *Apteryx rowi* | - (THW) | - | Takaka fossil cave, Takaka | Bone | AY713365 | AY713338 |
| 39 | *Apteryx rowi* | S.23211 | MNZ | Takaka Hill | Bone | AY713370 | AY713314 |
| 40 | *Apteryx rowi* | AV16697 | CM | Kiwi Hole, Caanan, Takaka | Bone | AY713359 | AY713320 |
| 41 | *Apteryx rowi* | DM7869 | MNZ | Martinborough | Bone | AY713345 | AY713341 |
| 42 | *Apteryx rowi* | DM7896* | MNZ | Martinborough | Bone | AY713351 | AY713342 |
| 43 | *Apteryx rowi* | DM7900 | MNZ | Martinborough | Bone | AY713343 | AY713340 |
| 44 | *Apteryx rowi* | S.009401 | MNZ | Poukawa | Bone | AY713366 | AY713316 |
| 45 | *Apteryx australis* | AV588 | CM | Te Anau | Toe pad | AY713352 | AY713317 |
| 46 | *Apteryx australis* | AV589 | CM | Te Anau | Toe pad | AY713355 | AY713318 |
| 47 | *Apteryx australis* | S.34531* | MNZ | Castle Rock, Southland | Bone | AY713344 | AY713334 |
| 48 | *Apteryx australis* | DM6489 | MNZ | Castle Rock, Southland | Bone | AY713346 | AY713333 |
| 49 | *Apteryx australis* | DM6555 | MNZ | Castle Rock, Southland | Bone | AY713347 | AY713313 |
| 50 | *Apteryx australis* | DM6557 | MNZ | Castle Rock, Southland | Bone | AY713360 | AY713321 |
| 51 | *Apteryx australis* | DM6498* | MNZ | Castle Rock, Southland | Bone | AY713356 | AY713322 |
| 52 | *Apteryx australis* | AV12651* | CM | Mt Somers Quarry, South Canterbury | Bone | AY713361 | AY713323 |
| 53 | *Apteryx australis* | S.33369.1 | MNZ | Mt Cookson | Bone | AY713362 | AY713326 |
| 54 | *Apteryx australis* | S.33369.2 | MNZ | Mt Cookson | Bone | AY713353 | AY713325 |
| 55 | *Apteryx australis* | S.33369.3 | MNZ | Mt Cookson | Bone | AY713354 | AY713327 |
| 56 | *Apteryx australis* | S.33369.5 | MNZ | Mt Cookson | Bone | AY713363 | AY713328 |
| 57 | *Apteryx australis* | S.33369.4 | MNZ | Mt Cookson | Bone | AY713357 | AY713324 |
| 58 | *Apteryx haastii* | S.23187* | MNZ | Charleston | Bone | AY713364 | AY713331 |
| 59 | *Apteryx haastii* | AV19163 | CM | Charleston | Bone | AY713358 | AY713330 |
| 60 | *Apteryx haastii* | S.34491 | MNZ | Mt Arthur | Bone | AY713350 | AY713312 |

mainly selected from regions where kiwi are now locally extinct, and comprised either subfossil bones (usually femora although in some cases rib bones) or skins. Subfossil bones of the 'Eastern' brown kiwi, the proposed extinct species from the eastern South Island, i.e., Castle Rock (samples 47-51) and Mt Somers (sample 52) (Worthy, 1997; 1998a; b); and possibly those from Mt Cookson (samples 53-57) (TH Worthy, pers. comm.), were included.

*Ancient DNA extractions*

To minimise the risk of contamination from modern DNA, ancient DNA extractions were prepared in a separate and dedicated ancient DNA laboratory at Massey University that undergoes regular decontamination with UV-irradiation and hypochloride treatment. Blank DNA extractions and PCRs were routinely screened for contamination.

Approximately 1 cm x 0.3 cm was cut from the centre of each kiwi bone, thus leaving the bone largely intact. The surface of this section was then cleaned using a Dremel grinder with a new Dremel wheel used for each sample. Samples were ground to a fine powder using a coffee grinder (cleaned with ethanol between each sample and regularly UV-irradiated). For skin samples, 4 $mm^2$ of kiwi footpad tissue was cut into several pieces using a clean razor blade.

Bone samples were decalcified in 7.5 ml of 500 mM EDTA pH 8.0 overnight at room temperature. The supernatant was removed following centrifugation. The remaining bone sediment, as well as the tissue samples, were incubated overnight at 50°C in 2.5 ml extraction buffer (10 mM Tris-HCl pH 8.0, 1 mM NaCl), 250 µl of 10% SDS, 15 µl of 200 mg/ml Dithiothreitol (DTT) and 25 µl of 50mg/ml Proteinase-K. DNA was extracted with Tris-saturated phenol followed by chloroform:isoamyl (24:1) and then concentrated to 200 µl on a Vivaspin-30 (Viva Science, U.K.) membrane. An additional clean-up step using the QIAGEN DNA mini kit was performed to remove co-purifying PCR-inhibitors from samples that did not initially amplifiy.

A subset of five samples was independently extracted and PCR amplified by L. Huynen in a dedicated ancient DNA facility at the University of Auckland in order to verify results.

*DNA primer design and sequencing*

Primer sequences for the mitochondrial cytochrome b gene were designed, from modern kiwi sequences available on GenBank, to amplify two overlapping DNA fragments, resulting in 641 base pairs (bp) of sequence. Kcytb1 (5'AAACATCTCCGCGTGATGAAACTTCGGAT) and Kcytb2 (5' AACTGTAGCCCCTCAGAATGATATTTGTCCTCA) amplify a product of length 300 bp. Kcytb3 (5'ACATCGGCCGAGGCTTTTACTACGGCTC) and Kcytb4 (5'TGGAGTGAAGTTCTCTGGGTCTCCTA) amplify a product 427 bp in length. The above four primers were designed by L. Huynen. Kiwi specific primers were also designed to amplify a 200 bp fragment from domain 1 of the mitochondrial control region (kcf 5'CAGTATGGTCACCGAACAC and kcr 5'ACAGGGGTTGCTGATTTCA).

PCR amplification was performed in 20 µl volumes with the following thermocycling profile: 94°C for 2 minutes; ten cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minute; 32 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 1 minute; and a final extension time of 5 minutes at 72°C. PCR products were purified through High Pure purification columns (Roche). Automated sequencing of all DNA was performed on an ABI-3730 (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit version 3.1. Both of the cytochrome b fragments and the control region fragment were sequenced in both directions from independent amplifications.

DNA sequences were aligned by eye to modern sequences from previously published studies (Baker et al., 1995; Burbidge et al., 2003; details in Table 3 of Appendix A). The ancient sequences were slightly shorter owing to primer set differences.

*Phylogenetic and population analyses*

Phylogenetic relationships of the concatenated sequence data were analysed using maximum parsimony (MP), maximum likelihood (ML), neighbour joining (NJ) and Quartet Puzzling (QP) criteria in PAUP* 4.0b10 (Swofford, 2002). Uncorrected p-distances were used to construct the NJ phylogeny because the sequences were closely related and this distance method has a small variance (Nei and Kumar, 2000). ML used the HKY + I + G model of sequence evolution as selected by the likelihood ratio test in

Modeltest v3.06 (Posada and Crandell, 1998) as most appropriate for these combined data. ML and MP analyses were performed using the heuristic search option with tree bisection-reconnection (TBR) branch swapping and 10 random addition replicates. Support for nodes was assessed using 1000 (NJ, MP) or 100 (ML) bootstrap replicates. In addition, a Bayesian inference approach that allows a different model of evolution to be employed for each partition in a combined data set (i.e., cytochrome b versus control region) was used to estimate phylogenetic relationships (MrBayes 3.0b4; Huelsenbeck and Ronquist, 2001). Modeltest v3.06 (Posada and Crandell, 1998) selected HKY+G and K81+G as the most appropriate models of evolution for cytochrome b and control region, respectively. Four Markov chains were run, each for 2 000 000 generations, sampling every 100$^{th}$ cycle from the chain. The initial 1000 samples were discarded as 'burn-in'. The Bayesian analysis was repeated three times.

A minimum spanning network (MSN) was constructed from p-distances in Arlequin 2000 (Schneider et al., 2000) by molecular variance partitioning. Network methods such as this have been suggested to better represent intraspecific sequence data whereby ancestral and descendent sequences may coexist (Posada and Crandell, 2001).

Measures of genetic diversity for each species were estimated by calculating the number of haplotypes, polymorphic sites, transitions and transversions using Arlequin version 2.001 (Schneider et al., 2000).

**Results**

The entire 841 bp of DNA sequence was obtained from twenty-eight of the forty-six ancient samples (deposited in GenBank; accession numbers AY713312-AY713370, AY749106-AY749107). Five other samples provided partial DNA sequences (Appendix 1). However, only full-length sequences were used in the analyses reported here. Three samples grouped with great spotted kiwi, while the remaining 25 clustered amongst brown kiwi. The five samples independently extracted and amplified at the ancient DNA facility at the University of Auckland produced identical sequences to those obtained at the ancient DNA laboratory at Massey University.

*Comparison of sequence variability within kiwi*

Two haplotypes were identified within great spotted kiwi. These haplotypes were characterised by three polymorphic sites, all of which differed by transitions (Table 3). The entire alignment of the cytochrome b and control region sequences of brown kiwi contained 86 variable sites (81 sites with transitions, four sites with transversions and one site that exhibited three different nucleotides) and defined 44 haplotypes (Table 4).

*The past range of great spotted kiwi*

The only sequences from subfossil bone samples to group with modern great spotted kiwi sequence were those from Mt. Arthur and Charleston, which fall within the recorded range of great spotted kiwi. Great spotted kiwi were not detected amongst the large bones from the North Island.

*Patterns of genetic variation in brown kiwi*

The phylogenetic analyses of the concatenated sequences produced largely concordant relationships and consequently only the Bayesian phylogeny is presented (Figure 1).

**Table 3**. Variable sites defining spotted kiwi mitochondrial DNA haplotypes. GSK is great spotted kiwi, LSK is little spotted kiwi. Nucleotide positions of the variable sites from 200 bp of domain 1 of the control region (CR) and 641 bp of cytochrome b are given at the top. Ancient samples sequenced in this study are shown in bold, with sample identifiers.

| | | CR | Cytochrome b |
|---|---|---|---|
| | | 1 | 123333444556 |
| | | 666 | 9790258566882 |
| | | 166 | 2002099257346 |
| 149 | GENBANK GSK | TAA | TCCAACCGACTCC |
| **58,59** | **Charleston1** | ... | ..... |
| **60** | **MtArthur1** | C.. | C.....T. |
| 150 | GENBANK LSK | .GG | CTTGGATTGTCTT |

**Table 4**. Variable sites defining brown kiwi mitochondrial DNA haplotypes. Nucleotide positions of the variable sites from 200 bp of domain 1 of the control region and 641 bp of cytochrome b are given at the top. Ancient samples sequenced in this study are shown in bold (note that some ancient haplotypes are identical to modern haplotypes but are reported for completeness).

| | | Control Region | Cytochrome b |
|---|---|---|---|
| | | 1111111111111 | 1111111222222222223333334444444555555566666 |
| | | 2234455566667777771222333333466 | 1246777890111788012445667891235893345677023688990012 3 |
| | | 0212657807891456786579012678426 | 2349421789830396370942870957673838514055360490171538805 |
| **NORTH ISLAND BROWN KIWI** | | | |
| 1 | NORTHLAND1 | AAACTATTACGCACTCTATATCCGCCCCCTA | TTCGGTCCCACTACACTCATCTTAGATACTGTCCACCAAGCCTGTCTATCCCATA |
| 2 | NORTHLAND2 | ......C..... | ..... |
| 3 | NORTHLAND3 | .G....C..... | ......G.....A..... |
| 4 | NORTHLAND4 | .G....C..... | ..... |
| 5 | LITTLEBARRIER1 | G.....C....T.....TT.. | .C.....G.....A...T..... |
| 6 | LITTLEBARRIER2 | G.....C.....TT.. | .C.....G.....T.....C..... |
| 7 | LITTLEBARRIER6 | G.....C.....TT.. | .C.....G.....T..... |
| 8 | BAYOFPLENTY3 | ......C.....T.....T... | .....C..GT.....G..T.....G..... |
| 9 | BAYOFPLENTY1 | .....T.....T... | .....G.....T..... |
| 10 | TARANAKI1 | G.....C....T.....TT.. | .C.....G.....A...T..... |
| 11 | TARANAKI3 | G.....TT.. | .C.....G.....T..... |
| 12 | TARANAKI2 | G.....C.....TT.. | .C.....G.....C..... |
| 13 | TARANAKI4 | G.....C.....TT.. | .C.....G.....T..... |
| 14 | TARANAKI6 | ......C..... | ..... |
| 15 | HAWKESBAY2 | ......C.....T.....T... | .....G.....T.....G..... |
| 16 | HAWKESBAY1 | .....T..T..T... | .....G.....T..... |
| **32** | **OHAKUNE1** | G.....TT.. | .C.....G.....T.....T..... |
| **33** | **WANGANUI1** | ......C..... | .....G.....T..... |
| **34** | **WAVERLEY1** | G.....C....T.....TT.. | .C.....G.....A...T..... |
| **ROWI** | | | |
| 17 | OKARITO8 | G....G.CGT..G.....C....TTT... | .....G.CT..T...A...T.....G.....T.A...G...T..C |
| 18 | OKARITO1 | G....G.CGT.....C....TTT... | .....G.CT..T...A...T.....G.....T.A...G...T..C |
| 19 | OKARITO7 | G....G.CGT.....C.....TT... | .....G.CT..T...A...T.....G.....T.A...G...T..C |
| **36,37** | **BULLERGORGE1** | G....G.CG.....CT...GC....A.T... | ..T....T....G.G.C..CT.....T.....T.A.....T..C |

Table 4 continued.

| | | Control Region | Cytochrome b |
|---|---|---|---|
| | | 1111111111111 | 1111111222222222223333334444444555555556666 |
| | | 2234455566667777771222333333466 | 1246777890111788012445667891235893345677023688990012 3 |
| | | 0212657807891456786579012678426 | 2349421789830396370942870957673838514055360490171538805 |
| **ROWI continued** | | | |
| **38** | **TAKAKA2** | G....G.CG.....CT...GC....A.T... | ..TA...T....G.G.C..CT.......T.....T.A.......T..C |
| **39** | **TAKAKA3** | G....G.C......CT....C....A.T... | ..T.........G.G.C..CT.......T.....T.A.......T..C |
| **40** | **TAKAKA1** | G....G.CG.....C.....C....A.T... | ..T.........G.G.C..CT.......T.....T.A.......T..C |
| **41-43** | **MARTINBOROUGH1** | G....G.CG.....CT.GG........T... | .....G.G.C.....T.....A...A......TTG.C |
| **44** | **POUKAWA1** | G..T.G.CG.....C...G........T... | .....G...C.....T.....G....A...A......TT..C |
| **TOKOEKA** | | | |
| 20 | HAAST4 | ....CG.CGT....C.....CTTA..... | C.....GTGT.....C.G..CTTCA.T.G....A...A....C....CC |
| 21 | HAAST1 | ....CG.CG.....C.....CTTA..... | C.....GTGT.....C.G..CTTCA.T.G....A...A....C....CC |
| 22 | HAAST3 | ....CG.CGT....C.....CTTA...T... | C.....GTGT.....C.G..CTTCA.T.G....A...A....C....CC |
| 23 | FIORDLAND3 | .....G.CG.A...C.....CTTA...T... | C........G..GTGT.....C....CTTCA.T.GT...A...A.....C |
| 24 | FIORDLAND1 | G....G.CG.....C....GCT.A...T.C. | C...AC..T...G.GT.....C....CTTC..T.G..G.A...A.....C |
| 25 | FIORDLAND5 | G....G.CG.....C.....CT.A...T.C. | C...AC..T...G.GT.....C....CTTC..T.G..G.A...A.....C |
| 26 | STEWART7 | .....G.......TC.C...C......T... | C...AC.....C..GT.....C....CTTC..TTG.T..A...A.....C |
| 27 | STEWART5 | .....G.C.....TC.C...C......T... | C...AC....TC..GT.....C....CTTC..TTG.T..AT..A.....C |
| 28 | STEWART4 | .....G.....T.TC.C...C......T... | C...AC.....C..GT.....C....CTTC..TTG.T..AT..A.....C |
| 29 | STEWART3 | .....G.....T.TC.C...C......T... | C.............G......C....CTTC..TTG.T..AT..A.....C |
| 30 | STEWART2 | .....G.....T.TC.C...C......T... | C.............G.....TTC..TTG.T..AT..A.....C |
| 31 | STEWART1 | .....G.C.....TC.C...C......T... | C.....G.G.....TTC..TTG.T..AT..A.....C |
| **45** | **TEANAU1** | .....G.CG.A...C.....CTTA...T... | C........G..GTGT.....C....CTTCA.T.GT...A...A.....C |
| **46** | **TEANAU2** | .....G.CG.A...C.....CTTA...T..G | C........G..GTGT.....C....CTTCA.T.GT...A...A.....C |
| **47,48** | **CASTLEROCK1** | G....G.CGT..G.C....GCT.A.T.T.C. | C...AC..T...G..T..G..C....CTTC..T.G..G.A...A.....C |
| **49** | **CASTLEROCK2** | G....G.CGT..G.C....GCT...T.T.C. | C...AC..T...G.GT..G..C....CTTC..T.G..G.A...A.....C |
| **50** | **CASTLEROCK3** | G....G.CG...G.C....GCT...T.T.C. | C...AC..T...G.GT..GC.C....CTTC..T.G..G.A...A.....C |
| **51** | **CASTLEROCK4** | G....G.CG...G.C....GCT...T.T.C. | C...AC..T...G.GT..G..C....CTTC..T.G..G.A...A..C.....C |
| **52** | **MTSOMERS1** | G.G..G.C.T..G.......CT...A.T... | C...A.......GTGT.....C....CTTC..T.G....A..CA.....C |
| **53,54** | **MTCOOKSON1** | .....G.CG....TCT....C....A.T... | C..A........G.G......CC...CTTC.C..G....A...A.....T....C |
| **55,56** | **MTCOOKSON2** | .......CG....TCT....C....A.T... | C..A........G.G......CC...CTTC.C..G....A...A.....T....C |
| **57** | **MTCOOKSON3** | .....G.CG....TCT....C....A.T... | C..A........G.G.....TCC...CTTC.C..G....A...A.....T....C |

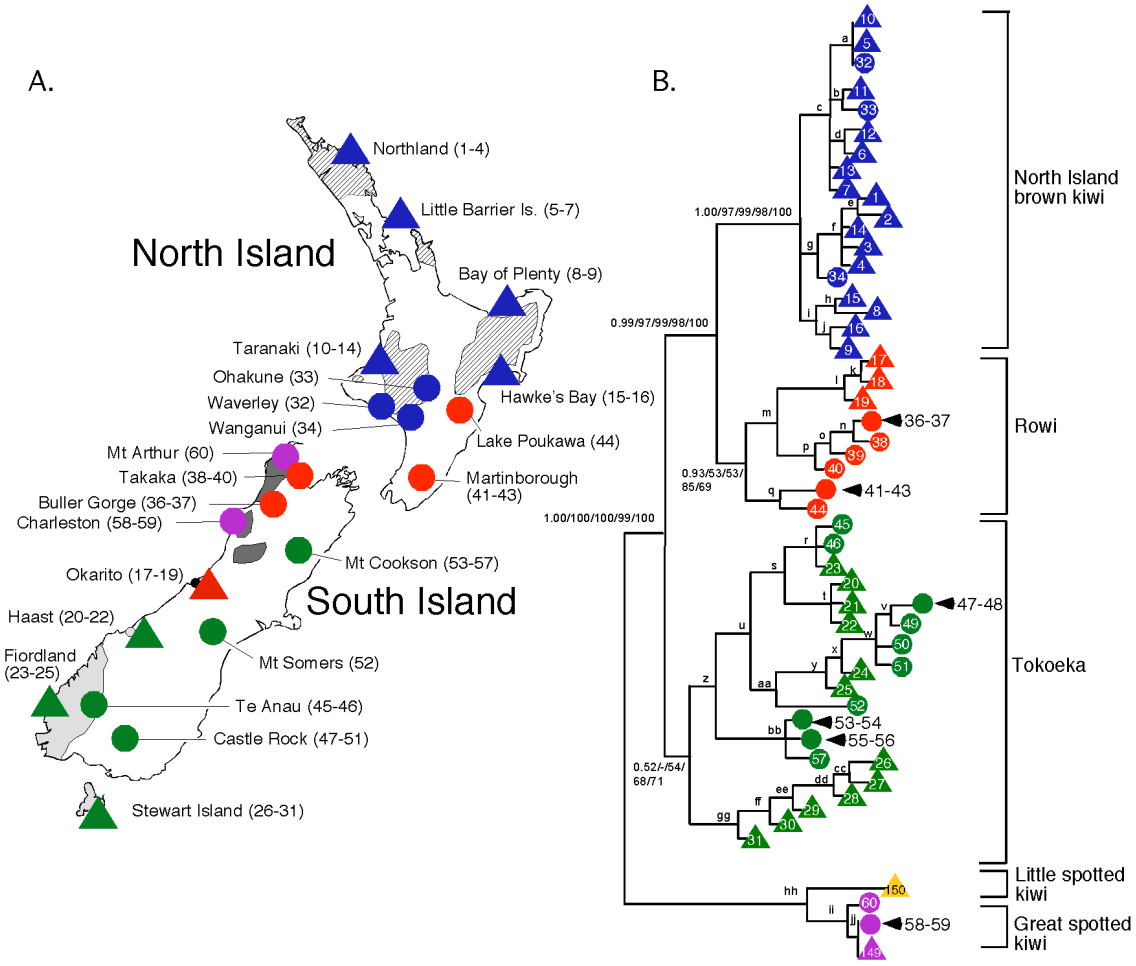


**Figure 1**. A. Locality of samples of kiwi taxa. The current distribution of North Island brown kiwi is striped, rowi is shown in black, tokoeka in light grey and great spotted kiwi in dark grey. Modern samples are indicated by triangles, ancient samples by circles. The colour of the symbol indicates with which kiwi species the ancient sequences group: blue indicates North Island brown kiwi, red represents rowi, green indicates tokoeka and purple represents a sequence most closely related to great spotted kiwi. The provenance of the modern little and great spotted kiwi samples is not shown because they were not recorded in GenBank. B. Bayesian phylogeny of modern and ancient brown kiwi control region and cytochrome b sequences. Support for major nodes are given on the tree in the following order: Bayesian posterior probabilities/ML bootstrap/MP bootstrap/QP values/NJ bootstrap. Support for nodes labelled a-jj are given in Appendix 2. The root of the tree was determined by midpoint rooting.

Nodal support values from all analyses are displayed on the Bayesian phylogeny. The ML analysis produced a single tree (log likelihood score = 2546.12270). The MP analysis yielded 2304 equally parsimonious trees (score of best trees = 186). One point of incongruence amongst the equally parsimonious trees was the relationship between the three major mitochondrial lineages; two thirds of the trees placed rowi and North Island brown kiwi as sister taxa, while the remainder had rowi as sister taxon to tokoeka. Another discrepancy amongst the MP trees was basal divergences within rowi. Two thirds of the MP trees supported the Okarito (samples 17-19) and Takaka and Buller Gorge (samples 36-40) as sister groups. Whereas the remaining trees supported Lake Poukawa (sample 44) and Martinborough (samples 41-43) as the sister group to Takaka and Buller Gorge (samples 36-40). A difference between the phylogenetic reconstruction methods was whether the basal divergences in tokoeka involved Mt Cookson (samples 53-57) or Stewart Island brown kiwi (samples 26-31) (however, there is little support for either). Three major mitochondrial lineages were recovered in the phylogenetic analyses (Figure 1). North Island brown kiwi were supported by high support values (1.00 Bayesian posterior probability (PP), 97 ML bootstrap (BS), 99 MP BS, 98 QP BS and 100 NJ BS) and, relative to spotted kiwi, by one synapomorphy (position 635 of cytochrome b). Tokoeka were supported by 0.52 PP, 54 MP BS, 68 QP BS and 71 NJ BS and by two synapomorphies (positions 287 and 317 of cytochrome b). In all analyses the ancient samples from Lake Poukawa (sample 44), Martinborough (samples 41-43), Takaka (samples 38-40), and Buller Gorge (samples 36-37) grouped with those from the extant population of rowi from Okarito (samples 17-19). This was supported by 0.93 PP, 53 ML BS, 53 MP BS, 85 QP BS, 69 NJ BS and by two synapomorphies (positions 183 and 608 of cytochrome b). Although overall support for this broadly circumscribed rowi was generally low, the sub-groups within it were well supported (i.e., Okarito – samples 17-19; Lake Poukawa and Martinborough – samples 41-44; and Takaka plus Buller Gorge – samples 36-40); see Figure 1 and Appendix 2.

The three brown kiwi groups were also apparent in the minimum spanning network (Figure 2) with 16 mutational steps separating the most closely related haplotypes of the North Island brown kiwi and rowi, and 18 steps between rowi and tokoeka. The level of variation within each of these principle groups differed. North Island brown kiwi comprised a number of closely related haplotypes connected by a maximum of four

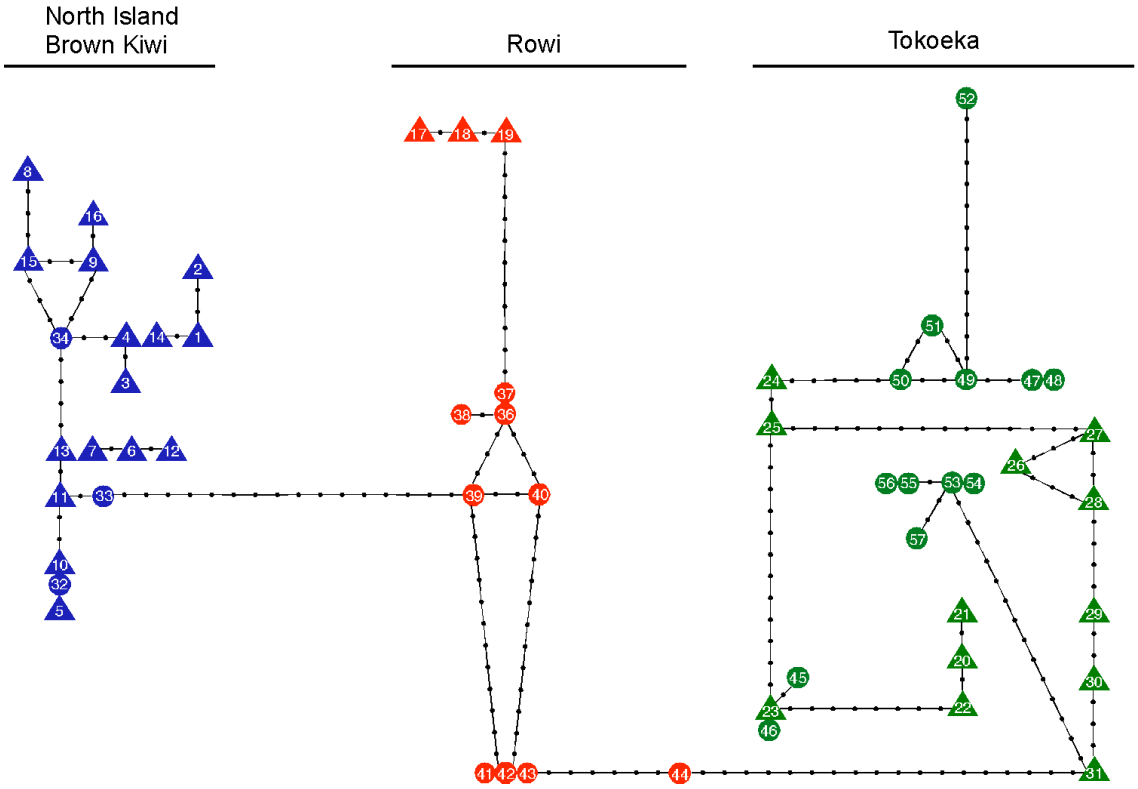


**Figure 2.** An unrooted minimum spanning network of brown kiwi haplotypes. The number of steps separating haplotypes is indicated by small black dots. Modern kiwi samples are indicated by triangles, ancient samples by circles. The colour of the symbol indicates with which kiwi species the ancient sequences group: blue indicates North Island brown kiwi, red represents rowi and green indicates tokoeka.

mutational steps. In contrast, rowi and tokoeka contained more divergent haplotypes with up to 12 mutational steps connecting haplotypes within rowi and 14 in tokoeka. Subfossil samples described from morphology as 'Eastern' brown kiwi did not form a monophyletic group and were nested within the haplotypic diversity of tokoeka. However, the Castle Rock and Mt Somers haplotypes grouped together in the minimum-spanning network.

*Loss of genetic diversity in brown kiwi*

Seventeen of the 44 brown kiwi haplotypes were only detected in ancient populations, indicating that 38.6% of the total haplotypic diversity detected here in brown kiwi, has

now been lost. However, this loss has not been equal amongst groups: 14.2% of the overall genetic variation in North Island brown kiwi has been lost; 66.6% from rowi and 42.8% from tokoeka.

**Discussion**

*Previous ranges of great spotted and brown kiwi*

Cytochrome b and control region sequences from the large ancient kiwi samples have allowed a better understanding of the previous distributions for great spotted and brown kiwi. Brown kiwi were previously more widespread in both the North and South Islands. In contrast, great spotted kiwi seem to have a pre-human distribution very similar to their current distribution; i.e. the north-west of the South Island (Figure 1). This information can now be used to select sites for re-introduction that are within species' past ranges.

These kiwi distributions also have implications for the study of patterns of kiwi extinction. Great spotted kiwi was one of the species used by Channell and Lomolino (2000) that led them to suggest that species contract into the periphery, rather than the centre, of their ranges. They assumed the past range of great spotted kiwi covered the entire South Island. The ancient DNA evidence presented here suggests that great spotted kiwi should not have been included in their study because it does not fit the criteria described in their methods (i.e. the present range of great spotted kiwi is not less than 25% of its historical distribution) (Channell and Lomolino, 2000).

*Delimiting species using genetic distance*

An aim was to investigate how the inclusion of DNA sequence from extinct, intermediate populations of brown kiwi affected the phylogeny and species' delimitations. The ancient samples attributed to 'Eastern' brown kiwi fall within the haplotypic diversity of tokoeka, providing little support for the recognition of the former as a separate species, as suggested by Worthy (1997; 1998a; b). The three major mitochondrial groups of Burbidge et al. (2003) are still recovered, - North Island brown kiwi, rowi and tokoeka - with the addition of ancient brown kiwi mitochondrial DNA sequences. Therefore, the distinctiveness of these lineages does not appear to be a result of recent anthropogenic fixation of characters, as has been found for tiger beetles (Goldstein and DeSalle, 2003). However, phylogenetic support for both rowi and

tokoeka lineages decreased with the addition of the ancient haplotypes. The following well-supported sub-groups were evident within rowi (Martinborough and Lake Poukawa, samples 41-44; Buller Gorge and Takaka, samples 36-40) and tokoeka (Stewart Island, samples 26-31; and Mt Cookson, samples 53-57). Using a 'genetic distance' approach for species delimitation, these groups could all be considered separate species because the genetic distance between them is greater than that separating the two reference species (great spotted and little spotted kiwi) used in previous studies (Baker et al., 1995; Burbidge et al., 2003). However, the use of genetic distance criteria to delimit species has long been criticised (e.g., Lambert and Paterson, 1982; Ferguson, 2002), in part because it is not linked to any particular species concept and is not directly related to the genetic processes involved in speciation. In addition, a single clonally inherited genome such as mitochondrial DNA may not reflect the species tree because of lineage sorting (Nichols, 2001) or male-biased gene flow. Moreover, deep phylogenetic breaks in mitochondrial phylogenies have recently been demonstrated to occur in species with low dispersal, even in the absence of barriers to gene flow (Irwin, 2002).

Given that the three brown kiwi species presently recognised are based primarily on mitochondrial DNA differences, further study is required to test their separate species status. Nuclear allozyme loci have been previously analysed for kiwi (Baker et al., 1995; Herbert and Daugherty, 2002) but the levels of variation were low. Only one of the six variable loci examined in these studies displayed a fixed allele difference separating North Island brown kiwi and rowi from tokoeka. Surprisingly, brown kiwi from Haast demonstrated substantial allelic differences relative to other tokoeka (more than the difference between North Island brown kiwi and rowi), contrasting with the mitochondrial phylogeny. Modern brown kiwi live in small isolated populations and may therefore be highly susceptible to genetic drift (Baker et al., 1995). This may explain the observed allozyme allele frequency changes. Therefore a large number of neutral nuclear loci, such as microsatellite DNA, are necessary to explore the relationships between these groups.

However, even if mitochondrial DNA differentiation of the modern brown kiwi populations is supported by nuclear data, this does not necessarily mean that they constitute separate species but merely that there has been long-term genetic isolation of

these groups. There are several means by which the specific status of these lineages might be examined. Firstly, a number of both North Island brown kiwi and tokoeka were introduced to Kapiti Island between 1908 and 1940 (Wilkinson and Wilkinson, 1952) and they now occur in sympatry. Examining whether they randomly interbreed under the relatively natural conditions of this island may constitute a test of the specific status of these two groups. Secondly, the geographic boundary between rowi and North Island brown kiwi identified in this study should be examined. Within this region rowi may possibly still exist in remote areas and may occur in sympatry with North Island brown kiwi. Genetic analysis of any populations within this region should be a conservation priority. This could potentially resolve whether North Island brown kiwi and rowi constitute different species and, if so, may reveal another, previously unrecognised, extant population of rowi.

*Loss of genetic diversity in the brown kiwi*

Brown kiwi ancient cytochrome b and control region sequences demonstrated that, as with almost all modern kiwi populations, nearly every ancient location examined was shown to possess unique haplotypes. The extinction of these ancient populations has resulted in nearly 40% of the known total brown kiwi haplotypes being lost. The loss of genetic diversity from rowi and tokoeka accounted for most of this loss. In addition, as demonstrated by the minimum spanning network, these two groups contained much more genetic variation than North Island brown kiwi. The high level of genetic variation and presence of unique haplotypes in tokoeka is not consistent with the suggestion that the South Island was repeatedly recolonised by brown kiwi from glacial refugia in the North Island (Lloyd, 2002).

In summary, these ancient DNA analyses of the New Zealand kiwi illustrate the importance of placing present day genetic diversity within the context of past patterns and levels of genetic variation. This demonstrates how a temporal perspective can contribute to conservation objectives.

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**Appendix 1**. Ancient kiwi samples that either failed to amplify or only provided partial sequence.

| Museum Number | Locality | Partial sequence amplification | GenBank number |
|---|---|---|---|
| DM1552 | Hawke's Bay | yes | AY749106 |
| DM14849 | Retaruke Valley, Katieke | yes | AY749107 |
| DM1212 | Waikoau, Hawke's Bay | yes | AY713315 |
| S.25792 | Oparara, West Coast | yes | AY713329 |
| AV800 | Okarito | yes | AY713319 |
| AV5835 | Pyramid Valley | no | - |
| DM9041 | Mangatepopo, Tongariro National Park | no | - |
| S.3782 | Kings Cave, South Canterbury | no | - |
| S.36314 | Lake Grassmere | no | - |
| S.7612 | Poukawa | no | - |
| S.23403 | Macraes, Otago | no | - |
| S.39039 | Pryde's Gully Rd Swamp, North Otago | no | - |
| S.23194 | Cave south of Greymouth | no | - |
| S.23492 | Oparara, West Coast | no | - |
| S.23488 | Oparara, West Coast | no | - |
| S.27785 | Takaka Hill | no | - |
| S.33505 | Takaka Hill | no | - |
| AV6414 | Pleasant Point | no | - |

**Appendix 2.** Nodal support values from Figure 1. These values are given in the following order: Bayesian posterior probabilities/ML bootstrap/MP bootstrap/QP values/NJ bootstrap.

a. 1.00/92/87/98/85
b. 0.69/-/-/96/57
c. 0.94/68/75/97/77
d. 0.95/65/62/94/62
e. 0.93/60/60/87/63
f. 1.00/75/74/49/75
g. 0.90/53/54/88/52
h. 0.98/67/76/89/81
i. 0.53/-/51/86/59
j. 0.58/-/-/71/80
k. 0.63/-/57/97/65
l. 1.00/100/100/95/100
m. 0.98/67/58/58/64
n. 1.00/82/89/93/93
o. 0.85/66/67/84/54
p. 1.00/89/94/76/96
q. 1.00/91/90/97/94
r. 1.00/98/96/98/100
s. 0.99/74/79/88/99
t. 1.00/96/96/95/99
u. 0.97/-/56/72/78
v. 0.68/-/-/56/62
w. 0.99/81/86/-/90
x. 0.68/-/52/-/-
y. 1.00/90/90/-/95
z. 0.85/-/-/-/-
aa. 0.82/-/-/81/78
bb. 1.00/98/100/99/100
cc. 0.85/-/-/-/ -
dd. 1.00/68/70/91/84

ee. 1.00/-/-/-/-

ff. 1.00/59/58/-/55

gg. 1.00/76/79/88/83

hh. 1.00/86/92/99/100

ii. 1.00/88/99/100/100

jj. 1.00/88/89/100/91

# CHAPTER THREE

## Ancient DNA analyses reveal contrasting phylogeography in the New Zealand kiwi (*Apteryx* spp.).[1]

**Abstract**

The endemic New Zealand little spotted kiwi (*Apteryx owenii*) is a previously widespread species now greatly restricted in distribution. Genetic variation was surveyed throughout the pre-human range of little spotted kiwi by obtaining mitochondrial DNA sequences from bones and skins. Genetic structuring in little spotted kiwi differed to that previously identified in brown kiwi species. Overall, little spotted kiwi exhibited lower levels of genetic diversity than brown kiwi. In brown kiwi, mitochondrial DNA haplotypes were often restricted to populations, particularly in the South Island. In contrast, little spotted kiwi from the South Island exhibited very little genetic variation and a single haplotype predominated. The differing patterns of genetic structuring between brown and little spotted kiwi are discussed and hypothesised to relate to differences in behaviour and/or population history.

### Introduction

Phylogeography is the study of processes determining the geographic distribution of genetic lineages (Avise, 2000). Comparative phylogeography involves the examination of the phylogeographic patterns in multiple, co-distributed species and, should concordant patterns be found, infers common historical influences. Most phylogeographic studies have been based on mitochondrial DNA sequences. Although this only traces the maternal line, it offers the advantage of a high rate of evolution, a large number of copies per cell and lower coalescent times.

New Zealand has experienced a dynamic geological history, which has likely had a significant influence on the history of the New Zealand biota. Major geological events that may have fashioned phylogeographic patterns in New Zealand taxa include the

[1] Shepherd, LD, Worthy, TH, Tennyson, AJD, Scofield, P and Lambert, DM. To be submitted to *Journal of Biogeography*.

reduction of the terrestrial landmass during the Oligocene (e.g., Cooper and Cooper, 1995), the formation of the Southern Alps during the Pliocene (e.g., Trewick et al., 2000; Lockhart et al., 2001), the Pleistocene glacial cycles (e.g., Gardner et al., 2004; Neiman and Lively, 2004) and volcanism in the North Island (e.g., Holzapfel et al., 2002; Lloyd 2003**).** Discrimination between alternative phylogeographic hypotheses has commonly been achieved by examining the geographic patterns of genetic variation and the application of a molecular clock to date divergences.

One of the most striking phylogeographic patterns observed in New Zealand, and in fact, in any bird worldwide (Avise, 2000), is that detected in the flightless endemic New Zealand brown kiwi (North Island brown kiwi, *Apteryx mantelli*; rowi, *A. rowi*; and tokoeka, *A. australis*). Mitochondrial DNA sequences from the reduced, disjunct modern populations of brown kiwi revealed an extremely high level of genetic structuring, with almost every population possessing private mitochondrial DNA haplotypes (Baker et al., 1995; Burbidge et al., 2003), a pattern more akin to that often seen in mammals rather than birds. Analysis of ancient brown kiwi samples from regions where they are now extinct indicated that this structuring, with even higher levels of genetic variation, also existed in the past (Chapter Two) and was not therefore the result of human-mediated extinction. Molecular dating with the application of the 'standard' avian rate of 2% sequence divergence per million years in cytochrome b led Baker et al. (1995) to propose that divergences of the brown kiwi species occurred during the Pleistocene. However, a later re-estimation using an emu fossil calibration calculated that the main brown kiwi lineages diverged in the Miocene and Pliocene (Burbidge et al., 2003).

A second morphological group of kiwi is also present in New Zealand, the spotted kiwi, which comprises two species differentiated from brown kiwi by their mottled grey plumage. Great spotted kiwi (*A. haastii*) now occupy the northwest of the North Island, a range that does not seem to have diminished in response to human arrival to the same extent as in other kiwi species (Chapter Two). In contrast, the distribution of subfossil bones of little spotted kiwi (*A. owenii*), which are significantly smaller than the bones of other kiwi, indicate that this species previously occurred throughout the North and South Islands (Worthy and Holdaway, 2002). The subfossil bones of little spotted and brown kiwi have been found in many of the same deposits on the mainland of New

Zealand (e.g., Worthy and Holdaway, 1995; Worthy, 1998a; b; Worthy et al., 2002) suggesting that they previously occurred in sympatry on the mainland. Therefore, ancient little spotted kiwi from across New Zealand might be expected to have been influenced by the same historical factors as brown kiwi and thus exhibit similar patterns of phylogeographic structure.

Since European settlement, only one little spotted kiwi specimen has been recorded in the North Island, from the Tararua Ranges in 1875 (Worthy and Holdaway, 2002). The last confirmed complete little spotted kiwi specimen from the South Island mainland was recovered in 1938 (Jolly, 1990). Since then there have been only two verified reports of little spotted kiwi (a feather, and recently deposited leg bones), both from Fiordland (Peat, 1990). However, there are a number of small spotted kiwi skins held in museums that were collected in the 1970s whose identities have been debated (e.g., Jolly, 1992; Colbourne, pers. comm. in McLennan and McCann, 2002). These specimens could either be little spotted kiwi or juvenile great spotted kiwi.

Little spotted kiwi managed to survive in two populations on offshore islands (Kapiti Island and D'Urville Island; Figure 1), although they have since been introduced to a number of other islands and a mainland sanctuary. There has, however, been debate regarding the origin of the little spotted kiwi on Kapiti Island. A number of bird species have been introduced to Kapiti Island since it was declared a sanctuary in 1897, including brown kiwi, and it has been suggested that the little spotted kiwi population derives from such a translocation (e.g., Wilkinson and Wilkinson, 1952; Heather and Robertson, 2000). However, Jolly and Daugherty (2002) examined historical records of such translocations and concluded that there was no evidence that little spotted kiwi were introduced. Instead, they suggested that little spotted kiwi might have become isolated on Kapiti Island as a result of rising sea levels following the end of the last glaciation. A further possibility is that Maori moved little spotted kiwi to Kapiti Island prior to European arrival (Jolly, 1990).

In this study, the genetic structure of little spotted kiwi across its former range was examined by isolating mitochondrial DNA sequences from museum skins, including two debatable little spotted skins from the1970s, and subfossil bones. The patterns and levels of genetic variation detected were also compared to the previously determined

phylogeographic structure of brown kiwi since these species may have been influenced by similar historical factors. Furthermore, the origin of the little spotted kiwi population on Kapiti Island was investigated.

## Methods

### *Samples*

A total of thirty-four little spotted kiwi specimens, consisting of either skins or subfossil bones, were obtained from a number of New Zealand museums (Table 1 and Appendix 1). Modern great spotted kiwi (n = 4, sample numbers 142-145, Table 2) and little spotted kiwi (n = 3 sample numbers 146-148, Table 2) blood samples were acquired from the National Tissue Collection, Victoria University.

### *DNA extraction*

All DNA extractions from ancient samples (bones, toe pads and feathers) were performed in a dedicated ancient DNA laboratory. This was regularly UV-irradiated and physically isolated from where PCR products were handled and modern DNA extractions performed. Negative controls were used throughout the extraction and PCR amplification processes.

For one little spotted kiwi sample (sample 62, Table 1) DNA was extracted from a whole vertebra. The remaining little spotted kiwi bones (femurs) were sampled for DNA by either removing a section using a Dremel grinder (Museum of New Zealand Te Papa Tongarewa samples) or by drilling (samples from Canterbury Museum and the Geology Department of University of Auckland). The surface layer of bones that were sampled with a Dremel grinder was removed by sanding with a Dremel wheel that was changed between each sample. Segments of 1cm x 0.3 cm were cut from the centre of each bone and finely ground in a coffee grinder that was cleaned between each sample with ethanol and regularly irradiated with UV light. The remaining bone samples were drilled using a 3 mm drill bit and the shavings collected. The drill bit was cleaned with bleach between each sample. All ancient bone samples were decalcified and a phenol-chloroform extraction method performed (Chapter Two).

Museum skins were sampled by removing a sliver of approximately 3 $mm^2$ of kiwi footpad tissue from the underside of the foot with a clean razor blade. For one museum

**Table 1.** Details of ancient little spotted kiwi samples from which full-length DNA sequence was obtained. Samples marked with an * were independently extracted at the University of Auckland. Museum abbreviations: CM - Canterbury Museum, MNZ – Museum of New Zealand Te Papa Tongarewa, WM – Waitomo Museum.

| Sample identifier | Museum Number | Museum | Provenance | Sample Type |
|---|---|---|---|---|
| 61 | AV22817 | CM | Helectite Hole, Raglan | Bone |
| 62 | WO255 | WM | Ann's Cavern, Waitomo | Bone |
| 63 | S.24478 | MNZ | Coonoor | Bone |
| 64 | NM23036 | MNZ | Banjo Creek, Westhaven Inlet, NW Nelson, 1978 | Skin |
| 65 | AV16713 | CM | Cave at Canaan, Takaka | Bone |
| 66 | S.27784.1 S.27784.2* | MNZ | Earl Grey Cave, Takaka | Bone |
| 67 | S.1174 | MNZ | Otuhuhu or Rough River (Westland), 1952 | Toe pad |
| 68 | S.23043 | MNZ | Smyth River, South Westland, 1978 | Toe pad |
| 69 | S. 22007* | MNZ | Karangarua River, South Westland, 1894 | Feather/Toe pad |
| 70 | S.2069 | MNZ | Lake Manapouri, 1888-96 | Toe pad |
| 71 | AV32392B | CM | Cave at Springhill, Southland | Bone |
| 72 | DM6672 | MNZ | Castle Rock, Southland | Bone |
| 73 | AV25301 | CM | King's Cave, South Canterbury | Bone |
| 74 | AV12648C | CM | Limestone fissure, Mt Somers Quarry | Bone |
| 75 | S.33365 | MNZ | Holocene Cave, Mt Cookson | Bone |
| 76 | AV25141 | CM | West Coast, South Island | Bone |

**Table 2.** Details of modern spotted kiwi blood samples used in this study.

| Species | Sample identifier | National Tissue Collection code | Provenance |
|---|---|---|---|
| Great spotted kiwi | 142 | GS14 | Ugly River, NW Nelson, coll. by J. McLennan. |
| | 143 | M3 | Heaphy, NW Nelson, coll. April 1987 by J. McLennan. |
| | 144 | FT2921 | Kahurangi, NW Nelson, coll. 10.10 90 by J. McLennan. |
| | 145 | GS21 | Taramakau River, Arthurs Pass, coll. by J. McLennan. |
| Little spotted kiwi | 146 | CD899 | Kapiti Island, coll. 27.10.84 by B. Reid and M. Finglan. |
| | 147 | CD1206 | Kapiti Island, coll. 28.5.85 |
| | 148 | WS1764 | D'Urville Island |

skin (sample 69, Table 1) a single feather was removed in addition to the toe pad for verification purpose (see below). The lowermost 2 mm of the feather shaft was used for DNA extraction. DNA was extracted from skin, feather and the modern blood samples by proteinase digestion followed by phenol-chloroform extraction (Sambrook et al., 1989).

*Ancient DNA verification*

The small size of little spotted kiwi bones prevented them from being sampled twice without substantially damaging the integrity of the bone structure. Two samples, a feather and a toe pad, were taken from one museum skin specimen (sample 69, Table 1). Femurs from two different individuals were sampled from Earl Grey Cave (sample 66, Table 1). DNA was extracted from one of the femurs and from the feather sample at the ancient DNA laboratory at Massey University. DNA from the remaining femur and the toe pad sample was independently extracted and 190 bp fragment of the control region amplified in the ancient DNA laboratory of the University of Auckland by L. Huynen.

*PCR and sequencing*

Kiwi-specific primers were designed from modern little spotted kiwi sequence available from GenBank to amplify mitochondrial control region and cytochrome b DNA sequence. Primers to amplify 190 bp of sequence from domain 1 of the control region were designed (kcf2 5'CCTTGTAGGCAAATACAGT and kcr2 5'GTGTTGAATCAGGAAATCC). Three short fragments of cytochrome b were also amplified using little spotted kiwi specific primers. Two of these fragments were overlapping and the third was non-contiguous resulting in a total of 471 bp of sequence. Kcytb1 (Chapter Two) and LSKcytA (5'GATGCTCCGTTTGCATGTAG) amplified a product 191 bp in length. LSKcytB (5'ATCCATCGCCCATATCTGTC) and LSKcytb2 (5'AACTGTAGCCCCCCAAAATGATATTTGTCCCCA) amplified a product 246 bp in length. LSKcytD (5'TCCCATACATCGGACAAACC) and LSKcytE (5'GTATGGGTGGAAGGGGATTT) amplified a product 214 bp in length.

PCR amplifications were performed in 20 µl volumes with 1× PCR buffer (500 mM Tris pH 8.8, 200 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 200 µl of each dNTP, 1 U Taq DNA polymerase (Roche) and 0.5 µM of each

primer. PCR amplification was performed in a Hybaid Omnigene Thermal Cycler with a PCR profile of 94°C for 2 minutes; ten cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minute; followed by 32 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 1 minute; and a final extension of 5 minutes at 72°C. PCR products were purified through High Pure purification columns (Roche). Automated sequencing of all PCR products was performed on an ABI-3730 (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit ver. 3.1. All DNA fragments were sequenced in both directions from independent PCR amplifications.

*Sequence alignment*

DNA sequences were edited using Sequencher$^{TM}$ 3.1.1 (Gene Codes Corporation). The ancient great spotted kiwi sequences previously presented in Chapter Two were included in the analyses. Levels of diversity in spotted kiwi were compared to those in brown kiwi using previously isolated sequences from both modern (Baker et al., 1995; Burbidge et al., 2003) and ancient samples (Chapter Two) were used to compare levels of diversity to that detected in spotted kiwi. In order to reduce computational time, only a subset of the brown kiwi DNA sequences were selected to represent the diversity previously detected in both modern and ancient brown kiwi lineages. The sequences contained no indels and were aligned manually.

*Phylogenetic analyses*

Support and conflict for each split (bipartition in the data) in the spotted kiwi sequences was examined using split decomposition (Bandelt and Dress, 1992) in SplitsTree 4.0b21 (Huson and Bryant, in prep.). Split decomposition was implemented using Hamming distances (observed number of differences ÷ total sequence length).

Phylogenetic analyses of the concatenated control region and cytochrome b sequences from the spotted kiwi and representative brown kiwi were conducted in PAUP* version 4.0b10 (Swofford, 2002). A neighbour-joining phylogeny was constructed with p-distances. Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using a heuristic search algorithm with 10 (ML) or 100 (MP) random addition sequences and tree bisection-reconnection (TBR) branch swapping. The maximum likelihood search was performed using HKY + I + G model of sequence

evolution as selected by the Akaike Information Criterion (AIC) in Modeltest v3.06 (Posada and Crandell, 1998) as most appropriate for these data. Nodal support was assessed by 100 (MP and ML) or 1000 (NJ) bootstrap replicates. A Bayesian inference approach was also used to estimate phylogenetic relationships (MrBayes 3.0b4; Huelsenbeck and Ronquist, 2001). Modeltest v3.06 (Posada and Crandell, 1998) was used to select the most appropriate models of evolution for the cytochrome b (TrN+I) and control region (K81+I+G) datasets and both models were incorporated in the analysis. Four Markov chains were run, each for 2 000 000 generations, sampling every $100^{th}$ cycle from the chain. The initial 1000 samples were discarded as 'burn-in'. The Bayesian analysis was repeated three times to confirm convergence had been reached.

Haplotype and nucleotide diversity (Tajima, 1983; Nei, 1987) were estimated for spotted and brown kiwi, as well as for each species in Arlequin 2.001 (Schneider et al., 2000). A comparison of the rates of evolution of the brown and spotted kiwi sequences was performed uisng a likelihood ratio test (LRT; Felsenstein, 1981). The LRT involved a comparison of the likelihood of the optimal tree to the likelihood of an ultrametric tree (i.e. with a molecular clock enforced). A $\chi^2$ distribution was used to assess whether the difference in likelihoods was significant.

*Divergence time estimates*

The timing of lineage divergences was estimated with two methods that have both previously been used in kiwi. Firstly, a 'standard' avian rate of evolution of 2% per million years was applied to net average genetic distances between species determined from the cytochrome b sequences in MEGA v3.0 (Kumar et al., 2004) following Baker et al. (1995). However, unlike Baker et al. (1995) who used uncorrected distances, cytochrome b distances were corrected using a Tamura Nei model as selected by Modeltest (Posada and Crandall, 1998).

Secondly, divergence dates of kiwi lineages were estimated in relation to an emu fossil calibration, following Burbidge et al. (2003). Burbidge et al. (2003) used the program r8s (Sanderson, 2002) to calculate a date for the divergence of spotted and brown kiwi from 13 protein-coding mitochondrial genes for single specimens of great spotted kiwi, North Island brown kiwi, emu, cassowary and ostrich. They fixed the root of the tree, where ostrich diverged from the remaining ratites, was fixed at 65 mya and the kiwi

split from emu/cassowary at 62 mya. They constrained the emu/cassowary split to a minimum of 25 mya and a maximum of 35 mya. All of the above dates were based on a single emu fossil dated at 25 mya. Using these constraints, Burbidge et al. (2003) calculated a date of 16.1 mya for the separation of great spotted kiwi and brown kiwi. This date was then used as a calibration point to determine divergence dates within kiwi for a dataset consisting of ATPase6, ATPase8 and cytochrome b.

In the present study, the root of the kiwi phylogeny was constrained to fall between brown and spotted kiwi. The exact placement of the root between these groups was determined from a phylogeny constructed from ATPase6, ATPase8 and cytochrome b sequences from a representative of each of the kiwi species, obtained from GenBank, in addition to the outgroups emu (*Dromaius novaehollandiae*, GenBank accession number AY016014) and cassowary (*Casuarius bennetti*, GenBank accession number AY016011). The inferred root position was then transferred to the Bayesian consensus phylogeny constructed from the cytochrome b and control region sequence data for kiwi. Estimates of divergence times were calculated with penalised likelihood in r8s version 1.70 with the truncated Newton algorithm (Sanderson, 2002) and a smoothing value of 1 as determined by cross-validation.

**Results**

DNA was successfully retrieved from seventeen of the thirty-four ancient samples. Full-length sequences comprising a total of 661 bp were obtained from sixteen of these samples (Table 1) and were used in subsequent analyses. Both of the samples extracted and amplified at the ancient DNA laboratory at the University of Auckland had identical sequences to the corresponding samples processed at Massey University.

*Sequence characteristics and diversity measures*

Twenty-seven variable sites were present in the combined spotted kiwi sequences, of which twelve were parsimony informative (Table 3). For great spotted kiwi, four haplotypes, defined by three variable sites, were detected. Little spotted kiwi exhibited twenty-one variable sites, nine of which were parsimony informative, and defined six haplotypes. The majority of the variation in little spotted kiwi occurred amongst the

**Table 3.** Variable sites defining spotted kiwi haplotypes. Sequences derived from ancient samples are shown in bold. Sequence alignment positions are indicated.

| | | Control Region | Cytochrome b |
|---|---|---|---|
| | | 1111 | 111222333333444 |
| | | 5788884478 | 59347689124799013 |
| | | 7612362671 | 92700010407702294 |
| GREAT SPOTTED KIWI | | | |
| 142 | NW NELSON | CTACGACATA | CCTCCGTCCATGACCTA |
| 143,144 | NW NELSON | ........C. | ..... |
| 145 | ARTHURS PASS | ..... | .T..... |
| **58,59** | **CHARLESTON** | **.....** | **.T.....** |
| **60** | **MT ARTHUR** | **.C.....** | **.....** |
| LITTLE SPOTTED KIWI | | | |
| 146,147 | KAPITI IS | ..G......G | ....T..TT..TGT... |
| 148 | D'URVILLE | ..GT.....G | ....T..TT..TGT... |
| **61** | **KARAMO** | **......TG..** | **...T...TTGCT..ACG** |
| **62** | **WAITOMO** | **.....G....** | **.....A.TT.CT..AC.** |
| **63** | **COONOOR** | **T..T.....G** | **T.C...CTT..TG....** |
| **64** | **WESTHAVEN** | **..G......G** | **....T..TT..TGT...** |
| **65** | **TAKAKA1** | **..GT.....G** | **....T..TT..TGT...** |
| **66** | **TAKAKA2** | **..G......G** | **....T..TT..TGT...** |
| **67** | **ROUGH RIVER** | **..G......G** | **....T..TT..TGT...** |
| **68** | **SMYTH RIVER** | **..G......G** | **....T..TT..TGT...** |
| **69** | **KARANGARUA RIVER** | **..G......G** | **....T..TT..TGT...** |
| **70** | **LAKE MANAPOURI** | **..G......G** | **....T..TT..TGT...** |
| **71** | **SPRINGHILLS** | **..G......G** | **....T..TT..TGT...** |
| **72** | **CASTLE ROCK** | **..G......G** | **....T..TT..TGT...** |
| **73** | **KINGS CAVE** | **..G......G** | **....T..TT..TGT...** |
| **74** | **MT SOMERS** | **..G.A....G** | **....T..TT..TGT...** |
| **75** | **MT COOKSON** | **..G......G** | **....T..TT..TGT...** |
| **76** | **WEST COAST** | **..G......G** | **....T..TT..TGT...** |

three North Island samples (sample numbers 61-63), with each having a different haplotype. In contrast, nearly all the little spotted kiwi from the South Island (11 of 13 samples) possessed the same haplotype. The modern little spotted kiwi population from Kapiti Island also had this haplotype.

*Phylogenetic relationships of spotted kiwi.*

The concatenated sequences from all spotted kiwi samples were used to construct a splits graph (Figure 1). The splitsgraph indicated only one source of conflict in the data, represented by a reticulation in the graph. The split separating the ancient South Island and modern Kapiti and D'Urville Island little spotted kiwi from the remaining samples was supported by three substitutions. Two of these were determined to be

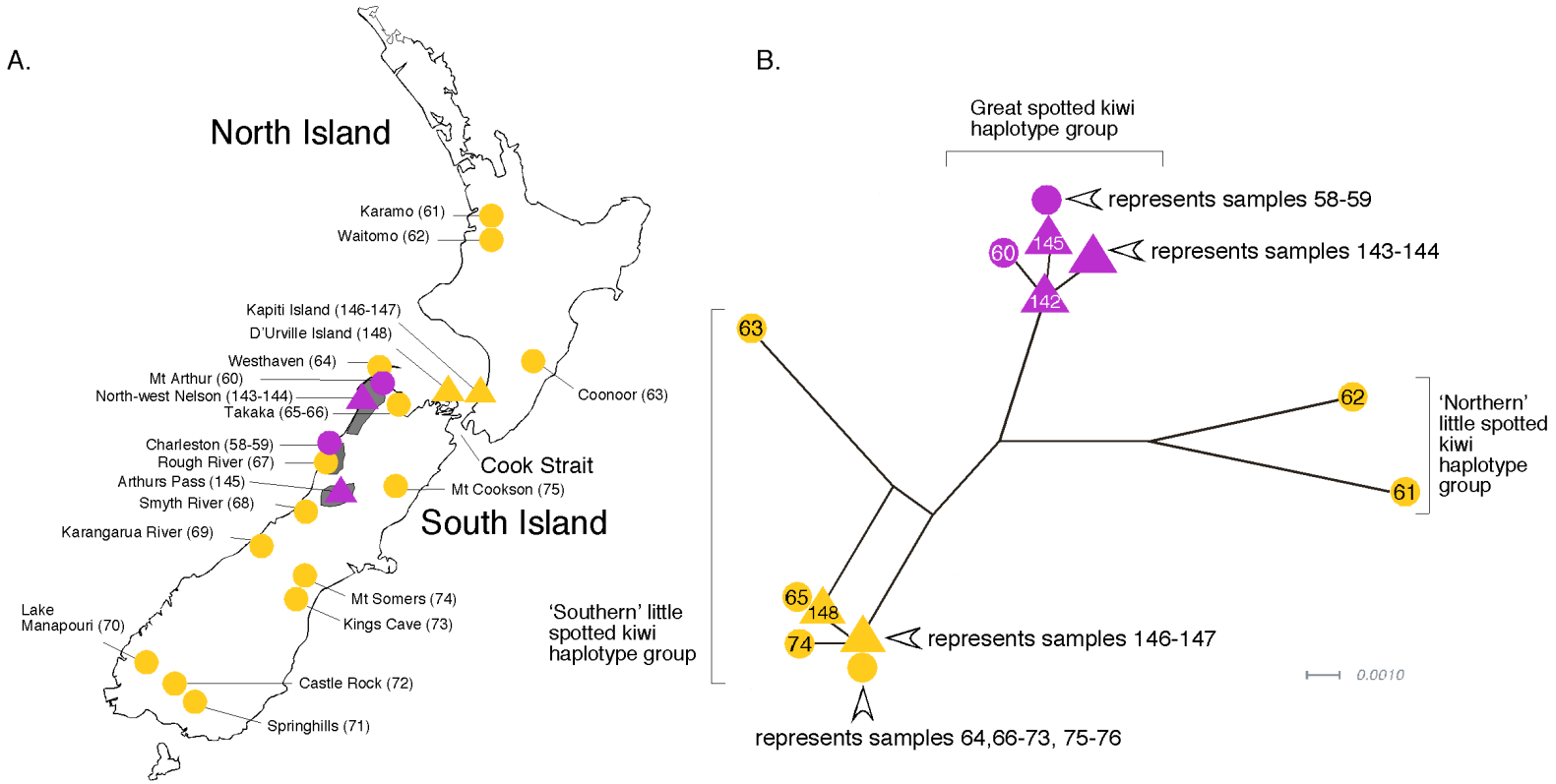


**Figure 1.** A. Locality of spotted kiwi samples. The current distribution of great spotted kiwi is shown in grey. Little spotted kiwi were reduced to populations on Kapiti and D'Urville Islands. Note that the position of sample 76 is not indicated on the map because of the imprecision of its recorded locality, 'West Coast, South Island'. B. Splits graph of cytochrome b and control region sequences. The fit of the data to the splits graph was 100 indicating that all splits in the data were represented. Triangles represent modern samples; circles represent ancient samples. Little spotted kiwi samples are shown in yellow; great spotted kiwi samples are shown in purple.

synapomorphies when using brown kiwi as outgroups (nucleotide position 81 in the control region and 170in cytochrome b, Table 3). Contradicting this split was that in nucleotide position 82 of the control region, which partitioned samples from Coonoor (sample 63), D'Urville Island (sample 148) and one of the Takaka samples (sample 65), from the rest. This is likely to be a homoplasious base change. The remaining two little spotted kiwi sequences from the North Island (samples 61 and 62) comprised a lineage (the 'northern' haplotype group) supported by three synapomorphies (nucleotide positions 347, 402 and 419 of cytochrome b, Table 3). Great spotted kiwi were defined by three synapomorphies in the cytochrome b DNA sequences: two transitions (nucleotide positions 290 and 314) and a transversion (nucleotide position 377). Furthermore, a split separating great spotted kiwi and the 'northern' haplotype group of little spotted kiwi from the remaining little spotted kiwi samples ('southern' haplotype group) was supported by two substitutions (nucleotide position 181 of the control region and 390 of cytochrome b, Table 3).

The different phylogenetic analyses of the spotted kiwi sequences and representative brown kiwi sequences produced largely concordant relationships. The Bayesian consensus phylogeny, presented as a chronogram from r8s (see below), and nodal support values for all phylogenetic analyses are presented in Figure 2. The relationships of brown kiwi, examined with longer sequences, have been discussed in more detail elsewhere (Chapter Two). Therefore, the present discussion will be limited to the relationships among spotted kiwi. The sequences isolated from bones identified by morphology as little spotted kiwi were not supported as monophyletic in any of the analyses. As in the splitsgraph, little spotted kiwi from the northern North Island grouped with great spotted kiwi sequences, although support was low with all tree-building methods.

Overall, brown kiwi exhibited higher haplotypic (t-test; $P < 0.01$) and nucleotide diversity (t-test; $P < 0.01$) than spotted kiwi (Table 4). Brown kiwi also exhibited much longer terminal branch lengths than those of spotted kiwi (Figure 2). However, the LRT indicated no significant rate variation between sequences ($2\Delta$log-likelihood = 12.70, critical $\chi^2_{24} = 36.42$, $P > 0.05$), suggesting that the difference is not related to rate variation.

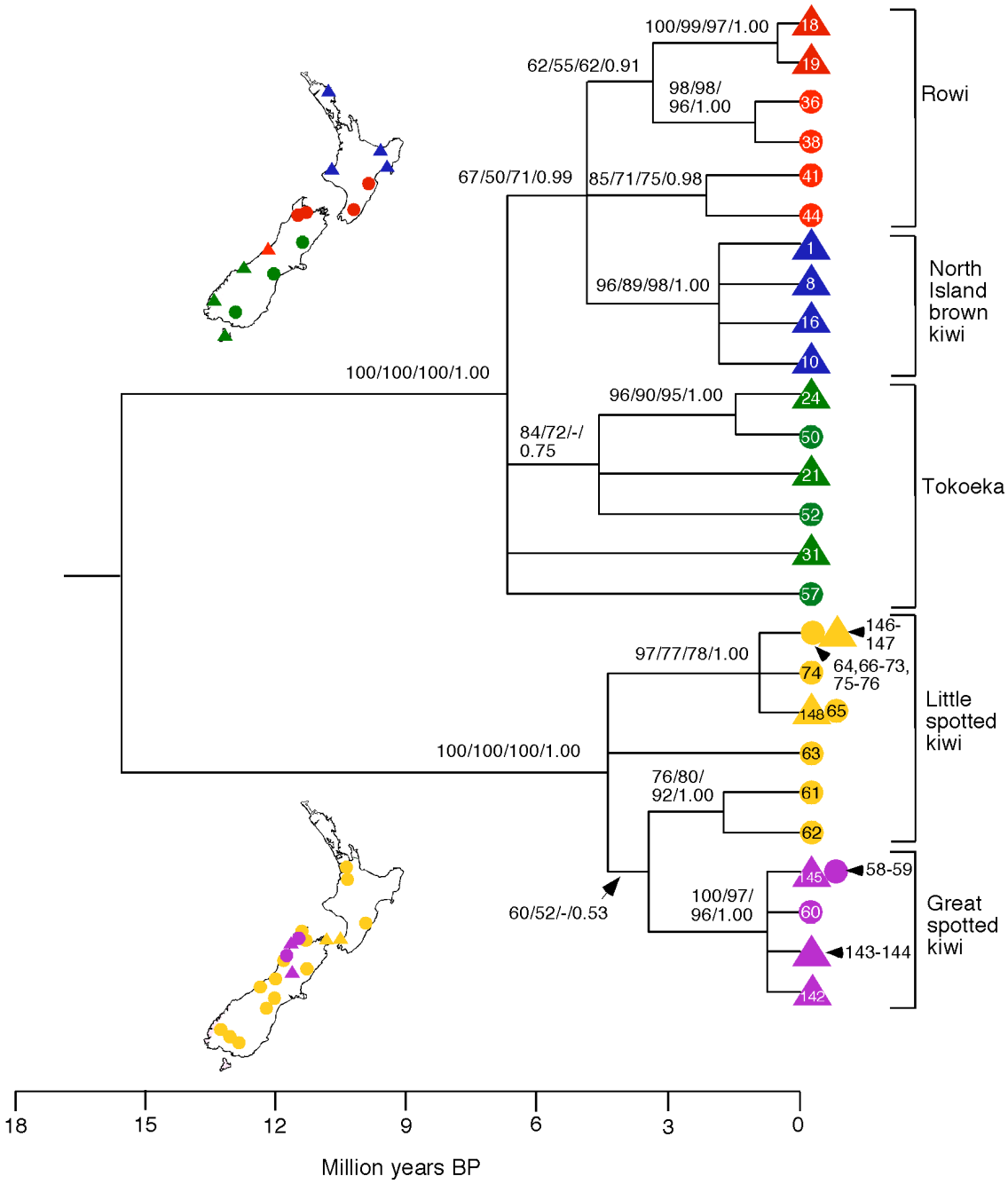


**Figure 2.** Bayesian consensus phylogeny presented as a r8s chronogram. Nodal support values from all analyses are presented in the following order NJ/MP/ML/PP. The distribution of brown kiwi samples are shown on the map at the top, with spotted kiwi samples on the lower map. Circles represent ancient samples; triangles represent modern samples. Brown kiwi sample details are given in Appendix A.

**Table 4.** Genetic diversity measures for mitochondrial DNA sequence data from brown and spotted kiwi species.

| Taxon | No. of samples | No. of haplotypes | Haplotype diversity ($h$) ± SD | Nucleotide diversity ($\pi$) ± SD |
|---|---|---|---|---|
| Brown kiwi | 75 | 47 | 0.978 ± 0.008 | 0.028 ± 0.014 |
| North Island brown kiwi | 19 | 16 | 0.982 ± 0.022 | 0.008 ± 0.004 |
| Rowi | 17 | 9 | 0.890 ± 0.054 | 0.015 ± 0.008 |
| Tokoeka | 39 | 22 | 0.943 ± 0.023 | 0.021 ± 0.010 |
| | | | | |
| Spotted kiwi | 26 | 10 | 0.745 ± 0.087 | 0.009 ± 0.005 |
| Great spotted kiwi | 7 | 4 | 0.809 ± 0.129 | 0.002 ± 0.002 |
| Little spotted kiwi | 19 | 6 | 0.538 ± 0.133 | 0.005 ± 0.003 |

*Divergence estimates*

Molecular dating using the 'standard' avian rate of cytochrome b sequence evolution of 2% per million years with corrected genetic distances resulted in a divergence date for the separation of brown and spotted kiwi in the Pliocene, whereas the species of kiwi within each morphological group diverged during the Pleistocene (Table 5). In contrast, divergence dates estimated by penalised likelihood rate-smoothing were considerably older (Figure 2, Table 5) with divergences within each morphological group dating to the Miocene and Pliocene.

**Discussion**

*Past genetic variation in little spotted kiwi*

The two little spotted kiwi samples from the northern part of the North Island ('northern' haplotype group) formed a strongly supported cluster more closely related to great spotted kiwi sequences than to the little spotted kiwi 'southern' haplotype group. However, this anomalous relationship may be a consequence of the short sequences used. Indeed, some relationships within brown kiwi were not recovered until longer sequences (over 2000 bp) were obtained (Burbidge et al., 2003). Furthermore,

**Table 5.** Estimates of divergence times for kiwi taxa as determined by two methods. The '2%' rate is based on a rate of 2% sequence divergence per million years for cytochrome b and is calculated with net average distances estimated under the Tamura Nei model. The r8s column refers to dates calculated with a fossil calibration in r8s and are also shown in Figure 2. The date marked '*' was used as a constraint**.**

| Taxa | Date (mya) | |
|---|---|---|
| | '2%' rate | r8s |
| North Island brown kiwi vs. rowi | 0.70 | 5.05 |
| Spotted kiwi root | 0.35 | 4.57 |
| Brown kiwi root | 1.00 | 6.96 |
| Kiwi root | 2.45 | 16.1* |

rowi was not recovered as a monophyletic group here, while it was in Chapter Two where longer sequences were analysed. Regardless of the exact relationships between these groups, the genetic distances between the 'northern' little spotted kiwi haplotype group, the 'southern' little spotted kiwi haplotype group and great spotted kiwi was similar to that previously used to delimit species in kiwi (Baker et al., 1995; Burbidge et al., 2003). However, delimiting species by mitochondrial genetic distance alone is not a reliable way to ascertain species boundaries (discussed in more detail in Chapter Two). Furthermore, there are no known morphological differences between the bones of North Island and South Island little spotted kiwi.

*Phylogeographic patterns in brown and spotted kiwi*

The analyses clearly show that the two major morphological groups in kiwi (i.e. brown and spotted kiwi) differed in their phylogeographic patterns. Spotted kiwi exhibited considerably less genetic variation overall than brown kiwi. A single haplotype was detected in the modern little spotted kiwi population on Kapiti Island and was also common in ancient samples throughout the South Island. The two other haplotypes detected in little spotted kiwi from the South Island differed from this common haplotype by one base pair each. In contrast, the three little spotted kiwi samples from the North Island all possessed divergent haplotypes. The sequence of the little spotted

kiwi from the bone from Coonoor was more closely related to South Island little spotted kiwi than to the other samples from mainland North Island, although support for this relationship was weak. This result may mirror brown kiwi where 'rowi' type mitochondrial DNA extends across Cook Strait into the North Island. However, more sequence from these samples is needed to further clarify their relationships.

In contrast to the widespread haplotype found in little spotted kiwi from the South Island, haplotypes detected in the brown kiwi species rowi and tokoeka tended to be restricted to a single locality (Chapter Two). Haplotypic variation in North Island brown kiwi also showed geographic partitioning, particularly into eastern and western regions (discussed further in Chapters Four and Five). Brown kiwi exhibited a higher level of variation in the South Island than in the North Island, whereas little spotted kiwi showed the opposite pattern.

The high level of genetic structuring in brown kiwi has previously been explained as resulting from their flightlessness, and thus presumed low dispersal power (Baker et al., 1995; McLennan and McCann, 2002). However, little spotted kiwi are also flightless but do not exhibit a similar high level of genetic structuring. There are a number of possible explanations for the differences in phylogeographic structure between the kiwi species. Firstly, brown and spotted kiwi may differ in their dispersal patterns. Adults of all species are generally monogamous and remain in the same territory year round (Jolly, 1990; McLennan, 1990; Taborsky and Taborsky, 1999; Wilson, 2004). However, juveniles of some species are known to disperse, although little data has been collected (Robertson, 2003). North Island brown kiwi juveniles have been recorded as dispersing as far as 25 km (Robertson, 2004) to find an unoccupied territory (Robertson, pers. comm. in Hutching, 2004). In contrast, rowi juveniles do not disperse beyond the current population boundary and will fight adults for a territory (Robertson, pers. comm. in Hutching, 2004). There is also little published data available on whether there is any sex bias in dispersal between species. Male-biased sex dispersal can result in strong geographic structure in mitochondrial phylogenies, whereas female biased dispersal leads to a lack of mitochondrial structuring (Avise, 2000). North Island brown kiwi juvenile females have been reported as dispersing further than males, although samples sizes are currently small (Basse and McLennan, 2003). Researching kiwi dispersal is a high priority objective in the current kiwi recovery plan (Robertson,

2003) and further differences between species may become apparent as more data become available. However, current dispersal measures may not accurately represent the levels of dispersal that occurred prior to human arrival when kiwi populations were much larger. Also, since little spotted kiwi are now mainly restricted to islands it may be difficult to ascertain what their dispersal behaviour may have been on the mainland.

Alternatively, the contrasting phylogeographic patterns of brown and spotted kiwi may suggest that the two kiwi groups responded differently to the Pleistocene glaciations. During the glacial cycles of the Pleistocene much of the Southern Alps of the South Island were covered in ice. Grasslands and shrublands dominated most of the remaining areas of the South Island, although a forested glacial refugium is believed to have existed in north-west Nelson (McGlone, 1985). In contrast, glaciation in the North Island was much less severe with only small, localised areas of ice (McGlone, 1985).

Under this scenario, South Island little spotted kiwi may have survived in a single glacial refugium during the last glacial maxima (LGM), thus reducing ancestral genetic diversity. Following the end of the LGM they could have expanded out of the refugium to occupy their pre-human range. Conversely, if brown kiwi were restricted to several refugia or simply occupied the areas of scrub and grassland present over much of the South Island at the time then it may explain their higher levels of variation.

There are several lines of evidence supporting this hypothesis. Firstly, little spotted kiwi bones of Holocene age have not been found in sub-alpine areas where great spotted kiwi are present (Worthy and Holdaway, 2002), suggesting that they may not tolerate the colder temperatures that would have been present during the LGM. Secondly, subfossil 'large' kiwi bones (i.e. brown or great spotted kiwi) dating to the last glaciation have been found on both the east and west of the South Island (Worthy and Holdaway, 1994; Worthy and Holdaway, 1995; Worthy, 1997; Worthy and Holdaway, 2002). However, no little spotted kiwi bones of this age are known. Lastly, brown kiwi but not little spotted kiwi presently occur on Stewart Island, which was connected to the South Island during the last glaciation but became isolated 12 000 yrs BP (Gibb, 1986). Brown kiwi subfossil bones have been found in Stewart Island bone deposits but little spotted kiwi bones have not, although deposits of landbirds are not common on Stewart Island (Worthy, 1998c; Worthy and Holdaway, 2002). This

suggests that little spotted kiwi may not have been present in the south of the South Island during the last glaciation.

*Estimation of divergence times*

Dating the time of divergence of the lineages and species within kiwi depends upon an assumed rate of evolution. Two methods have previously been used to calculate divergences in kiwi, both of which were employed here. Baker et al. (1995) used a rate of 2% sequence evolution per million years for cytochrome b to find that kiwi species within each morphological group diverged during the Pleistocene. In contrast, a rate based on an emu fossil indicated these divergences occurred during the late Miocene/Pliocene (Burbidge et al., 2003). A parallel example exists in moa. A standard rate of control region evolution was used to time divergences in the moa genus *Dinornis* to the mid Pleistocene (Bunce et al., 2003). However, Baker et al. (2005) criticised this method, suggesting that because the standard control region rate was calculated from a flying bird it was not appropriate for the "more slowly evolving ratites", although they give no justification for why ratites are slowly evolving. Presumably this is in reference to the slower metabolic rate and longer generation time of ratites relative to other birds. These are two life history traits suggested to correlate with evolutionary rates (e.g., Martin and Palumbi, 1993, but see Slowinski and Arbograst, 1999 and Gissi et al., 2000). Instead Baker et al. (2005) used a calibration date of 82 mya for the divergence of moa from other ratites (based on the separation of New Zealand from Gondwana). They claimed that the slower rate of evolution obtained was "in general accordance with the slow phylogenetic rate of evolution in mtDNA genes in kiwi". However, both estimates used deep divergence dates for calibration so it is perhaps unsurprising they are similar.

The most appropriate method for calculating divergence dates in ratites is unclear. Lovette (2004) suggested that using the calibration date of 82 mya is inappropriate for timing recent divergences in ratites because severe sequence saturation, which is difficult to correct, is likely to be present in the sequences. Similarly, using a 25 million year old emu fossil as a calibration and extrapolating backwards to the divergence of kiwi from the ratites (approximately 60 mya) may incur similar inaccuracies. There are also difficulties with applying the 2% rate. Comparisons of avian mitochondrial clock rates using different calibrations (reviewed in Lovette, 2004

and Garcia-Moreno, 2004) indicated that there was considerable variation amongst rate estimates although most cluster around the 2% level. A moa or kiwi specific rate would be ideal but ratites, and indeed birds in general, suffer from a lack of suitable fossils to use in calibrating the molecular clock. However, there are a number of Pliocene moa fossils that have yet to be exploited for dating (Worthy and Holdaway, 2002). A rate determined with ancient DNA from radiocarbon dated samples (e.g., Lambert et al., 2002) for kiwi may be most appropriate for examining recent divergences.

Correct divergence date estimates are not only important for relating geographic structuring to geological events but, in the case of kiwi, have implications for the recognition of species. For instance, Burbidge et al. (2003) suggested that since brown kiwi lineages diverged in the Pliocene, reproductive incompatibilities are likely to have arisen consequently.

*The recent history of little spotted kiwi*

Two skin samples collected from the South Island in 1978 (samples 64 and 68) were confirmed as possessing little spotted kiwi mitochondrial DNA. One of these samples, sample 68, was previously believed to be a great spotted kiwi (Jolly, 1992, but see Colbourne, pers. comm. in McLennan and McCann, 2002). This suggests that little spotted kiwi survived on the mainland until quite recently. However, the possibility that they were hybrids cannot be discounted with the present data (a hybrid between a little spotted kiwi and a rowi has previously been discovered at Okarito; Herbert and Daugherty, 2002).

The lack of sequence variation in the 'southern' haplotype group does not permit discrimination between hypotheses regarding the origin of the little spotted kiwi population on Kapiti Island. If this population derives from a translocation by Europeans then it would have been sourced from the South Island since only one little spotted kiwi from the North Island has been found in historical times (Worthy and Holdaway, 2002). Furthermore, Kapiti Island was connected to both the North and South Islands during glacial maxima (Newnham et al., 1999), although there may have been no link to the South Island during the last glacial maxima (LGM) (Worthy and Holdaway, 2002). Longer sequences, particularly of fast evolving DNA regions such as the control region, may help to identify variation within ancient little spotted kiwi from

the South Island and therefore potentially discriminate between translocation hypotheses.

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**Appendix 1.** Little spotted kiwi samples that either failed to amplify or only provided partial sequence. Museum abbreviations: MNZ – Museum of New Zealand Te Papa Tongarewa, CM - Canterbury Museum, WM – Waitomo Museum, AU – Auckland University Geology Department.

| Museum Number | Museum | Locality | Sample type | Partial sequence amplification |
|---|---|---|---|---|
| DM6663 | MNZ | Castle Rocks, Southland | bone | Control region + 299 bp cytochrome b |
| S.22175 | MNZ | Kupe Bay, D'Urville Island | skin | - |
| AV17079 | CM | Cave 30 miles from Napier | bone | - |
| AV2852 | CM | East Coast, South Island | bone | - |
| AV19575 | CM | Te Kuiti, North Island | bone | - |
| AV23470 | CM | Mahoenui, North Island | bone | - |
| AV15060 | CM | Pyramid Valley, Canterbury | bone | - |
| AV22701 | CM | Tom Bowling Bay, Northland | bone | - |
| AV32392A | CM | Springhill, Southland | bone | - |
| AV23067A | CM | Kings Cave, Canterbury | bone | - |
| AV23067B | CM | Kings Cave, Canterbury | bone | - |
| W0270.7 | WM | Junior Mudball Cave, Waitomo | bone | - |
| AU4073.60 (N02/f047) | AU | Tom Bowling Bay, Northland | bone | - |
| AU4935.2 (N02/f049) | AU | Tom Bowling Bay, Northland | bone | - |
| AU4712.9 (N02/f055) | AU | Whareana Beach, Northland | bone | - |
| AU4712.10 (N02/f055) | AU | Whareana Beach, Northland | bone | - |
| AU4917.1 (N02/f055) | AU | Whareana Beach, Northland | bone | - |
| AU917.3 (N02/f055) | AU | Whareana Beach, Northland | bone | - |

# CHAPTER FOUR

## Identification of kiwi (*Apteryx* spp.) museum specimens using ancient DNA analysis.[1]

**Abstract**

Species identification is a straightforward application of ancient DNA methodology that has been applied to archaeological specimens, commercial products of illegally harvested species and captive individuals of endangered species. This method was extended to identify unlabelled museum specimens. Museum specimens may lack detailed information for several reasons: collection details may not have been recorded or have been lost and/or there may be a lack of morphological characters with which to distinguish closely related species. DNA-based identification was used in conjunction with a reference database of both ancient and modern sequences to assign six museum specimens of kiwi to species. Four of the five species of kiwi exhibit a substantial overlap in bone morphology but can be identified with DNA sequences. In several cases the origin of the specimens was further narrowed to geographic region. For example, this method revealed that an articulated kiwi skeleton was likely to have originated from Stewart Island, a region that is currently poorly represented in museum collections.

**Introduction**

Museum collections of biological specimens represent a valuable source of information regarding aspects of species biology, such as historical changes in distribution and the range of variation in morphological characters within species. However, in the past, detailed information regarding the provenance of museum specimens was often not recorded during collection, or has subsequently been lost. Alternatively, recorded collection locality data may be unspecific; many early collections of New Zealand birds were labelled with 'New Zealand' as their sole locality identifier. A further problem arises in the situation of cryptic taxa where there are insufficient morphological

[1] Shepherd, LD and Lambert, DM. To be submitted to *Journal of Avian Biology*.

differences to distinguish between species; for example, the bones of sheep and goats (Loreille et al., 1997). Juvenile bones and bone fragments pose additional difficulties because they commonly lack the distinguishing taxonomic characteristics of the adults.

The use of DNA-based methods to identify organisms is becoming increasingly common and has predominantly focused on archaeological specimens (e.g., Matisoo-Smith and Allen, 2001; Newman et al., 2002), captive individuals of endangered species (e.g., Goldberg, 1997; Burns et al., 2003) and illegal harvesting of wildlife (e.g., Birstein et al., 1998; Roman and Bowen, 2000). Crucial for accurate DNA identification is an extensive database of DNA sequences with which to compare data from 'unknown' samples. The nature of these reference DNA sequences determines the level to which samples can be identified. Mitochondrial DNA (mtDNA) sequences have in many cases permitted discrimination between species (e.g., Baker and Palumbi, 1994; Barnes et al., 2000). In exceptional circumstances, where a very high level of genetic differentiation exists between populations, mtDNA may allow identification of unknown specimens to a geographic region within a species' distribution (e.g., Orlando et al., 2003). However, most studies that have achieved this level of resolution have relied on genotyping highly variable nuclear microsatellite DNA markers (e.g., Primmer et al. 2000; Manel et al., 2002), which are more difficult to amplify from the degraded DNA of ancient specimens (Pääbo et al., 2004).

In this study the feasibility of identifying the origin of kiwi (*Apteryx* spp.) museum specimens was explored. Museum specimens of kiwi offer examples of three situations where DNA may be able to provide information that cannot be obtained from morphological analyses. Firstly, the species identification of kiwi bones is complicated by a lack of distinguishing characters. Five kiwi species are currently recognised (Tennyson et al., 2003), and these fall into two morphological groups: brown kiwi and spotted kiwi. The bones of four of the kiwi species (North Island brown kiwi, *Apteryx mantelli;* rowi, *Apteryx rowi*; tokoeka, *Apteryx australis* and great spotted kiwi, *Apteryx haastii*) overlap considerably in size and are difficult to identify on the basis of morphometric criteria (Worthy, 1997). The bones of little spotted kiwi (*Apteryx owenii*) are identifiable because they are significantly smaller than those of other kiwi species. Secondly, bones of juvenile kiwi cannot be identified to any of the five species (A. Tennyson, pers comm.). Thirdly, many museum specimens of kiwi lack precise

provenance data. The three species of brown kiwi exhibit high levels of genetic structuring in their mitochondrial DNA (Baker et al., 1995; Burbidge et al., 2003; Chapter Two), potentially allowing DNA assignment of unknown samples from these species to geographic region or population.

Mitochondrial DNA from six kiwi specimens was isolated, sequenced and compared to a reference database of kiwi specimens. One specimen of particular interest was a mounted brown kiwi skin thought to have been collected from the Wairarapa, a region from which kiwi are extinct. If the collection information was correct then this specimen would represent the only known skin from this region. Kiwi subfossil bones from the Wairarapa have previously been shown to contain unique mitochondrial haplotypes with affinity to rowi (Chapter Two). A kiwi skin from this region would be important to investigate whether morphology is concordant with mtDNA patterns.

**Methods**

*Sampling and DNA extraction*

Five kiwi specimens of unknown, or dubious, origin were sampled from the Museum of New Zealand Te Papa Tongarewa (Table 1; Unknown 1-5). A further sample (Table 1; Unknown6) was obtained from the South Taranaki District Museum, where it was on loan from the Museum of New Zealand Te Papa Tongarewa. Specimens were sampled with the aim of minimising physical damage, with the method employed varying depending on the type of sample (Table 1). A whole toe bone from Unknown1 was sanded to remove potentially contaminating surface bone using a Dremel grinder and then ground to a fine powder in a coffee grinder. Two samples (Unknown2 and Unknown3) were drilled using a 3 mm drill bit (Figure 1) and the shavings collected. Unknown4 was sampled by removing a 0.5 $cm^2$ section from the centre of the rib bone using a Dremel grinder. The surface of this section was sanded with the Dremel grinder and the sample was then ground in a coffee grinder. The coffee grinder was cleaned thoroughly between each sample with ethanol and regularly sterilised with UV-light and a fresh Dremel wheel was used for each sample. Tissue samples (4 $mm^2$) from Unknown5 (tissue attached to skull) and Unknown6 (footpad) were removed using a clean razor blade and cut into several pieces.

A

B

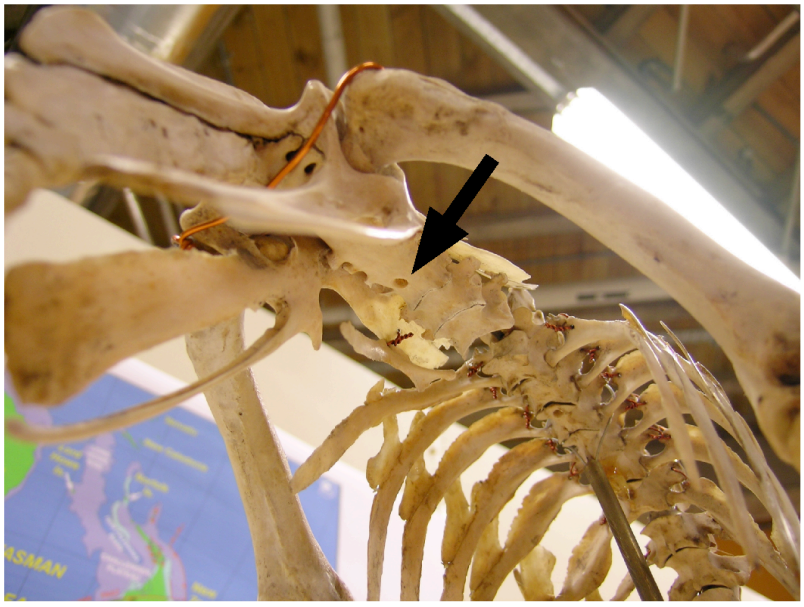

**Figure 1.** A. The articulated skeleton of Unknown3. B. Shavings from the hole drilled on the underside of the pelvis (arrowed) were used for DNA sampling. Photos by Leon Perrie.

**Table 1.** Kiwi samples of uncertain provenance analysed in this study. CR = control region, cyt b = cytochrome b.

| Sample Identifier | Museum number | Label information | Sample type | Sampling method | Sequence obtained |
|---|---|---|---|---|---|
| Unknown1 | S.1152 | none | Articulated foot | Whole toe bone | 200 bp CR; 96 bp cyt b |
| Unknown2 | S.1148 | none | Pelvis and femurs | Drilling of pelvis | 200 bp CR; 96 bp cyt b |
| Unknown3 | S.26398 | none | Articulated skeleton | Drilling of pelvis | 200 bp CR; 96 bp cyt b |
| Unknown4 | S.1145 | none | Juvenile rib | Dremel section | 190 bp CR; 299 bp cyt b |
| Unknown5 | S.966 | none | Skull | Tissue from edge of skull | 190 bp CR; 471 bp cyt b |
| Unknown6 | DM2135 | W. Smith Masterton. Coll, 1850s | Mounted skin | Footpad | 200 bp CR; 96 bp cyt b |

Ancient DNA extractions were performed in a dedicated ancient DNA laboratory physically separated from where PCR products and modern DNA were handled. Mock DNA extractions were also performed to screen for contamination. Ancient samples were extracted using the protocol described in Chapter Two.

A reference database of existing kiwi sequences was created containing modern (Baker et al., 1995; Burbidge et al., 2003) and ancient DNA sequences (Chapters Two and Three) that covered as wide a geographic area as possible (Figures 2 and 3). However, sequences of North Island brown kiwi from Little Barrier Island were omitted from the

database because they were thought to have been derived from translocations (Burbidge et al., 2003). DNA was also extracted from three additional modern North Island brown kiwi blood or tissue samples to increase the range of samples in the reference database. Tissue was obtained from one specimen from Tongariro National Park (sample 112, Appendix A). Blood samples from two individuals from Coromandel Peninsula were obtained from the National Tissue Collection, Victoria University of Wellington (samples 110 and 111, Appendix A). DNA was extracted from these three samples by proteinase digestion and phenol-chloroform extraction (Sambrook et al., 1989). Therefore, the entire reference database consisted of a total of 23 North Island brown kiwi samples (20 modern and 3 ancient), 18 rowi samples (9 modern and 9 ancient), 39 tokoeka samples (26 modern and 13 ancient), 19 little spotted kiwi samples (3 modern and 16 ancient) and 7 great spotted kiwi samples (4 modern and 3 ancient).

*DNA amplification and sequencing*

All PCR amplifications of ancient DNA were set up in the ancient DNA laboratory. A negative control was included with each batch of PCR amplifications to assess for contamination. An initial PCR amplification was performed to obtain a 190 bp fragment from domain 1 of the mitochondrial control region using the primer pair kcf2 and kcr2 (Chapter Three). Preliminary analyses were performed to establish whether each sample grouped with spotted or brown kiwi sequences. If the unknown sample grouped with brown kiwi then 200 bp of sequence from domain 1 of the control region was amplified with the primers kcf and kcr (Chapter Two). The latter primers were also used to obtain sequence from the modern North Island brown kiwi samples.

Sequence from the mitochondrial cytochrome b region was also obtained with the exact length of sequence amplified dependent upon the quality of the DNA. Two primer pairs: Kcytb1 (Chapter Two) + LSKcytbA and LSKcytB + LSKcytb2 (Chapter Three) were used to amplify two overlapping DNA fragments from Unknown4 to give a total of 299 bp of cytochrome b. The above two primer pairs and an additional primer pair LSKD + LSKE (Chapter Three) were used to amplify a total of 471 bp of cytochrome b sequence from Unknown5. From the remaining samples 96 bp of cytochrome b was amplified (Unknown 1-3, 6) using the primer LSKD combined with a novel primer Kcytb6 (5'TAGAAAGGTGAGGTGGATGA).

PCR amplifications were performed in 20µl volumes with 1x PCR buffer (500 mM Tris pH 8.8, 200 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 200 µl of each dNTP, 0.5 U Taq DNA polymerase (Roche) and 0.5 µM of each primer in a Hybaid thermocycler. The following thermocycling profile was used: 94°C for 2 minutes; ten cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minute; followed by 32 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 1 minute; and a final extension of 5 minutes at 72°C. PCR products were purified through High Pure purification columns (Roche). Automated sequencing of all PCR products was performed on an ABI-3730 (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit ver. 3.1. All DNA fragments were sequenced in both directions from independent PCR amplifications. DNA sequences were edited using Sequencher™ 3.1.1 (Gene Codes Corporation). The new sequences were easily aligned to the sequences in the reference database owing to a lack of indels.

*Phylogenetic Analyses*

Control region sequences were used to determine whether the unknown samples were spotted or brown kiwi. Then within each of these principle morphological groups the control region and cytochrome b sequences were concatenated and analysed using the neighbour joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) criteria as implemented in PAUP* 4.0b10 (Swofford, 2002). Uncorrected p-distances were used to construct NJ phylogenies. MP and ML analyses were performed using the heuristic search option with tree bisection-reconnection (TBR) branch swapping with 100 random additions of taxa. The most appropriate model of evolution for ML analyses were selected using the Akaike Information Criterion (AIC) implemented in Modeltest v3.06 (Posada and Crandell, 1998) (spotted kiwi, K81uf +I; brown kiwi, TIM + I + G). Support for the trees was assessed using 1000 (NJ) or 100 (MP, ML) bootstrap (BS) replicates.

A Bayesian approach was also applied to estimate phylogeny using MrBayes v3.04b (Huelsenback and Ronquist, 2001). The most appropriate model of evolution for the individual control region (spotted kiwi, K80 + I; brown kiwi, K81uf + I) and cytochrome b (spotted kiwi, TrN + I; brown kiwi, TrN + I) sequences was determined in Modeltest v3.06 (Posada and Crandell, 1998) and both were incorporated into a partitioned Bayesian analysis. Four Markov chains of 2 000 000 generations were run,

sampling trees every 1000 generations. The resulting log likelihood values were plotted against generation time to check that convergence had been obtained, and the initial 1000 trees were discarded as 'burn-in'. The remaining trees were used to construct a 50% majority rule consensus tree.

**Results**

All of the unidentified kiwi samples could be assigned to species with a high level of support. Three samples (Unknown1, Unknown2 and Unknown6) grouped with the reference sequences of North Island brown kiwi (Figure 2) with strong support (bootstrap support (BS) ≥ 88%; posterior probability (PP) 1.00). Although Unknown1 contained a novel haplotype not represented in the reference database, the sequences of Unknown2 and Unknown6 were identical to a haplotype presently known only in brown kiwi from Taranaki (samples 12, 13) and Tongariro (sample 112). The haplotype isolated from Unknown3 had not been previously detected but shared three synapomorphies with tokoeka haplotypes from Stewart Island (Table 2). Unknown3 and the Stewart Island samples (samples 26-31) formed a strongly supported monophyletic group in the phylogenetic analyses (BS ≥ 90%, PP 1.00) (Figure 2).

Two samples (Unknown4 and Unknown5) were nested within the spotted kiwi sequences (BS 100%, PP 0.95) (Figure 3). Furthermore, both unknown samples were identical to the most common little spotted kiwi haplotype detected in the reference sequences (Table 3). This haplotype has been detected in ancient little spotted kiwi samples from throughout the South Island (Nelson to Southland) and also occurs in the extant little spotted kiwi population on Kapiti Island.

**Discussion**

This study illustrates how ancient DNA methodology can be a powerful tool for resolving questions of sample identity and provenance. DNA was successfully retrieved and amplified from all six of the kiwi museum specimens. The high level of divergence amongst mitochondrial lineages within tokoeka permited the identification of the sample Unknown3 to a geographic region, Stewart Island, with high confidence. This is despite the lack of support for a monophyletic tokoeka, which probably relates to sequence length as tokoeka monophyly is strongly supported with the longer sequences used by Burbidge et al. (2003).

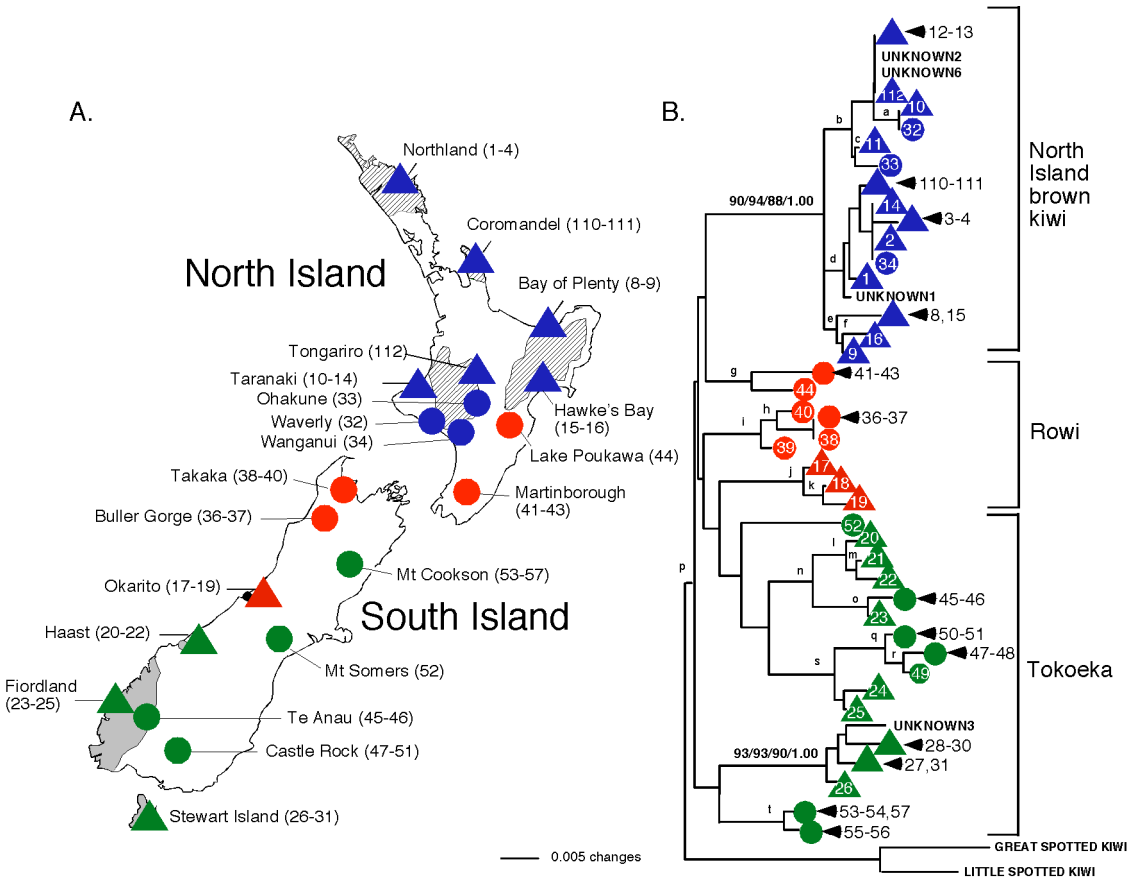


**Figure 2.** A. The current distribution of North Island brown kiwi is striped, rowi is shown in black and tokoeka in light grey. The colour of the symbols indicates the kiwi species: blue indicates North Island brown kiwi, red indicates rowi and tokoeka is represented by green. Ancient samples are represented by circles and modern samples by triangles. B. Neighbour-joining phylogeny of brown kiwi control region and cytochrome b sequences showing the position of four of the unknown kiwi samples. Support for nodes discussed in the text are given on the tree in the following order: NJ bootstrap/MP bootstrap/ML bootstrap/Bayesian posterior probabilities. Support for nodes labelled a-t are given in Appendix 1; support for remaining nodes was <50 %. Midpoint rooting was used to root the phylogeny.

**Table 2**. Variable sites defining brown kiwi mitochondrial DNA haplotypes. Nucleotide positions of the variable sites from 200bp of domain 1 of the control region and 96bp of cytochrome b are given at the top. The sample identifier on the left corresponds to Appendix A. Ancient samples are shown in bold. The three synapomorphies uniting Unknown3 with haplotypes from Stewart Island are highlighted in yellow.

| | | Control Region | Cytb |
|---|---|---|---|
| | | 1111111111111 | |
| | | 2223445556666777778122233333466 | 14557899 |
| | | 0121265780789145674657901267842 6 | 639283314 |
| **NORTH ISLAND BROWN KIWI** | | | |
| 1 | NORTHLAND1 | AAAACTATTACGCACTCTATATCCGCCCCCTA | CCCAAAGCC |
| 2 | NORTHLAND2 | .......C.......... | ......... |
| 3,4 | NORTHLAND3 | ..G....C.......... | ......... |
| 8 | BAYOFPLENTY3 | .......C.....T.....T... | .....G... |
| 9 | BAYOFPLENTY1 | ..........T.....T... | ......... |
| 10 | TARANAKI1 | G......C....T.....TT.. | ......... |
| 11 | TARANAKI3 | G..........TT.. | ......... |
| 12,13 | TARANAKI2 | G......C.....TT.. | ......... |
| 14 | TARANAKI6 | .......C.......... | ......... |
| 15 | HAWKESBAY2 | .......C.....T.....T... | .....G... |
| 16 | HAWKESBAY1 | ..........T..T..T... | ......... |
| 110,111 | COROMANDEL1 | G......C.......... | ......... |
| 112 | TONGARIRO1 | G......C.....TT.. | ......... |
| **32** | **WAVERLEY1** | G......C....T.....TT.. | ......... |
| **33** | **OHAKUNE1** | G..........TT.. | ........T |
| **34** | **WANGANUI1** | .......C.......... | ......... |
| ***Unknown1*** | | ***.......C.....T...*** | ********* |
| ***Unknown2*** | | ***G......C.....TT..*** | ********* |
| ***Unknown6*** | | ***G......C.....TT..*** | ********* |
| **ROWI** | | | |
| 17 | OKARITO8 | G.....G.CGT..G.......C....TTT... | .G......T |
| 18 | OKARITO1 | G.....G.CGT.........C....TTT... | .G......T |
| 19 | OKARITO7 | G.....G.CGT.........C.....TT... | .G......T |
| **36,37** | **BULLERGORGE** | G.....G.CG.....CT...GC....A.T... | ........T |
| **38** | **TAKAKA2** | G.....G.CG.....CT...GC....A.T... | ........T |
| **39** | **TAKAKA3** | G.....G.C......CT....C....A.T... | ........T |
| **40** | **TAKAKA1** | G.....G.CG.....C.....C....A.T... | ........T |
| **41-43** | **MARTINBOROUGH1** | G.....G.CG.....CT.GG........T... | ......A.. |
| **44** | **POUKAWA1** | G...T.G.CG.....C...G........T... | .G....A.. |
| **TOKOEKA** | | | |
| 20 | HAAST1 | .....CG.CG.....C.....CTTA..... | .G....A.. |
| 21 | HAAST3 | .....CG.CGT....C.....CTTA...T... | .G....A.. |
| 22 | HAAST4 | .....CG.CGT....C.....CTTA..... | .G....A.. |
| 23 | FIORDLAND3 | ......G.CG.A...C.....CTTA...T... | .GT...A.. |
| 24 | FIORDLAND1 | G.....G.CG.....C....GCT.A...T.C. | .G..G.A.. |
| 25 | FIORDLAND5 | G.....G.CG.....C.....CT.A...T.C. | .G..G.A.. |
| 26 | STEWART7 | ......G.......TC.C...C......T... | TG.T..A.. |
| 28-30 | STEWART2 | ......G.....T.TC.C...C......T... | TG.T..AT. |
| 27,31 | STEWART1 | ......G.C.....TC.C...C......T... | TG.T..AT. |
| ***Unknown3*** | | ***.G....G.......TC.C.....T...*** | ********* |
| **45,46** | **TEANAU2** | ......G.CG.A...C.....CTTA...T..G | .GT...A.. |
| **47,48** | **CASTLEROCK1** | G.....G.CGT..G.C....GCT.A.T.T.C. | .G..G.A.. |
| **49** | **CASTLEROCK2** | G.....G.CGT..G.C....GCT...T.T.C. | .G..G.A.. |
| **50,51** | **CASTLEROCK3** | G.....G.CG...G.C....GCT...T.T.C. | .G..G.A.. |
| **52** | **MTSOMERS1** | G..G..G.C.T..G.......CT...A.T... | .G....A.. |
| **53,54,57** | **MTCOOKSON1** | ......G.CG....TCT....C....A.T... | .G....A.. |
| **55,56** | **MTCOOKSON2** | ........CG....TCT....C....A.T... | .G....A.. |

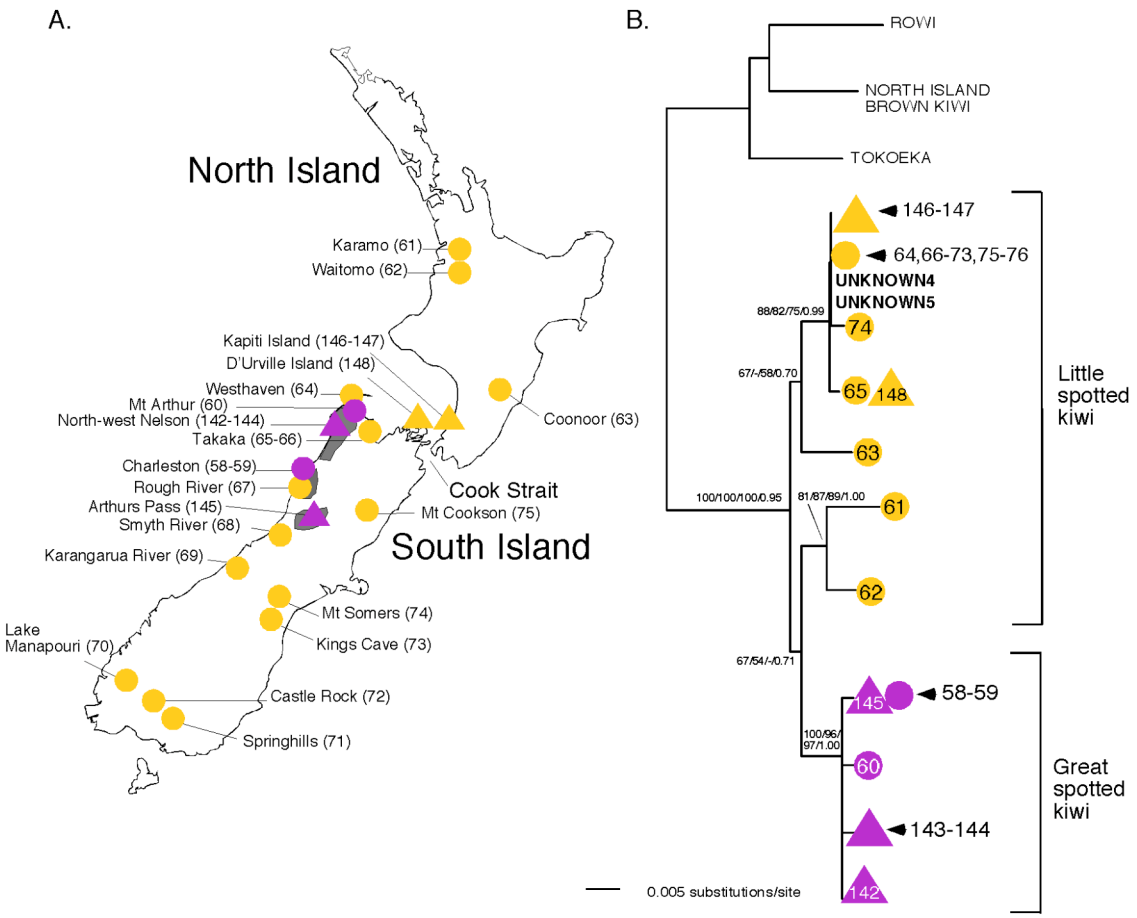


**Figure 3.** A. Map of the sample locations and the current distribution of great spotted kiwi (grey). All contemporary little spotted kiwi derive from populations on Kapiti and D'Urville Islands. Yellow symbols represent little spotted kiwi, purple symbols represent great spotted kiwi. The location of sample 76 is not mapped because of its imprecise provenance, ' West coast, South Island'. B. Neighbour-joining phylogeny of ancient (circles) and modern (triangles) spotted kiwi control region and cytochrome b sequences showing the position of two of the unknown kiwi samples. Note that Unknown4 has been placed on the phylogeny constructed from the longer sequences according to its position on the phylogeny constructed from the shorter sequences (not shown). Support for nodes are given on the tree in the following order: NJ bootstrap/MP bootstrap/ML bootstrap/Bayesian posterior probabilities. The phylogeny was midpoint rooted.

**Table 3.** Variable sites defining spotted kiwi mitochondrial DNA haplotypes. Nucleotide positions of the variable sites of 190 bp of domain 1 of the control region and 471 bp of cytochrome b are given at the top. The sample identifier on the left corresponds to Appendix A. Haplotypes detected in subfossil samples are shown in bold. * represents missing data. The three substitutions, two of which are synapomorphies, grouping Unknown4 and Unknown5 with Kapiti Island and South Island little spotted kiwi are highlighted in yellow.

| | | Control Region | Cytochrome b |
|---|---|---|---|
| | | 1111 | 111222333333444 |
| | | 5788884478 | 59347689124799013 |
| | | 7612362671 | 92700010407702294 |
| **GREAT SPOTTED KIWI** | | | |
| 142 | NW NELSON | CTACGACATA | CCTCCGTCCATGACCTA |
| 143,144 | NW NELSON | .....C. | ..... |
| 145 | ARTHURS PASS | ..... | .T..... |
| **58,59** | **CHARLESTON** | ..... | .T..... |
| **60** | **MT ARTHUR** | .C..... | ..... |
| **LITTLE SPOTTED KIWI** | | | |
| **61** | **KARAMO** | ......TG.. | ...T...TTGCT..ACG |
| **62** | **WAITOMO** | .....G.... | .....A.TT.CT..AC. |
| **63** | **COONOOR** | T..T.....G | T.C...CTT..TG.... |
| 146,147 | KAPITI | ..G......G | ....T..TT..TGT... |
| 148 | D'URVILLE | ..GT.....G | ....T..TT..TGT... |
| **64** | **WESTHAVEN** | ..G......G | ....T..TT..TGT... |
| **65** | **TAKAKA1** | ..GT.....G | ....T..TT..TGT... |
| **66** | **TAKAKA2** | ..G......G | ....T..TT..TGT... |
| **67** | **ROUGH RIVER** | ..G......G | ....T..TT..TGT... |
| **68** | **SMYTH RIVER** | ..G......G | ....T..TT..TGT... |
| **69** | **KARANGARUA RIVER** | ..G......G | ....T..TT..TGT... |
| **70** | **LAKE MANAPOURI** | ..G......G | ....T..TT..TGT... |
| **71** | **SPRINGHILLS** | ..G......G | ....T..TT..TGT... |
| **72** | **CASTLE ROCK** | ..G......G | ....T..TT..TGT... |
| **73** | **KINGS CAVE** | ..G......G | ....T..TT..TGT... |
| **74** | **MT SOMERS** | ..G.A....G | ....T..TT..TGT... |
| **75** | **MT COOKSON** | ..G......G | ....T..TT..TGT... |
| **76** | **WEST COAST** | ..G......G | ....T..TT..TGT... |
| ***Unknown4*** | | ***..G......G*** | ***....T..T*********** |
| ***Unknown5*** | | ***..G......G*** | ***....T..TT..TGT...*** |

This is a valuable finding because there are few samples of Stewart Island tokoeka in museums (A. Tennyson, pers. comm.).

The samples Unknown1, 2 and 6 can clearly be attributed to North Island brown kiwi. But in contrast to tokoeka, population assignment is limited by the lack of divergence between North Island brown kiwi populations. The situation is further complicated by mitochondrial haplotypes that do not 'fit' with the phylogeographic patterns proposed by Burbidge et al. (2003). In the reference database two identical haplotypes, from

Wanganui (sample 33) and Taranaki (sample 14), grouped more closely with Northland haplotypes than to geographically closer haplotypes. Although support for this grouping is weak in this dataset, analyses of additional sequence demonstrate that these haplotypes cluster with Northland haplotypes with strong support (Burbidge et al., 2003; Chapter Two). Burbidge et al. (2003) suggested that the sample from Taranaki (sample 14) was, or was descended from, a bird translocated from Northland. However, the skin from Wanganui (sample 33) analysed in this study was collected in 1968 and predates the translocations referred to by Burbidge et al. (2003). Possibly the skin was mislabelled or derives from an earlier unrecorded translocation. However, a subfossil kiwi bone from Waitomo (Chapter Five) also groups with Northland sequences and is likely to predate any possible translocations. Perhaps a more plausible explanation is that regional populations within North Island brown kiwi do not have monophyletic mtDNA lineages because of incomplete lineage sorting. More extensive sampling is required to provide a clearer picture of population structure within North Island brown kiwi. Nuclear markers, such as highly variable microsatellite DNA loci, may give greater resolution of relationships among North Island brown kiwi populations as well as provide further information regarding the provenance of unlabelled samples. However, the success rate of retrieving nuclear DNA from ancient specimens, particularly bone samples, is likely to be considerably less than for obtaining mtDNA and possibly restricted to only the most recent, well-preserved samples.

Unknown6 was thought to represent the only known brown kiwi skin from the Wairarapa region of the southern North Island. Kiwi are extinct from this area but ancient DNA sequences obtained from kiwi bones have revealed that it was previously occupied by kiwi with affinities to rowi and distinct from North Island brown kiwi (Chapter Two). However, the sequence obtained from Unknown6 was identical to those obtained from North Island brown kiwi from Taranaki and Tongariro. There are several possible explanations for this result. Firstly, Unknown6 may not have been collected from the Wairarapa but is instead mislabelled, or the label may refer to the residence of the collector or taxidermist rather than the site where the specimen was collected. This is supported by there being no other recent samples from the Wairarapa despite substantial searching of known kiwi collections (e.g., Museum of New Zealand Te Papa Tongarewa, Canterbury Museum, Auckland Museum, American Museum of Natural History, Vienna Natural History Museum). This suggests that brown kiwi may

have been extinct in the Wairarapa when European collectors arrived in New Zealand. Alternatively, if Unknown6 was indeed collected from the Wairarapa, then its haplotype may have co-existed with rowi-like haplotypes, although it has not been detected in the sampling of ancient diversity carried out to date.

The haplotype recovered from Unknown1 had not been sampled from any modern North Island brown kiwi. However, it has been isolated from brown kiwi feathers on Maori cloaks (Kahu kiwi) and baskets (kete) (Chapter Five). Provenance details associated with some of these Maori artefacts indicate that this haplotype was present in the Hawke's Bay and Bay of Plenty. This haplotype may be present in extant North Island brown kiwi that have not yet been sampled or it may be an extinct haplotype.

Two of the unknown samples were little spotted kiwi but the lack of variation within little spotted kiwi mitochondrial haplotypes (Chapter Three) restricts the precise identification of their provenance. Nearly all of the little spotted kiwi bones from the South Island, as well as the extant population on Kapiti Island, share an identical haplotype. This haplotype has not been detected in the reference sequences from the North Island, although sampling here has been limited. Genetic distinctions amongst regional populations of little spotted kiwi may be able to be detected with increased sequence length. Generating a reference database covering the past geographic range of little spotted kiwi would require considerable effort because the degraded DNA of these ancient samples would necessitate that the sequence be constructed from a number of short overlapping fragments.

The little spotted kiwi sequences provide an example of the importance of an extensive reference database. If the reference database only included sequences from extant little spotted kiwi populations (i.e. those derived from kiwi from Kapiti and D'Urville Islands) then it would have appeared that Unknown4 and Unknown5 originated from Kapiti Island (the D'Urville Island kiwi sampled here had a different haplotype). However, when sequences from little spotted kiwi subfossil samples that encompass the past distribution are included it becomes evident that the unlabelled specimens may have derived from anywhere in the South Island.

It has been noted that museums can be a valuable source of DNA for genetic studies (e.g., Graves and Braun, 1992; Payne and Sorenson, 2002; Winker, 2004). This research demonstrates that genetic analyses can also benefit museum collections. Species identification is a simple, yet under appreciated, application of ancient DNA methodology that could potentially be extended to identify bones of other cryptic New Zealand taxa. For example, it may allow distinction between samples of the red-crowned (*Cyanoramphus novaezelandiae*) and yellow-crowned (*C. auriceps*) parakeets, whose bones are morphologically indistinguishable (Worthy and Holdaway, 1994). DNA identification could also determine the species used, and potentially their provenance, in biological components of cultural artefacts (e.g., Borson et al., 1998; Chapter Five).

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Those who supplied kiwi samples are gratefully acknowledged: C. Daugherty, Victoria University; the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University; A. Tennyson, G. Stone and T. Worthy, Museum of New Zealand Te Papa Tongarewa and the South Taranaki District Museum. L. Perrie provided comments on the manuscript.

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**Appendix 1.** Nodal support values for Figure 2.

Nodal support for the branches indicated in Figure 2 with the analyses given in the order: neighbour joining bootstrap/maximum parsimony bootstrap/maximum likelihood bootstrap/Bayesian posterior probability. Dashes indicate bootstrap values below 50% or posterior probabilities below 0.5.

a. 71/68/69/0.98
b. 56/57/-/-
c. 52/-/-/-
d. -/-/53/0.54
e. 54/-/-/0.82
f. 64/-/54/0.87
g. 70/65/69/1.00
h. -/-/-/0.93
i. 64/51/-/0.90
j. 88/84/80/0.99
k. 66/-/54/0.89
l. 78/62/59/0.97
m. 52/-/-/0.5
n. 88/54/53/0.95
o. 95/89/89/1.00
p. 99/100/96/1.00
q. 75/70/69/0.97
r. 62/-/-/-
s. 76/74/70/0.97
t. 88/71/72/0.97

# CHAPTER FIVE

## The provenance of brown kiwi feathers in Maori cloaks and kete from DNA analysis.[1]

**Abstract**

The source species and geographic provenance of brown kiwi (*Apteryx* spp.) feathers in eighteen Maori cloaks and kete were examined using DNA analysis. A total of 48 mitochondrial control region DNA sequences comprising six haplotypes were successfully retrieved from fifteen of the artefacts. The haplotypes obtained from the feathers indicated that all fifteen artefacts were constructed using North Island brown kiwi feathers. The majority of the haplotypes obtained were identical to those detected in modern and ancient populations from the Hawke's Bay and Bay of Plenty regions. However, the most common haplotype isolated from the artefact feathers has not been detected in modern kiwi populations. Artefacts of known provenance suggested that this haplotype derives from the Hawke's Bay region. Three cloaks possessed a mixture of haplotypes that potentially derive from a number of different geographic regions. However, a more extensive reference database is required to build an accurate map of haplotype distributions.

### Introduction

New Zealand Maori made extensive use of biological materials from their surroundings (Orbell, 1996). For example, marine mammal teeth were made into necklaces and bones were made into fishhooks (Buck, 1987). Similarly, Maori cloaks (kakahu) and baskets (kete) were constructed largely from biological materials. The earliest written records of Maori cloaks are from Captain Cook's first visit to New Zealand in 1769-1770 (Pendergrast, 1997). At this time the flax (*Phormium* spp.) rain cape was the most common cloak type. Finely woven flax cloaks, often covered in dog skin, were worn only by chiefs. Feathered garments are commonly mentioned in the mythology of Maori and other Polynesian groups, but were not a common feature of Maori culture when the first European explorers reached New Zealand (Pendergrast, 1997). Early

[1] Shepherd, LD and Lambert, DM. To be submitted to *Molecular Ecology*.

flax cloaks sometimes had feathers, or skin with feathers attached, scattered across the cloak surface or woven into cloak borders (Ling Roth, 1923; Pendergrast, 1987). Maori cloaks completely covered in feathers began to be produced in the second half of the nineteenth century, and quickly became established. Kahu kiwi, cloaks covered with the feathers of brown kiwi, became, and are still considered, the most prestigious type of Maori cloak (Pendergrast, 1987). Feathers were attached in groups to the flax fabric by weaving the feather bases into the fabric, and then bending them back so that the ends could be secured by weaving them in a second time, thus preventing the feathers from falling out (Figure 1). It has been suggested that a full kiwi feather cloak required at least 12 birds (Te Kanawa, 1992).

Kete are small woven baskets or bags used by Maori for carrying different items, e.g., foods such as shellfish (Orbell, 1996). Like cloaks, kete were commonly woven from flax, although other plant species were also employed (Buck, 1987). Unadorned kete were used for practical purposes, whereas decorated kete, for example, with kiwi feathers, served as personal ornamentation.

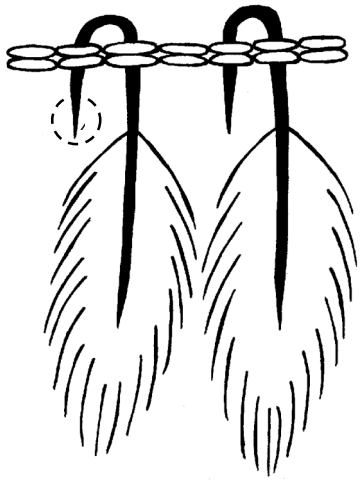

**Figure 1.** Feathers were attached to Maori cloaks and kete by being woven in twice (adapted from Pendergrast, 1987). Feather-bases (circled) were removed for DNA extraction leaving the remainder of the feather still firmly attached.

Many Maori kiwi feather cloaks and kete are held in museums, both in New Zealand and worldwide, but in many cases information regarding their provenance is unknown. Mitochondrial DNA analysis may enable determination of the provenance and species of individual brown kiwi feathers from such artefacts. Three brown kiwi species are currently recognised: North Island brown kiwi (*Apteryx mantelli*), rowi (*A. rowi*) and tokoeka (*A. australis*) (Tennyson et al., 2003). Brown kiwi, in particular rowi and tokoeka, exhibit high levels of mitochondrial DNA structuring in both modern (Baker et al., 1995; Burbidge et al., 2003) and extinct populations (Chapter Two). In this study DNA sequence was obtained from brown kiwi feathers on Maori cloaks and kete with the aim of identifying the species and provenance from which they were sourced.

**Methods**

*Sampling and DNA extraction*

Fifteen brown kiwi feather cloaks and three kete from Hawke's Bay Museum were sampled (Table 1). The majority of cloaks had no provenance information, although the donor had been recorded for fourteen of the fifteen cloaks. In contrast, all three kete had associated provenance information. The MacLean family, who were early settlers to the Hawke's Bay, donated eight of the cloaks to the Hawke's Bay museum, most likely in the 1930-40s (Fea and Pishief, 1996).

Between four and twenty-five feather bases, each one to three millimetres in length, were removed from the cloaks and kete using tweezers. In many cases the main part of the feathers had been previously detached and lost from the artefact and the feather-base was all that remained attached to the fabric. For the remaining samples the feather-base was carefully detached leaving the rest of the feather still woven into the cloak or kete (Figure 1), thus minimising damage to the artefacts. DNA was extracted from each feather-base in a physically isolated ancient DNA laboratory at Massey University where no modern DNA or PCR products have been handled. DNA was extracted with a proteinase digestion followed by phenol-chloroform extraction (Huynen et al., 2003). A negative extraction control was included with each batch of extractions.

An accurate and substantial reference database of sequences from samples of known origin is an essential tool in determining the provenance of 'unknown' samples. The

**Table 1.** Details of kiwi feather cloaks and kete from Hawke's Bay Museum.

| Sample identifier | Museum accession number | Feathers yielding DNA sequence/ number of feathers tested | Accompanying information |
|---|---|---|---|
| Cloak 1 | 2647 | 10/25 | Unknown acquisition source. |
| Cloak 2 | 2649 | 3/7 | Donated by Mrs G. Chapman (Havelock North). |
| Cloak 3 | 2644 | 4/4 | Donated by Mr A.R. Wilkie (Napier). |
| Cloak 4 | 2639 | 5/7 | Lent by Miss A.H. Bibby (Hawke's Bay). Given by the Waipawa Maori to Miss Bibby's grandfather. |
| Cloak 5 | 2641 | 2/9 | Donated by P.S. MacLean. |
| Cloak 6 | 2554 | 3/14 | Donated by Lady Florence MacLean. |
| Cloak 7 | 2624 | 4/7 | Donated by Mrs M.L. Smith (Napier). |
| Cloak 8 | 2642 | 2/8 | Donated by Mrs E.A. Navin (Hawke's Bay). |
| Cloak 9 | 2643 | 2/10 | Donated by J. Kelly (Hastings). |
| Cloak 10 | 2637 | 0/4 | Donated by Lady Florence MacLean. |
| Cloak 11 | 2653 | 0/8 | Donated by Lady Florence MacLean. |
| Cloak 12 | 26077 | 2/8 | Donated by Lady Florence MacLean. |
| Cloak 13 | 2646 | 0/4 | Donated by Lady Florence MacLean. |
| Cloak 14 | 2654 | 2/4 | Donated by Lady Florence MacLean. |
| Cloak 15 | 4909 | 2/9 | Donated by Lady Florence MacLean. Purchased from Masterton. |
| Kete 1 | 4917 | 4/9 | Made by Joyce Grace, Tarawera, 1930s. |
| Kete 2 | 4914 | 1/7 | Made about 1910 at Te Pohue, Hawke's Bay. |
| Kete 3 | 4908 | 2/5 | Made about 1887 at Tangoio, Hawke's Bay. |
| | | Total = 48/149 | |

brown kiwi reference database sequences, to which cloak feather sequences were compared, consisted of previously obtained modern (Burbidge et al., 2003) and ancient brown kiwi DNA sequences (Chapters Two and Four; Appendix A). Five additional samples of known provenance, four modern and one subfossil (Table 2), were included to supplement those already in the reference database. DNA was obtained from the modern samples (4 $\mu$l whole blood, 2-3 feather bulbs or 100 mg of hard tissue) by proteinase digestion and phenol-chloroform extraction (Sambrook et al., 1989) in a 'modern' DNA laboratory. DNA was extracted from the kiwi subfossil, a toe bone. The entire bone was ground in a clean coffee grinder and decalcified, followed by proteinase digestion and phenol-chloroform extraction (protocol described in Chapter Two). DNA extraction and PCR set-up from this sample was performed in the Massey University ancient DNA laboratory.

*DNA amplification and sequencing*

All PCRs of ancient DNA were set up in a dedicated ancient DNA laboratory. A negative control was included with each batch of PCR amplifications to control for contamination. Two hundred base pairs (bp) of sequence from domain 1 of the mitochondrial control region was obtained from the feather, blood and bone samples through PCR amplification with the primer pair kcf and kcr (Chapter Two).

**Table 2.** Details of additional samples of known provenance used in the reference database.

| Sample identifier | Sample type | Specimen code | Collection details |
|---|---|---|---|
| 77 | Feather | NTU75 | Opotiki, Bay of Plenty coll. R. Burns |
| 78 | Footpad | 33806 | Opotiki, Bay of Plenty coll. R. Burns |
| 81 | Blood | CD1212 | Ex. Whakatane, Bay of Plenty (3.7.85) coll. C. Daugherty |
| 84 | Blood | CD889 | Ex. Gisborne, Hawke's Bay (20.10.84) coll. B. Reid and A. Billing |
| 35 | Bone | W080 | Maniapoto cave, Te Kuiti. |

PCR amplifications were performed in a Hybaid thermocycler in 20 µl volumes with 1× PCR buffer (500 mM Tris pH 8.8, 200 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 200 µl of each dNTP, 0.5 U Taq DNA polymerase (Roche) and 0.5 µM of each primer. The following thermocycling profile was used: 94°C for 2 minutes; ten cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minute; followed by 32 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 1 minute; and a final extension of 5 minutes at 72°C. PCR products were purified through High Pure purification columns (Roche). PCR products were sequenced on an ABI-3730 (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit ver. 3.1. All DNA fragments were sequenced in both directions from independent PCR amplifications. The sequences produced here were aligned by eye. The DNA sequences derived from cloaks and kete were compared to a DNA database. The control region reference database used here comprised a total of 28 North Island brown kiwi samples (24 modern and 4 ancient), 18 rowi samples (9 modern and 9 ancient) and 39 tokoeka (26 modern and 13 ancient).

*Sequence analysis*

Initial phylogenetic analyses were performed to determine from which brown kiwi species the cloak feathers were sourced. A phylogeny was constructed using uncorrected p-distances and the neighbour joining method as implemented in PAUP* 4.0b10 (Swofford, 2002). Support for the phylogeny was estimated using 1000 bootstrap replicates. All of the haplotypes from the cloak and kete feathers grouped with North Island brown kiwi (*Apteryx mantelli*), so subsequent analyses only involved reference sequences from this species.

North Island brown kiwi mitochondrial control region sequences are closely related (Chapter Two). Therefore, relationships between these sequences were represented with a network (Posada and Crandall, 2001). An advantage of network methods is that they can accurately represent coexisting ancestral and descendent haplotypes, whereas methods that construct trees, such as maximum parsimony and neighbour joining, do not. Also, networks can display equally parsimonious relationships simultaneously. A network of North Island brown kiwi sequence relationships was constructed using the statistical parsimony method (Templeton et al., 1992) as implemented in TCS version 1.13 (Clement et al., 2000). This method allows missing (i.e. extinct or undetected)

intermediate haplotypes to act as branch points in the network, providing an advantage over other available methods.

**Results**

The success of DNA amplification varied among cloaks and kete and ranged from a 100% success rate (4/4 samples amplified from Cloak 3) to 0% for three of the cloaks (Cloaks 10, 11 and 13; Table 1). All of the haplotypes from the cloaks and kete grouped with strong support with those of North Island brown kiwi in the neighbour joining phylogeny (bootstrap support 84%; phylogeny not shown).

The 200 bp of control region sequence included eight variable sites within all of the North Island brown kiwi analysed (Table 3), and these defined twelve haplotypes. Six haplotypes were present in the cloaks; two of these were also found in the kete. Up to three different haplotypes were detected from a single cloak and two from a kete. Five of the haplotypes from the artefacts had previously been isolated from modern North Island brown kiwi samples (Burbidge et al., 2003). The sixth haplotype (haplotype 8) had not been detected from modern North Island brown kiwi, but had been sequenced from a subfossil bone sample of unknown provenance from the Museum of New Zealand Te Papa Tongarewa (Chapter Four).

Figure 2A shows a statistical parsimony network of North Island brown kiwi DNA sequence relationships constructed from the reference database of sequences of known provenance. A map of known haplotype distributions was produced from the reference database sequences (Figure 2B). The DNA sequence obtained from sample 81, a modern reference sample sequenced specifically for this study, extended the distribution of haplotype 11 beyond Hawke's Bay to include Bay of Plenty. The distribution of the most common artefact haplotype (haplotype 8, Table 3), which had not been detected in modern North Island brown kiwi, is mapped according to its occurrence in cloak 4, kete 1 and 3, which have associated provenance data. The haplotypes detected within each cloak and kete, as well as the known distributions of these haplotypes, as determined from the reference database, are presented in Figure 3.

The majority of the cloaks and all three of the kete contained haplotypes that have been

**Table 3**. The twelve North Island brown kiwi mitochondrial control region haplotypes (the same colours and numbering system is used in Figures 2 and 3) and their distributions and occurrence in artefacts. Variable sites of 200 bp of domain 1 of the mitochondrial control region and their nucleotide position are given. * haplotype 8 was not detected in the reference database but was isolated from artefacts known from the Hawke's Bay.

| Haplotype | Distribution of haplotype from reference database samples | Artefacts possessing haplotype | 1111<br>22562334<br>02799284 |
|---|---|---|---|
| 1 | Northland | - | AATCCCCC |
| 2 | Northland | - | .GC..... |
| 3 | Northland, Taranaki, Wanganui, Waitomo | Cloak 5 | ..C..... |
| 4 | Coromandel | - | G.C..... |
| 5 | Taranaki, Tongariro | Cloak 5 | G.C...TT |
| 6 | Taranaki, Waverley | - | G.CT..TT |
| 7 | Taranaki, Ohakune | - | G.....TT |
| 8 | -* | Cloak 1, Cloak 2, Cloak 3, Cloak 4, Cloak 6, Cloak 7, Cloak 8, Cloak 9, Cloak 12, Cloak 14, Kete 1, Kete 3 | ..C...T. |
| 9 | Bay of Plenty, Hawke's Bay | Cloak 1, Cloak 2, Cloak 7, Cloak 15, Kete 1, Kete 2 | ..C.T.T. |
| 10 | Bay of Plenty | Cloak 1, Cloak 2, Cloak 4 | ....T.T. |
| 11 | Hawke's Bay, Bay of Plenty | Cloak 3, Cloak 6, Cloak 7, Cloak 14 | ....TTT. |
| 12 | Bay of Plenty | - | .T..T.T. |

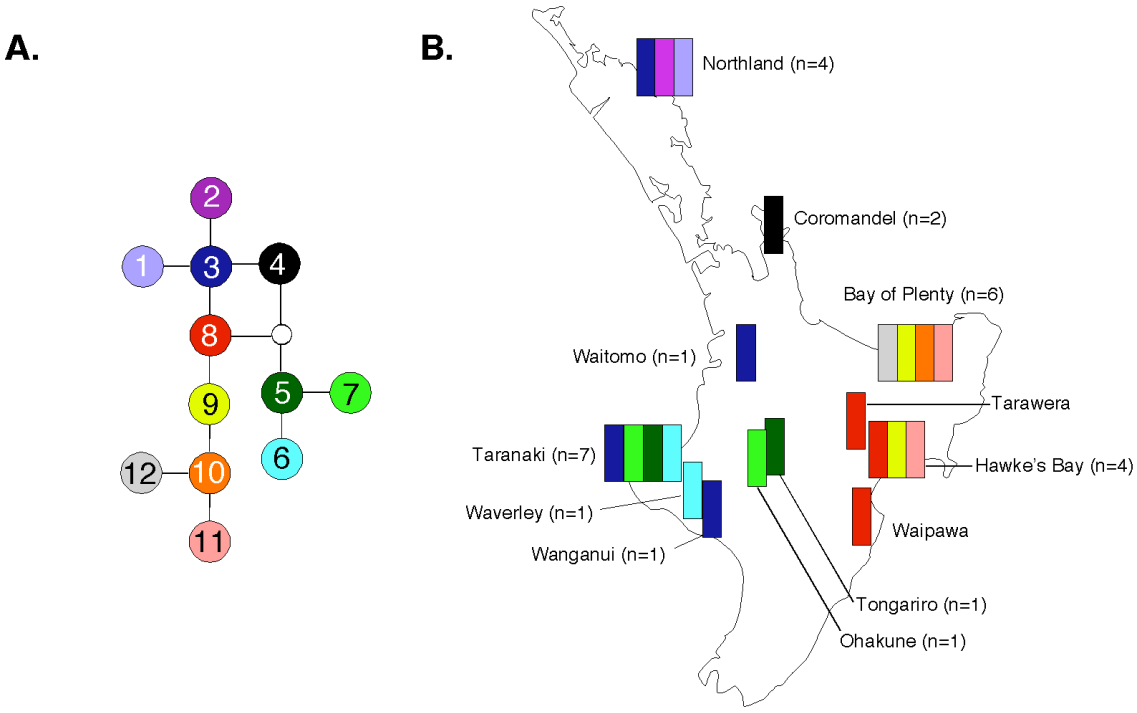


**Figure 2.** A. Statistical parsimony network of the twelve North Island brown kiwi control region haplotypes. Each haplotype is represented by a numbered, coloured circle. The smaller, open circle in the network represents a missing intermediate haplotype. The sequence of each haplotype is given in Table 2. B. Map of the North Island of New Zealand illustrating the sites where North Island brown kiwi control region haplotypes have been detected as coloured bars, with the colour corresponding to the colour of the haplotype circles in Figure 2A and Table 2. The distributions of the haplotypes were determined from the reference database, except for haplotype 8 (in red) which is mapped according to its detection in three of the artefacts of known provenance.

previously only found in the Bay of Plenty and Hawke's Bay regions. One cloak (cloak 5) had haplotypes that have previously been detected from only western New Zealand (Taranaki and Northland). No cloaks or kete contained a mixture of haplotypes from eastern and western North Island. However, three cloaks (cloaks 1, 2 and 4) comprised feathers whose haplotypes have not been found together from the same site in the reference database samples. All of these cloaks possessed haplotype 8, detected only

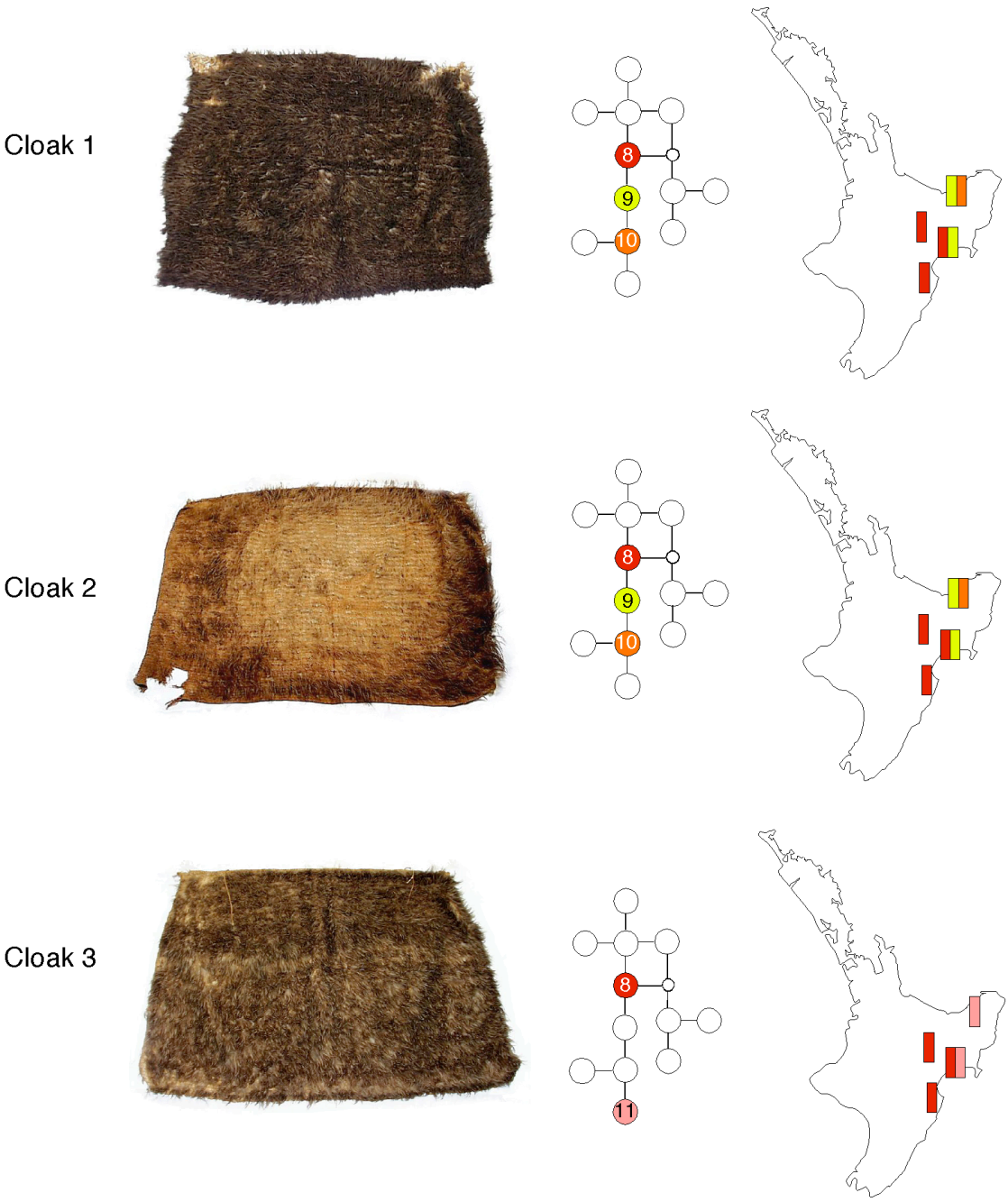


**Figure 3**. The haplotypes (numbered, coloured circles) detected in each cloak and kete, and their position in the statistical parsimony network of all North Island brown kiwi mitochondrial control region haplotypes. The haplotype numbers and colours match those used in Figure 2 and Table 2. A map of the known distribution of each haplotype (coloured bars), determined from the reference database, is also given for each cloak and kete. No photos were available for cloak 5 and cloak 6.

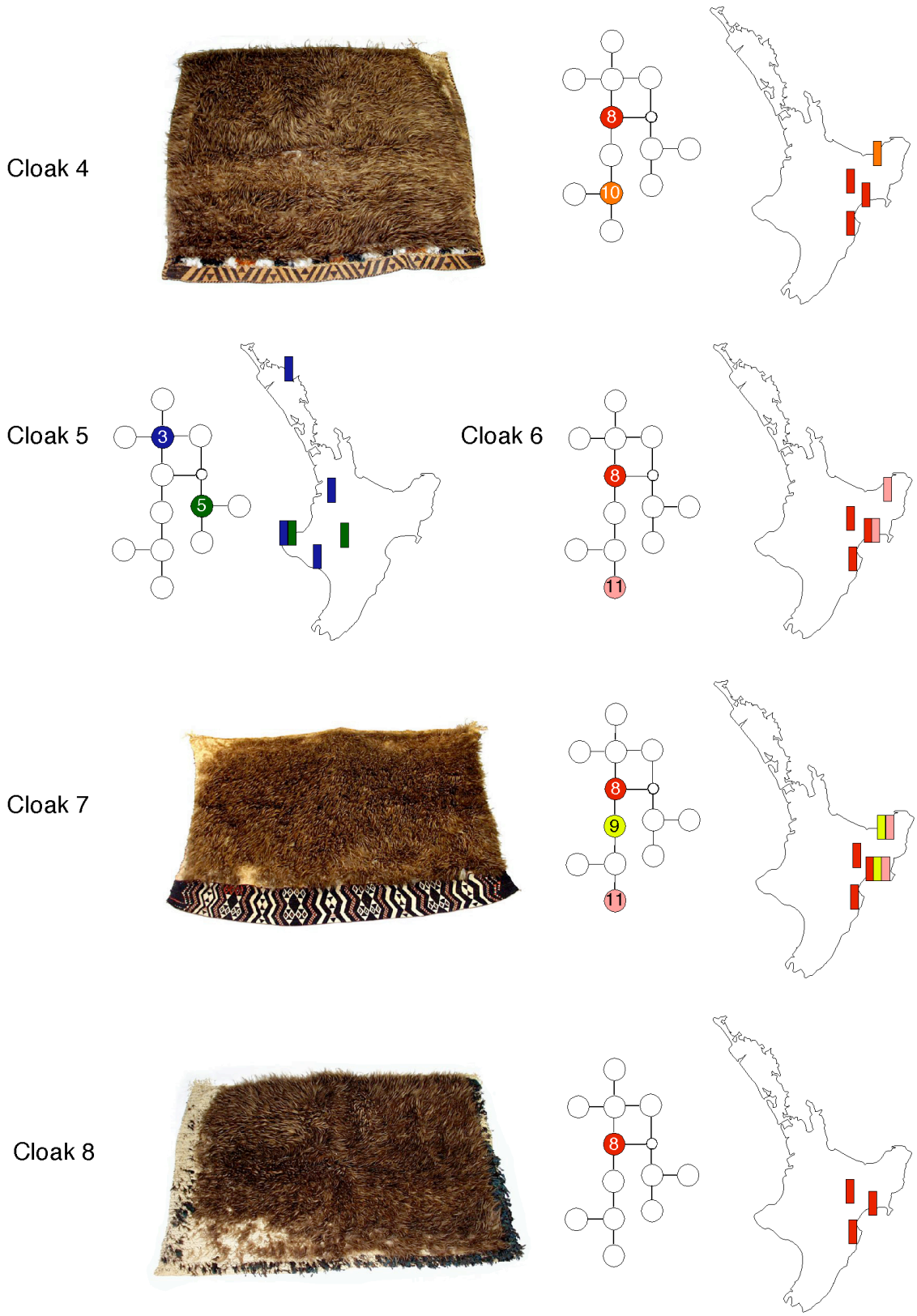


**Figure 3** continued.

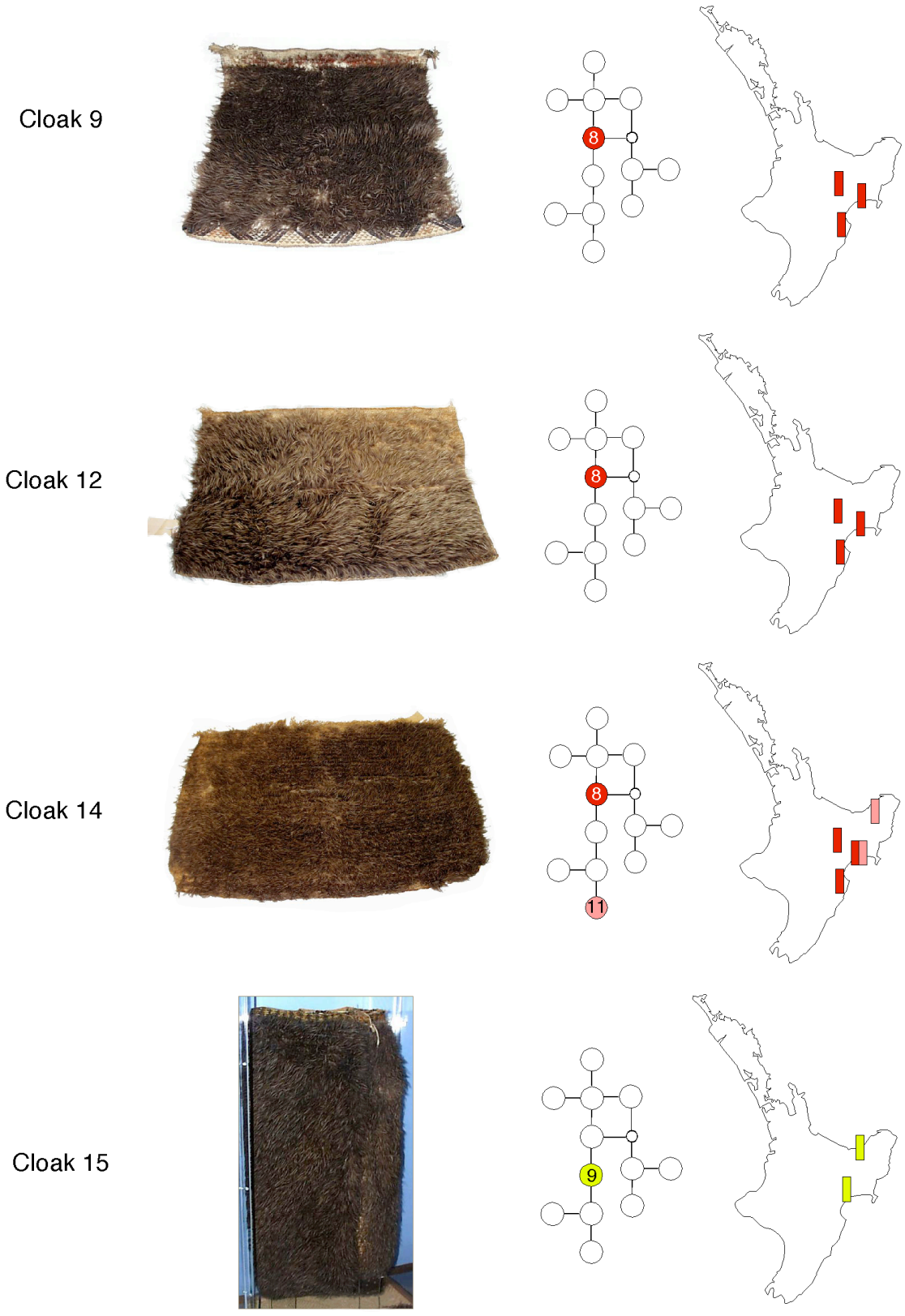


**Figure 3** continued.

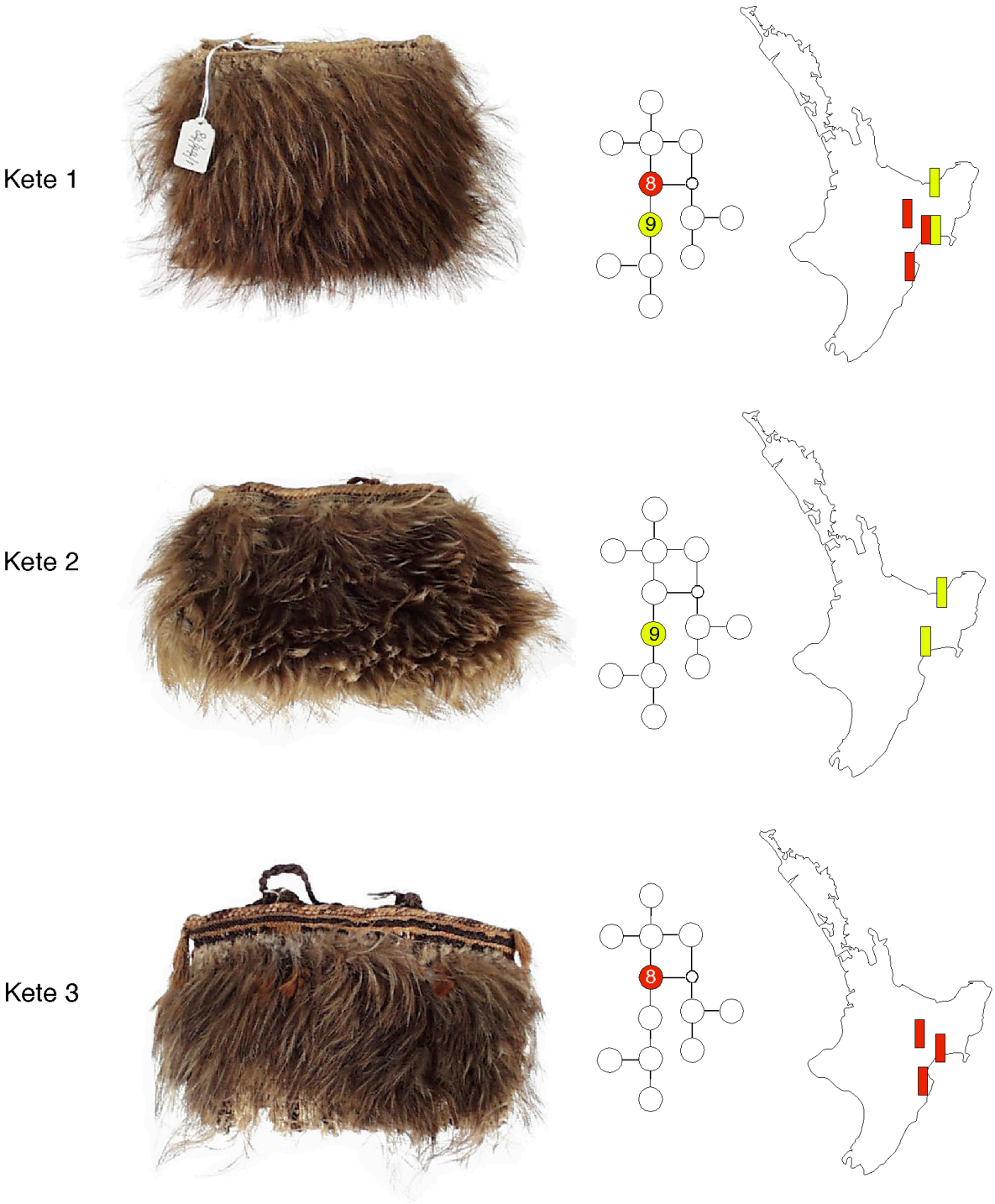


**Figure 3** continued.

in North Island brown kiwi from Hawke's Bay and haplotype 10, found only from North Island brown kiwi from the Bay of Plenty (Figure 2).

**Discussion**

Ancient DNA has provided insight into a number of evolutionary questions including past levels of genetic variation (e.g., Miller and Waits, 2003), the systematics of extinct organisms (e.g., Paxinos et al., 2002) and rates of evolution (e.g., Lambert et al., 2002). It also has the potential to determine the origins of biological components of cultural artefacts, although this application has been little explored (but see Borson et al., 1998; Burger et al., 2000). The present study demonstrates that DNA sequence can be successfully obtained from kiwi feathers from Maori cloaks and kete and that, by comparison to a reference database, these sequences can be used to investigate provenance.

Fourteen of the fifteen Maori artefacts analysed possessed haplotypes that have only been detected from North Island brown kiwi in the Hawke's Bay and Bay of Plenty. This is perhaps unsurprising considering that all of the cloaks were part of the Hawke's Bay Museum collection and are likely to have been originally produced by local Maori.

The most common haplotype identified from the cloaks and kete, haplotype 8, has not been detected in modern North Island brown kiwi. Therefore, modern samples do not help place the provenance of this haplotype. However, haplotype 8 was identified from one cloak and two kete that do have associated provenance information. The cloak was made in Waipawa (cloak 4) and the kete are from Tarawera and Tangoio (kete 1 and kete 3, respectively). Assuming that these artefacts were constructed from locally-obtained kiwi, the Hawke's Bay region can be considered the putative provenance for the unlabelled artefacts bearing haplotype 8. This haplotype may have become extinct in modern North Island brown kiwi, or it may still be extant but has not been detected because of insufficient sampling.

Three cloaks (cloaks 1, 2 and 4) were found to comprise a mixture of haplotypes that, as indicated by the reference database, are not known to occur together at the same site. One of these haplotypes, haplotype 8, was only detected from artefacts made in Hawke's Bay, as discussed in the previous paragraph. The other haplotype, haplotype

10, has only been detected from modern North Island brown kiwi from the Bay of Plenty. There are a number of possible explanations for this result. Firstly, Maori may have hunted kiwi over a considerable area, for example Hawke's Bay and Bay of Plenty, when obtaining feathers for artefacts. Secondly, kiwi feathers may have been traded between Maori from Hawke's Bay and Bay of Plenty. However, perhaps the most plausible explanation is that this result is a consequence of an insufficient number of samples in the reference database. The reference database contained a total of four sequences from the Hawke's Bay and six from the Bay of Plenty. Adding further reference samples from these regions may provide more accurate haplotype distributions and thus determine whether haplotypes 8 and 10 really are restricted to the Bay of Plenty and Hawke's Bay respectively; possibly both of these haplotypes did occur in one or both of these regions.

The occurrence of haplotypes in these cloaks and kete is consistent with the separation of North Island brown kiwi haplotypes into Eastern and Western haplotype groups. That is, no artefacts with admixed Eastern and Western haplotypes were found**.** An east/west genetic split in the North Island has been observed for a number of New Zealand taxa including two species of moa (Baker et al., 2005), short-tailed bat (Lloyd, 2003) and the parasitic plant *Dactylanthus taylorii* (Holzapfel et al., 2002). It has been proposed that these east/west genetic differences have resulted from recurrent large-scale volcanic eruptions in the central North Island (Holzapfel et al., 2002; Lloyd, 2003). Within each of these Western and Eastern regions, some North Island brown kiwi haplotypes are widespread such that few populations are diagnosable by a particular haplotype or haplotype cluster. This contrasts with the situation found in rowi and tokoeka, where populations are diagnosable. Therefore, evidence for trading of kiwi feathers by Maori might be better obtained from kiwi feather cloaks constructed in the South Island.

The cloaks exhibited considerable variation in the PCR amplification success rate, possibly owing to variation in age, feather preparation, the amount the cloaks were worn or how they have been stored. However, overall there was a high success rate with DNA obtained from feathers in fifteen of the eighteen artefacts. This demonstration of the feasibility of provenance-determination through DNA analysis of feather-containing cloaks and kete indicates that this technique could be applied to other

feathered Maori artefacts. Moreover, feathered artefacts and garments are made by a number of other cultures, particularly in the Pacific. Brightly coloured featherwork artefacts (e.g., cloaks and helmets) from Hawaii have been particularly sought after by museums worldwide (Holt, 1985). DNA analysis of such objects could potentially be applied to questions regarding the species identity and provenance of their feather components.

Many Maori cloaks, particularly early examples, are presently held in museums in Europe. However, in many cases information regarding the precise origin of these cloaks has been lost (Bruce McFadgen, Department of Conservation, pers. comm.). Knowledge of the provenance of such cloaks may aid repatriation efforts by allowing particular iwi (tribes) to claim ownership of cloaks produced with kiwi feathers from their region.

**Acknowledgements**

The staff of the Hawke's Bay museum, particularly R. Mulvay, K. Mitcalfe and D. McKnight generously allowed access to kiwi feather cloaks and kete. C. Daugherty provided access to the National Tissue Collection and K. Wilde (Waitomo Museum) permitted sampling of the kiwi toe bone sample. L. Huynen kindly sequenced three of the modern reference samples. H. Lawrence and L. Perrie provided valuable comments. K. Mitcalfe and H. Taukamo generously made their cloak and kete photographs available.

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# CHAPTER SIX

## Nuclear microsatellite DNA markers for New Zealand kiwi (*Apteryx* spp.).[1]

**Abstract**

Genetic differentiation in the endemic New Zealand kiwi was investigated using five polymorphic microsatellite DNA loci and a preliminary set of available samples. Statistical analyses (exact tests, $F_{ST}$ and $R_{ST}$ values) revealed high levels of divergence among species, and a Bayesian assignment test suggested that subdivision was also present within two of the kiwi species examined. These nuclear data provided discordant results regarding the relationships between the brown kiwi species compared to previously published mitochondrial DNA sequences. Possible reasons for this discordance are discussed.

### Introduction

Kiwi (Apterygidae) are an endemic New Zealand family of ratite birds. They have a number of features that are unusual for birds; for example, two functional ovaries, external nostrils at the end of their bill, marrow filled bones, and they live in burrows (Peat, 1990). These characteristics led Calder (1978) to describe kiwi as, in some respects, more similar to mammals than birds. Two morphological groups and five allopatric species of kiwi are currently recognised (Tennyson et al., 2003). The brown kiwi group consists of North Island brown kiwi (*Apteryx mantelli*), rowi (*A. rowi*) and tokoeka (*A. australis*), with tokoeka divided into two subspecies: *A. a. australis* from Haast and Fiordland, and *A. a. lawryi* found on Stewart Island. The spotted kiwi group comprises the great spotted kiwi (*A. haastii*) and the little spotted kiwi (*A. owenii*). The ranges of all kiwi species have contracted through habitat clearance and predation by introduced mammals. All five species are considered threatened (Robertson, 2003) and are actively conserved.

[1] A reduced version of this chapter has been published: Shepherd, LD and Lambert, DM (2006) Nuclear microsatellite DNA markers for New Zealand kiwi (*Apteryx* spp.). *Molecular Ecology Notes* 6 (1), 227.

Genetic studies of brown kiwi using mitochondrial DNA (mtDNA) sequences have demonstrated extremely high levels of divergence among both modern (Baker et al., 1995; Burbidge et al., 2003) and ancient brown kiwi populations (Chapter Two). In addition, mitochondrial DNA sequences from rowi suggested a closer relationship with North Island brown kiwi rather than the geographically closer tokoeka (Baker et al., 1995; Burbidge et al., 2003). This relationship was unexpected because it is discordant with morphology and behaviour. However, the mitochondrial genome is inherited maternally as a single unit, and therefore represents a gene genealogy that may not be identical to the species phylogeny (Nichols, 2001). There are many published examples of discordance between the relationships suggested by mitochondrial versus nuclear markers (e.g., Shaw, 2002; Monsen and Blouin, 2003). Hence, data from bi-parentally inherited nuclear markers are necessary to further clarify relationships and taxonomic boundaries in kiwi. Initial studies of nuclear markers (allozymes) provided little resolution because of low levels of variation (Baker et al., 1995; Herbert and Daugherty, 2002). Microsatellite DNA markers are usually more variable than allozymes (Hedrick, 1999), and their development for kiwi may allow verification of the relationships suggested from the mtDNA analysis. Such markers may also be valuable in assessing individual identity, parentage and levels of relatedness in captive-bred kiwi.

In this study, five nuclear microsatellite DNA markers isolated from North Island brown kiwi are presented. These markers were used for a preliminary investigation of genetic differentiation between kiwi species and to compare the partitioning of variation with that of mitochondrial DNA.

## Methods

*Sample collection and DNA extraction*

Kiwi tissue samples were collected from dead birds sent to the Institute of Veterinary, Animal and Biomedical Sciences, Massey University. Kiwi blood and feather samples were obtained from the National Tissue Collection and the Department of Conservation respectively. Overall a total of 66 modern kiwi samples from a range of locations were obtained (Table 1 and Figure 1).

**Table 1.** Numbers and locations of kiwi taxa sampled in this study. Further sample details are given in Appendix A.

| Species | Region | Number of samples | Sample identifier |
|---|---|---|---|
| North Island brown kiwi | Bay of Plenty | 7 | 77-83 |
| | Hawke's Bay | 5 | 84-88 |
| | Northland | 21 | 89-109 |
| | Coromandel | 2 | 110-111 |
| Rowi | Okarito | 9 | 113-121 |
| Tokoeka | Fiordland | 2 | 122-123 |
| | Stewart Island | 1 | 124 |
| | Haast | 8 | 125-132 |
| GSK | West Coast | 9 | 133-141 |
| LSK | Kapiti Island | 2 | 142-143 |

Genomic DNA was extracted from 4 µl whole blood, 2-3 feather bulbs or 100 mg of hard tissue by proteinase digestion and phenol-chloroform extraction (Sambrook et al., 1989). DNA samples were resuspended in 10 mM Tris, 1 mM EDTA pH 8.0 and stored at 4°C.

*Microsatellite DNA loci isolation*

A genomic library enriched for CA/GT repeats was constructed for North Island brown kiwi from Tangiteroria, Northland, following a modified protocol of Armour et al. (1994) as described by Berry et al. (2003). Eight hundred and sixty four clones were screened for the presence of microsatellite containing inserts with $^{32}$P-labelled $(CA/GT)_n$ dinucleotides. All 30 positive clones were sequenced using M13 primers. Primers were designed to microsatellite repeat flanking regions either manually or using

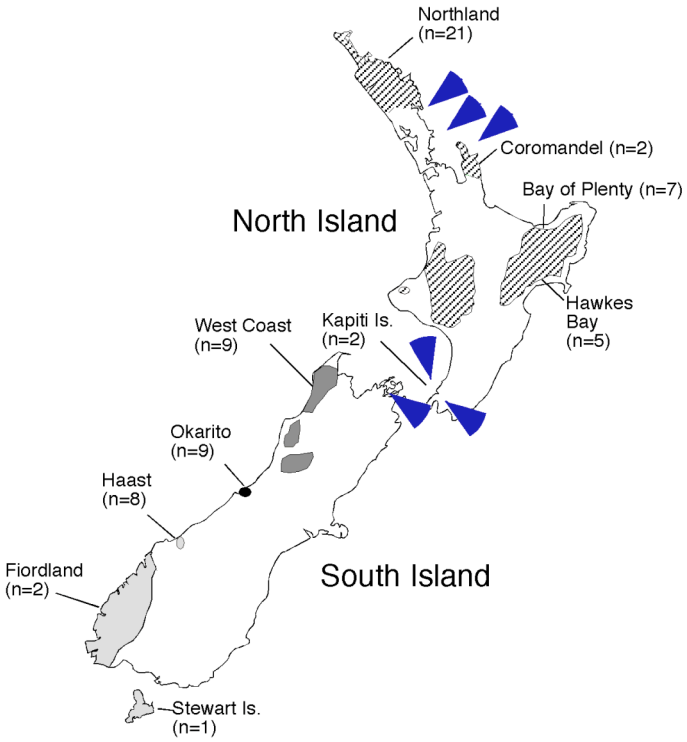


**Figure 1.** Location of kiwi samples used in this study. The number of samples obtained from each location is given in brackets. The current distributions of kiwi species are shown: North Island brown kiwi is striped, tokoeka in light grey, rowi in black, great spotted kiwi is shown in dark grey and little spotted kiwi populations are indicated by blue arrows.

Primer3 (Rozen and Skaletsky, 2000). Five loci amplified consistently and were polymorphic in North Island brown kiwi (Table 2).

*Microsatellite PCR and genotyping*

The five microsatellite loci were amplified from samples of North Island brown kiwi (*Apteryx mantelli*), from which they were isolated, as well as from samples of the other kiwi species (*A. australis*, *A. rowi*, *A. haastii* and *A. owenii*).

PCR amplifications were performed in a Hybaid Omnigene Thermal Cycler and conditions were optimised for each set of primers (Table 2). Primers were fluorescently labelled with 6-FAM (Apt 29 and Apt 68), PET (Apt 37) or NED (Apt 59 and Apt 35)

**Table 2.** Microsatellite DNA loci isolated from North Island brown kiwi. Primer sequences, the repeat sequence, length of the cloned allele and annealing temperature used in PCR are given.

| Locus | Repeat motif in clone | Primer Sequences (5' to 3') | Clone size (bp) | Annealing temp. (°C) |
|---|---|---|---|---|
| Apt29 | $(AC)_{12}(AG)_2(AC)_2$ | F-AGTAGCTACATGCGTACGTGTC | 103 | 56 |
| | | R-TGGCCCACCTGGAGATGTGCA | | |
| Apt35 | $G_6(TG)_{14}$ | F-CAGCTTGTCTCAGGGAGCATTTGT | 153 | 58 |
| | | R-CTATCTCAAGCGGCATCACAAAAG | | |
| Apt37 | $(AC)_4T(CA)_3TG(CA)_8$ | F- CTGATTTGGCTTACTGCTGAC | 151 | 56 |
| | | R-AAGGCTGAATCCAGGCCAA | | |
| Apt59 | $AAAACAAAAAC(AAAC)_3(AC)_{14}$ | F-TCTGTGCCTTGGAAGCAGTC | 142 | 56 |
| | | R-GGAAGCTTGGGATCACTGGG | | |
| Apt68 | $(TG)_{11}$ | F-GGACCAGTGTGTTTATATATTCTGC | 206 | 56 |
| | | R-TGCAGATTCAGCCAGTAACG | | |

dyes. PCR amplification was performed in 10 µl volumes containing 0.5 units Taq polymerase (Roche), 200 µM of each dNTP, 0.8 pmol of each primer, 1.5 mM $MgCl_2$, 1× buffer (Roche) and 1 µl extracted DNA. For locus Apt 35, 1 µl of 10% dimethylsulfoxide (BDH Laboratory Supplies) was added to PCR reactions. Samples were amplified with the following thermocycling profile: 94°C for 4 minutes; followed by 30 cycles of denaturation at 94°C for 45 seconds, annealment at 56-58°C for 50 seconds, elongation at 72°C for 1 minute; and a final elongation of 5 minutes.

Microsatellite allele sizes were determined on an ABI 3730 automatic sequencer using GeneMapper version 3.0 software with an internal LIZ size standard (Applied Biosystems).

*Statistical analysis*

Microsatellite loci were tested for linkage disequilibrium and departure from Hardy-Weinberg equilibrium within each species using Genepop version 3.4 (Raymond and Rousset, 1995). Observed and expected heterozygosities were calculated with Arlequin 2.001 (Schneider et al., 2000). Allelic richness, which provides a measure of the number of alleles per population independent of population size, was estimated using rarefaction in FSTAT version 2.9.3 (Goudet, 1995). Little spotted kiwi was omitted from the allelic richness calculation because only two samples were available. A minimum sample size of seven was used for calculating allelic richness from the remaining species; this was the smallest number of individuals typed at a locus.

Population differentiation between kiwi species was investigated by performing pairwise Fisher's exact test using Genepop version 3.4 (Raymond and Rousset, 1995). The level of structuring was assessed by calculating pairwise $F_{ST}$ (Weir and Cockerham, 1984) and $R_{ST}$ (Michalakis and Excoffier, 1996) estimates using SPAGeDi version 1.1 (Hardy and Vekemans, 2002). The relative importance of genetic drift versus mutation in the genetic differentiation of kiwi was determined by an allele size permutation test in SPAGeDi version 1.1 (Hardy and Vekemans, 2002). This test determines whether allele size contributes to population differentiation by randomly changing the allele sizes in a data set but maintaining allele identity, and then computing $R_{ST}$ for this simulated data set. This randomisation procedure is performed a number of times (10 000 in this case) to determine $\rho R_{ST}$. If the observed $R_{ST}$ is significantly higher than

$\rho R_{ST}$, then stepwise mutations have contributed to differentiation and $R_{ST}$ is a more appropriate statistic to use than $F_{ST}$ (Hardy and Vekemans, 2002).

Shared allele distances ($D_{AS}$) between individuals were calculated and a UPGMA tree was constructed in POPULATIONS version 1.2.28 (Langella, 2000), and visualised in TREEVIEW version 1.6.6 (Page, 1996).

An assignment test was performed using the Doh calculator (Paetkau et al., 1995; Brzustowski, 2002) to assess whether individuals could be accurately assigned to species. In addition, a Bayesian assignment test was implemented in STRUCTURE version 2.1 (Pritchard et al., 2000; Falush et al., 2003) to determine the most likely number of clusters (K) in the data set. STRUCTURE simultaneously infers population structure and assigns individuals to populations. The program was run for $10^6$ MCMC repetitions with a 'burnin' of 30 000 iterations. No prior population information was used and admixture was assumed. Allele frequencies among clusters were assumed to be independent to prevent overestimation of the number of clusters (Falush et al., 2003). K, the number of sampled populations, was set at 1-8. The posterior probabilities of the data, P(X/K), were calculated from the mean estimated log likelihood of each K, lnP(X/K), in order to select the optimal K. At the optimum K, samples were assigned to each cluster based upon the highest percentage of membership (q). A threshold value of $q \geq 0.9$ was chosen for determining an accurate assignment (after Cegelski et al., 2003).

**Results**

*Loci characterisation*

Five microsatellite DNA loci were used to genotype a total of 66 kiwi from five species (2-35 individuals per species). Allele frequencies, expected ($H_E$) and observed ($H_O$) heterozygosity values are presented in Table 3. Each pair of microsatellite loci was tested for linkage disequilibrium and independence was confirmed. Hardy-Weinberg tests were performed for all loci in all species. Two tests showed deviation from Hardy-Weinberg equilibrium ($P < 0.0001$) following a sequential Bonferroni correction (Rice, 1989). Both tests involved a single locus (Apt 29), in North Island brown kiwi and tokoeka.

**Table 3**. Microsatellite DNA allele frequencies, expected ($H_E$) and observed ($H_O$) heterozygosities and allelic richness (A) for five putative kiwi species.

| Locus | Allele length (bp) | NI brown kiwi (n=35) | Rowi (n=9) | Tokoeka (n=11) | Great spotted kiwi (n=9) | Little spotted kiwi (n=2) |
|---|---|---|---|---|---|---|
| Apt37 | 147 | 0.015 | 0.000 | 0.000 | 1.000 | 1.000 |
| | 149 | 0.000 | 1.000 | 0.864 | 0.000 | 0.000 |
| | 151 | 0.956 | 0.000 | 0.136 | 0.000 | 0.000 |
| | 155 | 0.029 | 0.000 | 0.000 | 0.000 | 0.000 |
| | $H_O$ | 0.088 | - | 0.091 | - | - |
| | $H_E$ | 0.115 | - | 0.325 | - | - |
| | A | 1.578 | 1.000 | 1.964 | 1.000 | - |
| Apt59 | 124 | 0.000 | 0.000 | 0.000 | 0.000 | 0.250 |
| | 130 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 131 | 0.000 | 0.000 | 0.000 | 0.333 | 0.000 |
| | 133 | 0.000 | 0.000 | 0.000 | 0.500 | 0.000 |
| | 135 | 0.000 | 0.000 | 0.000 | 0.111 | 0.750 |
| | 137 | 0.071 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 142 | 0.243 | 0.000 | 0.045 | 0.000 | 0.000 |
| | 143 | 0.000 | 0.000 | 0.000 | 0.056 | 0.000 |
| | 144 | 0.443 | 0.000 | 0.182 | 0.000 | 0.000 |
| | 146 | 0.129 | 0.000 | 0.636 | 0.000 | 0.000 |
| | 148 | 0.043 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 150 | 0.014 | 0.111 | 0.000 | 0.000 | 0.000 |
| | 152 | 0.029 | 0.056 | 0.045 | 0.000 | 0.000 |
| | 154 | 0.000 | 0.333 | 0.000 | 0.000 | 0.000 |
| | 156 | 0.000 | 0.500 | 0.091 | 0.000 | 0.000 |
| | 158 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | $H_O$ | 0.686 | 0.555 | 0.455 | 0.444 | 0.500 |
| | $H_E$ | 0.733 | 0.712 | 0.580 | 0.745 | 0.833 |
| | A | 5.038 | 3.739 | 4.142 | 3.739 | - |
| Apt68 | 202 | 0.000 | 0.000 | 0.000 | 0.812 | 0.000 |
| | 206 | 0.632 | 0.444 | 0.000 | 0.000 | 0.000 |
| | 208 | 0.250 | 0.278 | 0.450 | 0.000 | 0.000 |
| | 209 | 0.000 | 0.000 | 0.000 | 0.062 | 1.000 |
| | 210 | 0.059 | 0.000 | 0.500 | 0.000 | 0.000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 212 | 0.059 | 0.278 | 0.050 | 0.125 | 0.000 |
| | $H_O$ | 0.588 | 0.778 | 0.500 | 0.250 | - |
| | $H_E$ | 0.568 | 0.699 | 0.574 | 0.442 | - |
| | A | 3.106 | 3.000 | 2.700 | 2.867 | - |
| Apt35 | 139 | 0.000 | 0.000 | 0.000 | 1.000 | 1.000 |
| | 145 | 0.000 | 0.056 | 0.000 | 0.000 | 0.000 |
| | 151 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 153 | 0.286 | 0.278 | 0.500 | 0.000 | 0.000 |
| | 155 | 0.071 | 0.056 | 0.409 | 0.000 | 0.000 |
| | 157 | 0.314 | 0.556 | 0.091 | 0.000 | 0.000 |
| | 159 | 0.157 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 161 | 0.086 | 0.056 | 0.000 | 0.000 | 0.000 |
| | 163 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 165 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 167 | 0.043 | 0.000 | 0.000 | 0.000 | 0.000 |
| | $H_O$ | 0.686 | 0.788 | 0.455 | - | - |
| | $H_E$ | 0.795 | 0.641 | 0.602 | - | - |
| | A | 5.434 | 4.333 | 2.879 | 1.000 | - |
| Apt29 | 85 | 0.014 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 91 | 0.029 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 93 | 0.100 | 0.000 | 0.000 | 0.111 | 0.000 |
| | 95 | 0.000 | 0.000 | 0.000 | 0.000 | 1.000 |
| | 97 | 0.029 | 0.000 | 0.000 | 0.444 | 0.000 |
| | 99 | 0.143 | 0.000 | 0.000 | 0.444 | 0.000 |
| | 101 | 0.186 | 0.125 | 0.000 | 0.000 | 0.000 |
| | 103 | 0.343 | 0.000 | 0.000 | 0.000 | 0.000 |
| | 105 | 0.086 | 0.000 | 0.091 | 0.000 | 0.000 |
| | 107 | 0.000 | 0.000 | 0.136 | 0.000 | 0.000 |
| | 109 | 0.029 | 0.000 | 0.045 | 0.000 | 0.000 |
| | 111 | 0.043 | 0.875 | 0.318 | 0.000 | 0.000 |
| | 113 | 0.000 | 0.000 | 0.091 | 0.000 | 0.000 |
| | 115 | 0.000 | 0.000 | 0.091 | 0.000 | 0.000 |
| | 119 | 0.000 | 0.000 | 0.227 | 0.000 | 0.000 |
| | $H_O$ | 0.571 | 0.000 | 0.364 | 0.222 | - |
| | $H_E$ | 0.839 | 0.350 | 0.900 | 0.634 | - |
| | A | 6.225 | 1.992 | 6.234 | 2.961 | - |

The number of microsatellite alleles per locus ranged from 4 to 16, with a total of 52 alleles identified (Table 3). Each species exhibited at least one private allele. North Island brown kiwi had the greatest number of alleles, as measured by allelic richness (Table 3), although this difference was not significant (Wilcoxon signed rank test, $P > 0.05$). However, the comparisons of North Island brown kiwi with both rowi and great spotted kiwi approached significance (Wilcoxon signed rank test, $P = 0.0625$).

*Genetic differentiation between kiwi species*

Pairwise exact tests of genic differentiation between species revealed that all comparisons demonstrated highly significant differences in allele frequencies ($P < 0.0001$). $F_{ST}$ and $R_{ST}$ estimates were also high for all pairwise comparisons of species (Table 4). Pairwise tests suggested that the observed $R_{ST}$ was not significantly different than the simulated $R_{ST}$ ($\rho R_{ST}$) for three comparisons: rowi versus tokoeka, little spotted kiwi versus rowi and great spotted kiwi versus little spotted kiwi (Table 4). However, regardless of which estimate was used, both $F_{ST}$ and $R_{ST}$ indicated that rowi and tokoeka are more closely related to one another than either is to North Island brown kiwi.

**Table 4.** $R_{ST}$ and $pR_{ST}$ and $F_{ST}$ values for pairwise comparisons between kiwi species. The significance values refer to the allele size permutation test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

| Comparison | $R_{ST}$ | $pR_{ST}$ (95% C.I.) | $F_{ST}$ |
|---|---|---|---|
| N.I.B. vs. Rowi | 0.664** | 0.259 (0.010-0.562) | 0.372 |
| N.I.B. vs. Tokoeka | 0.582**** | 0.177 (0.018-0.410) | 0.323 |
| N.I.B. vs. GSK | 0.684** | 0.336 (0.069-0.618) | 0.482 |
| N.I.B. vs. LSK | 0.681* | 0.358 (-0.010-0.688) | 0.504 |
| Rowi vs. Tokoeka | 0.467 | 0.252 (0.001-0.535) | 0.253 |
| Rowi vs. GSK | 0.918*** | 0.414 (0.047-0.796) | 0.598 |
| Rowi vs. LSK | 0.914 | 0.481 (0.021-0.895) | 0.632 |
| Tokoeka vs. GSK | 0.875*** | 0.350 (0.032-0.683) | 0.530 |
| Tokoeka vs. LSK | 0.870** | 0.368 (-0.083-0.787) | 0.528 |
| GSK vs. LSK | 0.268 | 0.467 (-0.023-0.752) | 0.506 |

In the dendrogram of shared allele distance ($D_{AS}$) between individual kiwi (Figure 2), all rowi samples formed a cluster with the sample from Stewart Island. The remaining tokoeka samples (i.e. those from Fiordland and Haast) formed another cluster. The $D_{AS}$ tree also supported the genetic distinctiveness of both North Island brown kiwi and the spotted kiwi. Some subdivision was evident within North Island brown kiwi in the $D_{AS}$ tree. The two samples from Coromandel grouped together. Samples from Northland largely clustered together, although some grouped with Hawke's Bay and Bay of Plenty samples.

The doh assignment test correctly assigned all individuals to species, except for one sample from the Bay of Plenty (sample 86) that was assigned to Tokoeka. STRUCTURE divided kiwi into five clusters (posterior probability = 0.999; all other values of K resulted in posterior probabilities < 0.001). North Island brown kiwi were assigned to two clusters, suggesting that there may be subdivision within this species. This is not unexpected considering samples were collected from a number of geographic locations. 55.1% of samples from Northland were assigned with high probability to a single cluster ($q > 0.9$) and a total of 81.8% of the Northland samples had $q > 0.82$. The samples from the Bay of Plenty tended to be assigned to a different cluster than those from Northland but had lower q values (23.1% had $q > 0.9$ and 53.8% had $q > 0.82$). The q values of the two samples from Coromandel separated them between the two North Island clusters.

Genetic structuring was also evident within the tokoeka samples with all individuals from the Haast population of tokoeka assigned to a separate cluster ($q > 0.95$). The remaining tokoeka samples (i.e. those from Fiordland and Stewart Island) had low q values that divided them between the other brown kiwi clusters. Seven of the nine rowi samples were assigned to a single cluster with high probability ($q > 0.95$), and the probability that the remaining two rowi samples belonged to the same cluster was also high ($q > 0.85$). All great spotted and little spotted kiwi samples grouped together in a single cluster ($q > 0.96$).

**Discussion**

The analyses of the five microsatellite DNA loci isolated in this study indicated a high level of divergence amongst the kiwi species. All of the currently recognised species

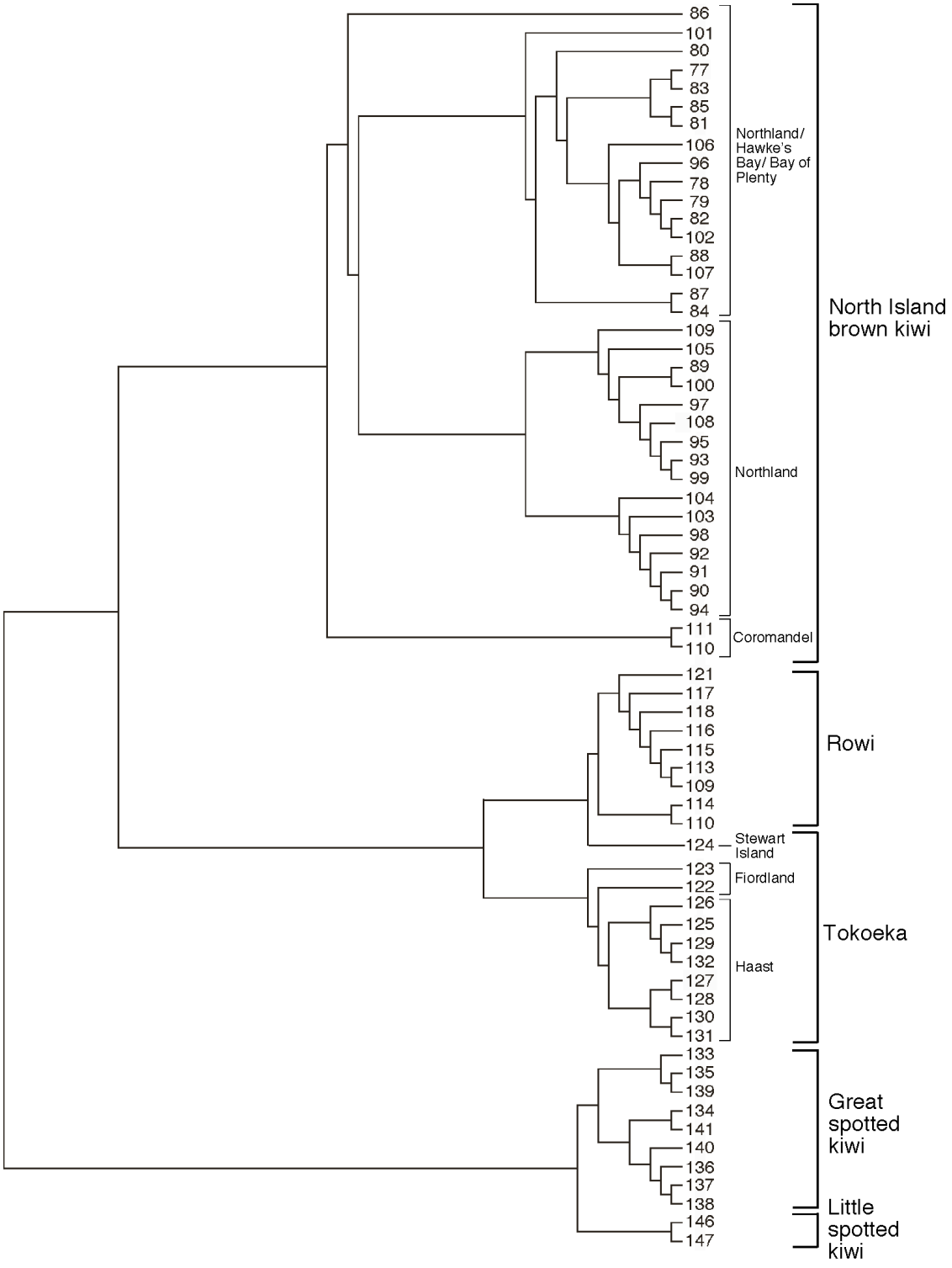


**Figure 2.** UPGMA tree of shared allele distances ($D_{AS}$) between individual kiwi. Species and regional populations of North Island brown kiwi and tokoeka are indicated.

exhibited private alleles, and individuals were assigned to their species of origin with high accuracy (only one was misassigned) using the doh assignment test calculator. This was despite the relatively low number of microsatellite DNA loci used. In addition, all pairwise exact tests were significant, and $F_{ST}$ and $R_{ST}$ estimates were high. Therefore, these preliminary data supported the high level of divergence amongst kiwi observed in mitochondrial DNA (Baker et al., 1995; Burbidge et al., 2003; Chapter Two).

The results of the Bayesian clustering assignment test performed in STRUCTURE suggested there may also be genetic subdivision within some of the currently recognised species. Both tokoeka and North Island brown kiwi samples were divided into more than one cluster. Further evidence that both tokoeka and North Island brown kiwi contain a number of different populations may be provided by the observed departure from Hardy-Weinberg equilibrium at locus Apt29 for these two species. However, other populations also approached significance for this locus. Therefore, this result may be a consequence of null alleles at Apt 29.

Within tokoeka, the Haast population was particularly well supported as a differentiated group. All individuals from this population grouped together in both the Bayesian assignment test and the dendrogram of shared allele distance ($D_{AS}$). Interestingly, allozyme analysis also recognised the genetic distinctiveness of the kiwi population at Haast (Herbert and Daugherty, 2002), and, in part, this distinctiveness has led to the conservation of this population in one of five intensively managed kiwi sanctuaries. However, mitochondrial DNA sequences from individuals from the Haast population are nested within mitochondrial DNA sequences from other tokoeka populations (Baker et al., 1995; Burbidge et al., 2003; Chapter Two). The population size of tokoeka at Haast was estimated to be approximately 225 birds in 1996. The small size and geographically isolated nature of this population may have led to microsatellite DNA differentiation and loss of genetic diversity through random genetic drift. A greater number of microsatellite DNA loci and further kiwi samples from Haast may allow testing for the genetic effects of a bottleneck (e.g., Cornuet and Luikart, 1996; Luikart et al., 1998). Furthermore, an investigation of the relationship between Haast tokoeka and other populations of tokoeka from Fiordland and Stewart Island is required using a greater number of samples from these populations.

The results presented here provide a different perspective from the mitochondrial data published for brown kiwi (Baker et al., 1995; Burbidge et al., 2003), particularly with regard to the relationships between the three species. Population genetic structure analyses ($F_{ST}$, $R_{ST}$, and $D_{AS}$) indicated that rowi and tokoeka are more closely related to each other than either is to North Island brown kiwi, which conflicts the mitochondrial DNA results. However, caution should be taken in interpreting these results, as each species may contain a number of differentiated populations that may influence analyses. Further, only a small number of microsatellite loci were used in this study; Schlotterer (2001) suggested that 20-40 or even up to 100 microsatellite DNA loci may be necessary for accurate genealogical inference. Ancestral polymorphism, which has been used to explain the discordance been morphological and behavioural relationships with those determined from mitochondrial DNA data in kiwi (Baker et al., 1995; Burbidge et al., 2003), may also be a complicating factor. For example, samples from rowi and the Haast population of tokoeka were fixed for the same allele at locus Apt 37. This may be a shared ancestral character state rather than indicate a close relationship between these groups.

The non-significance of the simulated $R_{ST}$ ($\rho R_{ST}$) for the pairwise comparison between rowi and tokoeka indicated that drift has had more of a contribution to differentiation than stepwise mutation. This result is surprising if rowi and tokoeka have been separated for 8.2 million years as suggested by Burbidge et al. (2003). However, Hardy et al. (2003) stated that, when sample size and number of loci is low, the accuracy of allele size permutation tests is uncertain. Although Hardy et al. (2003) did not give an indication of what constitutes an adequate number of alleles, it is unlikely that five loci are sufficient, especially since one locus had low variation (Apt37). Similarly, more kiwi samples are needed, particularly from Fiordland and Stewart Island tokoeka. Overall these results suggest a complex relationship between these groups, and more research is needed to clarify their exact positions.

In summary, nuclear microsatellite DNA markers indicated a high level of genetic differentiation amongst the five currently recognised species of kiwi. However, the relationships between the species are still uncertain and a greater understanding may be

gained from further sampling of individuals and a larger number of microsatellite markers.

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# CHAPTER SEVEN

## The relationships and origins of the New Zealand wattlebirds (Passeriformes, Callaeatidae) from DNA sequence analyses.[1]

**Abstract**

The monophyly of the endemic New Zealand wattlebirds (Callaeatidae) was examined through the sequencing of nuclear RAG-1 and *c-mos* genes and comparison to other passerine sequences. The New Zealand wattlebirds were strongly supported to be monophyletic and were nested within Corvida. An estimate for the time of divergence of the New Zealand wattlebirds indicated that the ancestors of this family arrived via transoceanic dispersal after the separation of New Zealand from Gondwana. Long branches separated the three New Zealand wattlebird genera from one another and relationships among them were unresolved, even in analyses including a further 1.5 kb of mitochondrial DNA sequences. However, most of the analyses supported either a basally-diverging huia or kokako.

**Introduction**

The Callaeatidae, or New Zealand wattlebirds, is one of the few avian families endemic to New Zealand. The New Zealand wattlebirds have limited powers of flight, and lack obvious close relatives, either in New Zealand or overseas. This has led to the suggestion that they have had a long history of isolation on the New Zealand landmass (Fuller, 2000). In fact, the New Zealand wattlebirds have been suggested to be one of the few extant avian families that were present when New Zealand split from Gondwana (Stevens, 1980; Newton, 2003), some 82-85 mya (Cooper and Millener, 1993).

Following the taxonomy of Dickinson (2003), we recognise the following three monotypic genera in the New Zealand wattlebirds: saddleback (*Philesturnus carunculatus*), kokako (*Callaeas cinereus*) and huia (*Heteralocha acutirostris*). Two

[1] Shepherd, LD and Lambert, DM. Submitted to *Molecular Phylogenetics and Evolution*.

allopatric subspecies are recognised in both saddleback and kokako, although recently it has been suggested that each should be raised to full species (Holdaway et al., 2001). The huia is extinct, with the last confirmed sighting in 1907 (Heather and Robertson, 2000). Since human arrival, the saddleback and kokako have declined to the extent that the saddleback is considered 'near threatened' and the kokako 'endangered' (IUCN, 2003). All three species have, or had in the case of the huia, colourful fleshy wattles at the gape, long, drooping tails and limited powers of flight (Heather and Robertson, 2000). A feature that has drawn attention to this family is the diverse range of bill morphologies (e.g., Ricklefs, 2004). In particular, huia have attracted much interest in theoretical ecology because they exhibited extreme reverse sexual dimorphism in bill morphology. The huia has been described numerous times as unique because of the difference in bill length between the sexes (e.g., Phillips, 1963; Chambers, 1989; Gill and Martinson, 1991). Female huia had a slender, curved bill averaging 96 mm in length, whereas male huia had a stouter, straighter bill usually less than 60 mm in length (Burton, 1974). This difference led to the male and female initially being described as separate species (Gould, 1837). However, several recent papers have pointed out that whilst huia are distinctive in the magnitude of their bill sexual dimorphism, they are not unique in displaying this character state (Jamieson and Spencer 1996; Frith, 1997). It has been suggested that the difference between the sexes was an adaptation to reduce competition between the sexes for food (the 'niche separation hypothesis'; Selander, 1966; Moorhouse, 1996), rather than sexual selection, because the difference was only in the feeding apparatus.

The morphological distinctiveness of the New Zealand wattlebirds has made it difficult to assess their relationships to other passerine birds using traditional morphological taxonomy. Many authors have described the position of the New Zealand wattlebirds as "obscure" or "unresolved" (e.g., Williams, 1976; Heather and Robertson, 2000). Recently two studies have used DNA sequences to examine the phylogenetic position of the New Zealand wattlebirds. Tebbutt and Simons (2002) sequenced small portions of the mitochondrial 12S and nuclear *c-mos* genes in huia. Their analyses confirmed the placement of the huia within the passerines but, because of the short length of DNA sequence obtained and the lack of other passerine sequences available for comparison, could not elucidate its exact position. Barker et al. (2004) included saddleback as part of a much larger project analysing the phylogenetic relationships of the majority of

passerine families. Their analyses of nuclear RAG-1 and RAG-2 DNA sequences indicated that saddleback, together with the cnemophiline birds of paradise (*Cnemophilus* and *Loboparadisaea*) and the berrypeckers (Melanocharitidae), both of which are endemic to New Guinea, are nested within the Corvida and form a sister group to their 'core Corvoidea'. Barker et al. (2004) also estimated divergence times for the major passerine groups but did not present a time of divergence for saddleback. Furthermore, their dates were based on a single calibration that assumed that divergence of the New Zealand wrens (Acanthisittidae) coincided with the rifting of New Zealand from Gondwana.

To date, no study has used DNA analyses to examine the relationships among the New Zealand wattlebirds. An accurate phylogeny of the relationships of the New Zealand wattlebirds is important for examining whether the sexual dimorphism in bill shape exhibited by huia is an ancestral or derived character state. Although it is now widely accepted that the New Zealand wattlebirds are monophyletic, past taxonomies have classified kokako within the Corvidae, whereas huia and saddleback have been placed within the Sturnidae (Buller, 1888). Furthermore, similarities in head and neck morphology have led to the suggestion that saddleback and huia are more closely related to each other than either is to kokako (Burton, 1974). Williams (1976) also assumed kokako to be most similar to the ancestral New Zealand wattlebird because it is least specialised in its nest construction and feeding strategy; it feeds primarily on fruit and leaves, whereas saddleback are, and huia were, insectivorous.

In this study, the monophyly of the New Zealand wattlebirds was examined by sequencing portions of the nuclear genes *c-mos* and RAG-1. These genes were selected because DNA sequences were available for comparison from a large number of passerine families (Barker et al., 2002). However, these nuclear data did not resolve relationships among kokako, saddleback and huia. Therefore, more rapidly evolving mitochondrial DNA sequences were obtained for the 12S, cytochrome b and ND2 genes. A number of alternative hypotheses regarding the date of divergence of the New Zealand wattlebirds from their closest known relatives were also examined using previously published DNA sequences (Barker et al., 2004).

**Methods**

*Taxon sampling and DNA extraction*

DNA was extracted from whole blood of North Island saddleback (*P. c. rufusater*), hereafter referred to as saddleback, North Island kokako (*C. c. wilsoni*), hereafter referred to as kokako, and an outgroup taxon, New Zealand robin (Petroicidae: *Petroica australis australis*), by proteinase digestion and phenol-chloroform extraction (Sambrook et al., 1989). Four $mm^2$ of footpad tissue was sampled from two huia skins held at Canterbury Museum (museum numbers AV2283 and AV1126) using a sterile razor blade. The huia DNA extractions were set up in a separate and dedicated ancient DNA laboratory to reduce the risk of contamination from modern DNA. This laboratory undergoes regular decontamination with UV-irradiation and hypochloride treatment. Negative extraction and PCR controls were used throughout the extraction and amplification process. Huia footpads were cut into several pieces using sterile razor blades and incubated overnight at 50°C in 2.5 ml extraction buffer (10 mM Tris-HCl pH 8.0, 1 mM NaCl), 250 µl of 10% SDS, 15 µl of 200 mg/ml Dithiothreitol (DTT) and 25 µl of 50 mg/ml Proteinase-K. DNA was extracted with Tris-saturated phenol followed by chloroform:isoamyl (24:1). The resulting solution was then concentrated to 200 µl on a Vivaspin-30 (Viva Science, U.K.) membrane.

*Primer design*

Initial primers (hm1F and hm6R; Table 1) to amplify the proto-oncogene *c-mos* exon were designed to conserved regions in alignments of *c-mos* avian sequences available on GenBank. These primers were used to amplify and sequence *c-mos* in saddleback and kokako. These sequences were then used to design internal primers (Table 1) enabling amplification of the degraded huia ancient DNA. A novel primer pair (hr1F and hr6R; Table 1) was designed to conserved passerine RAG-1 sequences available on GenBank and used to amplify and sequence a 838 base pair (bp) fragment of RAG-1 in kokako and saddleback. The sequences obtained were then used to design novel internal primers (Table 1) enabling amplification of short overlapping DNA fragments from huia.

A 985 bp fragment of mitochondrial cytochrome b (cyt b) was amplified and sequenced in the kokako, saddleback and New Zealand robin using the primers L14841 (Kocher et

**Table 1.** Novel primers designed to amplify DNA sequence in the New Zealand wattlebirds. Amplicon length refers to sequence from the New Zealand wattlebirds and includes primer sequences.

| DNA locus | Primer name | Primer sequence (5' to 3') | Amplicon length (bp) |
|---|---|---|---|
| *c-mos* | hm1 | F- GCCTGGTGCTCCATCGACTGGGA | 269 |
| | | R- TCCATGATGATGGTGCCCAGGCTGTT | |
| | hm2 | F- TGGCCCAGCTCCAGCACGATAATGT | 291 |
| | | R- CTCCCTGCTCAGTGATGAAAAC | |
| | hm3 | F- TCTTGTGACATCATGACAGGCTTA | 231 |
| | | R- GGGTGATGGCAAAGGAGTAGATGTC | |
| RAG-1 | hr1 | F- ATCCTTCCAGAACATCCTTGATAA | 217 |
| | | R- TCAGTTCCCTCAGACGATGTT | |
| | hr2 | F- AGATAGAGAGCTCTATAGCTAC | 228 |
| | | R- AGCTTCCAGTTCATCTGCTT | |
| | hr3 | F- ACAAGCAGATGAACTGGA | 229 |
| | | R- GAGGTTTCCACTCAAATGGGT | |
| | hr4 | F- CAGTAAAAGCTGTCTCTGGGAG | 246 |
| | | R- TTTAAGGCACAAACCAAGGC | |
| | hr5 | F- TTATAGATGGACTATCAGGAC | 223 |
| | | R- CTCACTGACATCTCCCATTCC | |
| | hr6 | F- CTGGATGACTATTTGAATGGC | 233 |
| | | R- CAGCATAAGGCACAAGGGCTT | |
| Cyt b | watcytb1 | F- CCATACATTACACAGCAGACACATCCC | 387 |
| | | R- GGGCGAAGAATCGGGTTAATGTGGGGT | |
| | watcytb2 | F- AGCCTTCGTAGGCTACGTACTGCCA | 345 |
| | | R- GACAAGTGGGATGAGTATTAGTGCGA | |
| | watcytb3 | F- CAACCCCCTAGGAATTCCGTCAGA | 344 |
| | | R- GATAGAGGTCGGAAGGTTATTGAGC | |
| ND2 | ND2A | F- GGAAGTGTGATTGTTGCGCAGT | 262 |
| | | R- CCCTAAACTCCATAAAAGTCCTCAA | |
| | ND2B | F- AATATGGTTAGTATCAGTATAG | 257 |
| | | R- CTAGGAGGATGAATAGGAC | |

al 1989) and H15767 (Edwards et al., 1991). The resulting sequences were used to design novel primers (Table 1) to amplify short, overlapping DNA fragments from huia to give a total sequence length of 756 bp. The primers L5216 and H6313 (Sorenson et al., 1999) were used to amplify and sequence the mitochondrial NADH dehydrogenase subunit 2 (ND2) in saddleback, kokako and New Zealand robin. Novel internal primers (Table 1) were designed from these sequences to amplify two overlapping fragments giving a total of 384 bp of sequence. The huia 12S rDNA sequence used in these

analyses is from Tebbutt and Simons (2002; GenBank number AF470618). The equivalent section of 12S sequence was also amplified from kokako, saddleback and New Zealand robin using the same primers used by Tebbutt and Simons (2002).

*DNA amplification and sequencing*

All PCR amplifications were performed in 20 µl reactions using 1× PCR buffer (500 mM Tris pH 8.8, 200 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 200 µl of each dNTP, 0.5 U Taq DNA polymerase (Roche) and 1 µM of each primer. Amplifications were performed in a Hybaid OmniGene thermocycler with the following thermocycling profile: 94°C for 2 minutes; ten cycles of 94°C for 20 seconds, 55°C for 20 seconds and 72°C for 1 minute; followed by 32 cycles of 94°C for 20 seconds, 50°C for 20 seconds to 1 minute and 72°C for 1 minute; and a final extension of 5 minutes. DNA sequencing was performed on an ABI-3730 (Applied Biosystems) using the BigDye Terminator Cycle Sequencing Kit version 3.1. PCR amplifications of huia DNA were set up in an ancient DNA laboratory. The cytochrome b gene fragment was sequenced for both huia samples (AV2283 and AV1126), the nuclear gene sequences were obtained from sample AV2283 and the ND2 sequence from sample AV1126. All PCR products were sequenced in both directions from independent PCR amplifications.

*Ancient DNA verification*

DNA was independently extracted and PCR amplified from huia specimen AV2283 at the University of Auckland ancient DNA laboratory by L. Huynen. A 690 bp region of RAG-1 sequence was replicated by amplification and sequencing using the primer pairs: hr2, hr3, hr4 and hr5. In order to verify the authenticity of the huia 12S sequence of Tebbutt and Simons (2002), which was not extracted in an ancient DNA laboratory, a 321 bp fragment of 12S was obtained for huia by T. King at the ancient DNA laboratory at Massey University, Palmerston North. In addition, we compared the huia *c-mos* sequence obtained in this study with the 182 bp fragment of huia *c-mos* (GenBank accession number AF470617) sequenced by Tebbutt and Simons (2002).

*Sequence alignment and outgroup selection*

Wattlebird *c-mos* and RAG-1 sequences were aligned manually to the matrices provided by Barker et al. (2002; accessed from the European Molecular Biology Laboratory, *c-mos*: accession ALIGN_000207; RAG-1: accession ALIGN_000206). In

order to reduce the computational time, their data sets were reduced to forty-one representative taxa. These taxa were selected both to cover the range of diversity within the passerines and to include the closest relatives of the New Zealand wattlebirds, as determined in a previous study (Barker et al., 2004). In the alignment, 36 bp of the *c-mos* sequence (nucleotide positions 320-355) was excluded from the analyses owing to uncertainty regarding primary homology (following Barker et al., 2002). This resulted in an alignment comprising 1424 bp (586 bp *c-mos*; 838 bp RAG-1) of sequence for the forty-four taxa.

The mitochondrial sequence data were used to examine the relationships among the New Zealand wattlebirds. Four outgroups, including the New Zealand robin, were selected resulting in a sample size of seven taxa. Outgroup taxa were chosen because they were reasonably closely related to the New Zealand wattlebirds in the nuclear data phylogenetic analysis and because of the availability of the sequences required. The outgroup families were: Maluridae (*Malurus splendens*: 12S – AY488257; ND2 – AY488327; cytochrome b – AY488403), Meliphagidae (*Xanthotis flaviventer*: 12S – AY488245; ND2 – AY488315; cytochrome b – AY488391), Corvidae (*Corvus frugilegus*: complete mitochondrial genome NC002069) and Petroicidae (New Zealand robin; *Petroica australis australis)*.

Cytochrome b and ND2 sequences contained no indels and were aligned manually. The 12S sequences were aligned manually using the secondary structure and conserved motifs approach of Mindell et al. (1997). Ambiguous 12S nucleotide positions in loop regions totalling 30 bp of sequence were omitted from the phylogenetic analysis following Mindell et al. (1997).

*Sequence characterisation*

Mitochondrial sequences were tested for saturation by graphing, for each gene, the number of observed substitutions between pairs of taxa against their observed pairwise distances. This approach has the advantage of allowing transitions and transversions to be considered independently. Separate graphs were produced for each codon position of ND2 and cytochrome b. Data were considered saturated if the graphs asymptoted or if the number of substitutions between ingroup taxa was similar to the number of substitutions between ingroup and outgroup taxa (Griffiths, 1997). Homogeneity of

base frequencies in individual and combined data sets was tested using PAUP* version 4.0b10 (Swofford, 2002).

Gene partitions within the combined nuclear and combined mitochondrial data sets were tested for congruence using a partition homogeneity test (Farris et al., 1994) as implemented in PAUP* version 4.0b10 (Swofford, 2002). Tests were conducted with 10 random addition sequences and 100 replicates. The accuracy of this test has been questioned (Cunningham, 1997; Barker and Lutzoni, 2002; Darlu and Lecointre, 2002); therefore, topologies of separate gene sequences were also investigated for incongruence.

*Phylogenetic analysis*

Tree building searches were performed separately for each gene and for the combined nuclear DNA sequences and combined mitochondrial DNA sequences. Sequence data was analysed in PAUP* version 4.0b10 (Swofford, 2002) using maximum parsimony (MP) and maximum likelihood (ML) criteria. MP analysis was performed using the heuristic search option with tree bisection-reconnection (TBR) branch swapping with 100 random sequence additions of taxa. Heuristic ML analysis used 10 random sequence additions of taxa with nearest-neighbour interchange (NNI) branch swapping for the nuclear sequence data and TBR branch swapping for the mitochondrial sequence data. Branch swapping was limited to 15 minutes for each random addition sequence with the separate *c-mos* dataset in order to reduce search times. Analyses of combined datasets were performed both with all sites weighted equally, and with saturated sites (as determined from saturation plots) removed (ML and MP analyses only) or recoded as purines and pyrimidines and appended onto the end of the dataset. The most appropriate models of evolution for ML analyses were selected using the Akaike Information Criteria (AIC) as implemented in Modeltest v3.06 (Posada and Crandell, 1998). Support for the trees was assessed using 100 bootstrap (BS) replicates.

Phylogeny was also estimated using the Bayesian approach implemented in MrBayes v3.04b (Huelsenback and Ronquist, 2001). Modeltest v3.06 was used to analyse the individual gene sequences separately and determine the most appropriate model for each of them, which were then incorporated into a partitioned Bayesian analysis. Four Markov chains of 2 000 000 generations were run with re-sampling of trees and ML

parameters every 1000 generations. The initial 1000 trees were discarded as 'burn-in'. Log likelihood values were plotted against generation time to confirm that convergence had been obtained. The remaining trees were used to construct a 50% majority rule consensus tree. Two independent runs were performed for each dataset and the posterior probabilities averaged across runs.

*Molecular dating of the New Zealand wattlebirds*

Estimates of divergence times for the New Zealand wattlebirds were determined using previously published nuclear RAG-1 and RAG-2 sequence data for 40 taxa (Barker et al., 2004). These data were selected over the RAG-1 and *c-mos* data set because they produce a more resolved phylogeny of the passerines, and because sequences were available from the closest known relatives of the New Zealand wattlebirds, the cnemophiline birds of paradise (Barker et al., 2004). *Coracias caudata* was chosen as the outgroup and the remaining taxa were selected to encompass the range of diversity within the passerines.

The divergence times of taxa were estimated using penalised likelihood in r8s version 1.70 (Sanderson, 2002) with the TN algorithm and log penalty. The branch lengths generated by a partitioned Bayesian analysis of these data were used for the r8s analysis**.** An optimal smoothing parameter was estimated for each calibration using cross-validation. To account for errors in branch length estimation standard deviations were calculated using 100 trees generated with PAUP* by bootstrapping on the Bayesian consensus tree.

A calibration date of 82 million years (for the separation of New Zealand from Gondwana) was used to estimate divergence times under three scenarios. Firstly, this date was assigned to the divergence of the most basally-diverging passerine lineage, the New Zealand wrens, represented in the analysis by *Acanthisitta*, as used by Ericson et al. (2002) and Barker et al. (2004). Secondly, in order to explore the suggestion that the New Zealand wattlebirds have a vicariant origin, this date was applied to the node where the saddleback diverges from the cnemphiline birds of paradise. However, this node, and the preceding node placing saddleback and the cnemophiline birds of paradise as sister taxa to the berrypeckers, was weakly supported in previous phylogenies (Barker et al., 2004). Therefore, a third analysis was performed in which a

date of 82 mya was applied to the node where saddleback would diverge if the weakly supported nodes above were collapsed to a polytomy. The log penalty function was applied when calculating r8s estimates for the latter two scenarios because it is thought to produce more accurate results when using recent calibrations to extrapolate backwards in time (Sanderson, 2004).

*Estimating relationships among the New Zealand wattlebirds with mitochondrial DNA*

Signal within the mitochondrial DNA sequence data was visualised through spectral analysis (Hendy and Penny, 1993), as implemented in Spectronet version 1.2 (Huber et al., 2002), and split decomposition (Bandelt and Dress, 1992), as implemented in Splitstree 4.0b21 (Huson and Bryant, in prep.). Both methods allow representation of the support and conflict for a particular split (a split is any bipartition in the data). Spectral analysis was performed independently for the separate and combined mitochondrial DNA sequences. Splits were obtained both directly and via the Hadamard conjugation (Hendy et al., 1994), which corrects for multiple substitutions, and visualised as Lento plots. Split decomposition was used to construct a splits graph of the combined mitochondrial DNA sequence data. Split decomposition was implemented under a distance criterion with distances calculated under the optimal model of evolution selected for this data by Modeltest v3.06 (Posada and Crandell, 1998). Support for splits was assessed by performing 100 bootstrap replicates.

The placement of outgroups is important in determining the ancestral beak form of the New Zealand wattlebirds. The robustness of the outgroup position was examined using the test of Shimodaira-Hasegawa (SH test; Shimodaira and Hasegawa, 1999) and the less conservative Kishino and Hasegawa test (KH test, Kishino and Hasegawa, 1989) as implemented in PAUP*. The four possible taxon topologies (Figure 1), including a polytomy, were compared and all outgroups were trialled separately. The tests were performed with 1000 RELL bootstrap replicates for the independent and combined mitochondrial DNA sequence data using the maximum likelihood models identified as most appropriate with Modeltest. The Bonferroni correction (Rice, 1989) was used to correct for the multiple comparisons of the KH test (the SH test automatically corrects for the comparison of multiple topologies).

A likelihood ratio test (LRT) was performed on the mitochondrial DNA sequence data in PAUP* to test the null hypothesis that the internal New Zealand wattlebird branch was zero-length (i.e. such a topology corresponds to Tree4 in Figure 1). This test compares the likelihood of the optimal tree to the likelihood with the internal New Zealand wattlebird branch collapsed. The significance at the 5% level was determined under a mixed $\chi^2$ distribution (Table 2 of Goldman and Whelan, 2000) as recommended by Slowinski (2001). This test was performed separately for each mitochondrial gene and for the combined mitochondrial DNA sequence data, with and without the saturated sites removed.

**Results**

*Ancient DNA verification*

The DNA sequence of the 690 bp of the huia RAG-1 gene that was independently extracted and amplified at the University of Auckland ancient DNA facility, and the 182 bp of huia *c-mos* sequence of Tebbutt and Simon (2002), were identical to those

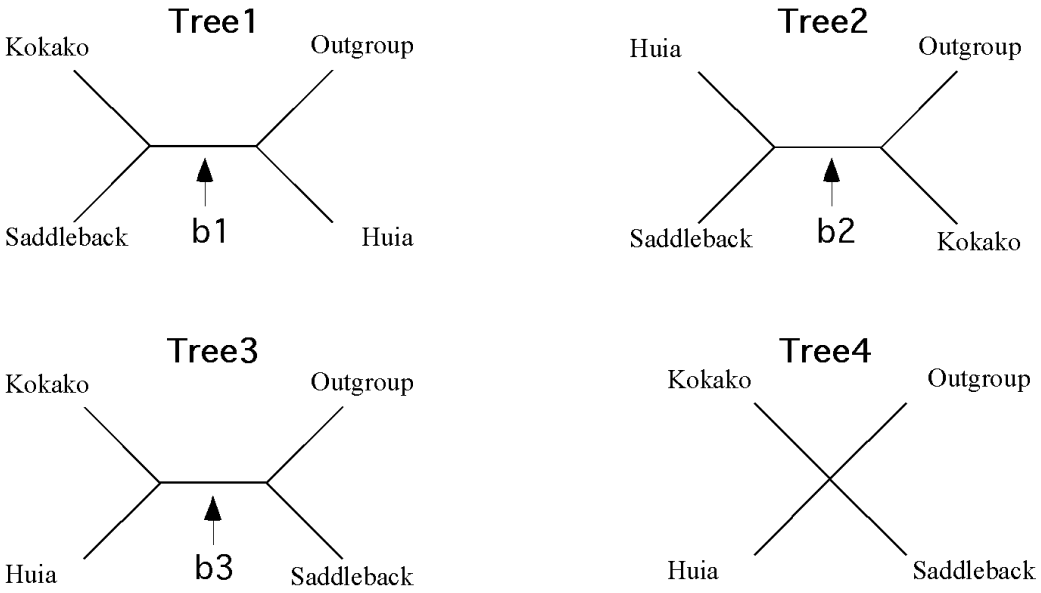


**Figure 1.** The four possible trees representing relationships between the three New Zealand wattlebird taxa and an outgroup. Tree4 is a polytomy, (i.e. it has no internal branch); the other three trees differ in the position of the internal branch, with branches labelled b1 to b3

produced in the present study. Furthermore, the 321 bp of huia 12S sequence generated independently by T. King was identical to that produced by Tebbutt and Simons (2002).

*Sequence characteristics*

Sequence lengths and levels of variation for the individual and combined *c-mos* and RAG1 regions are reported in Table 2. The sequence characteristics for the mitochondrial genes are shown in Table 3. The mitochondrial sequences appeared to correspond to functional mitochondrial genes rather than nuclear pseudogenes. Cytochrome b and ND2 sequences exhibited no stop codons or frameshift mutations and the 12S rDNA sequences contained conserved secondary structure motifs. Base frequencies of ND2 and cytochrome b (Table 3) were similar to those reported for mitochondrial sequences in other avian taxa, in particular the deficiency of guanine. Homogeneity of nucleotide base frequencies was not rejected by a $\chi^2$ test for the nuclear or mitochondrial gene sequences indicating that base composition bias should not mislead tree-building (Sanderson and Shaffer, 2002).

Within the New Zealand wattlebirds, uncorrected pairwise divergences were low for the nuclear (*c-mos*: 0.2 to 0.7%; RAG1: 0.6 to 1.0%) and 12S sequences (2.2 to 2.5%).

**Table 2.** Sequence characteristics for the nuclear data set comprising the three New Zealand wattlebirds and 41 other passerine taxa.

| | *c-mos* | RAG-1 | combined |
|---|---|---|---|
| Aligned sequence length (bp) | 586 | 838 | 1424 |
| Characters: variable/PI[a] | 235/143 | 296/167 | 531/310 |
| %A | 0.2345 | 0.3045 | 0.2758 |
| %C | 0.2575 | 0.2078 | 0.2282 |
| %G | 0.2990 | 0.2547 | 0.2728 |
| %T | 0.2090 | 0.2330 | 0.2232 |
| Number MP trees | 19 | 27403 | 551 |
| Model selected[b] | GTR+I+G | TrN+I+G | GTR+I+G |
| $\chi^2$ test of p-value[c] | 1.000 | 1.000 | 1.000 |

[a]PI = parsimony informative

[b]Model selected as most representative of sequence evolution by ModelTest

[c]test of homogeneity of base frequencies

**Table 3**. Sequence characteristics of the mitochondrial genes included in the analysis of the three New Zealand wattlebirds and four outgroup taxa.

| | 12S | ND2 | Cytochrome b | Combined mtDNA |
|---|---|---|---|---|
| Aligned sequence length (bp) | 363 | 384 | 756 | 1503 |
| Characters: variable/PI[a] | 69/32 | 196/120 | 226/128 | 482/272 |
| %A | 0.285 | 0.293 | 0.272 | 0.279 |
| %C | 0.260 | 0.328 | 0.340 | 0.319 |
| %G | 0.242 | 0.105 | 0.141 | 0.156 |
| %T | 0.213 | 0.273 | 0.247 | 0.244 |
| Number MP trees | 1 | 2 | 1 | 1 |
| Model selected[b] | K80 +G | GTR+G | TVM+G | TVM+I+G |
| $\chi^2$ test p-value[c] | 1.000 | 0.999 | 0.874 | 0.976 |

[a]PI = parsimony informative

[b]by ModelTest

[c]test of homogeneity of base frequencies

Divergences were much higher for ND2 (14.3% to 19.3%) and cytochrome b (8.9 to 10.6%) sequences. Saturation plots (Figure 2) indicated that transitions at all codon positions in cytochrome b, transitions at second codon positions in ND2, and both transitions and transversions at third codon positions of ND2, were saturated and therefore weighting schemes were applied only to these sites.

*Nuclear DNA sequence analyses*

The partition homogeneity test indicated that the *c-mos* and RAG-1 sequence data could be combined (P = 0.24). Furthermore, analysis of the independent nuclear sequences yielded trees with no conflict in the placement of the New Zealand wattlebirds, although *c-mos* trees showed little resolution. MP analysis of the combined *c-mos* and RAG-1 DNA sequences recovered 551 equally parsimonious trees (score of best trees = 1391 steps). In all MP trees huia, saddleback and kokako formed a monophyletic group. ML analysis of this data set selected a single most likely tree (–ln likelihood score of 9429.11475). Support for New Zealand wattlebird monophyly was high with all tree-building methods (1.00 Bayesian posterior probability (PP), 100 % maximum likelihood bootstrap (BS ML), 100% maximum parsimony bootstrap (BS MP))

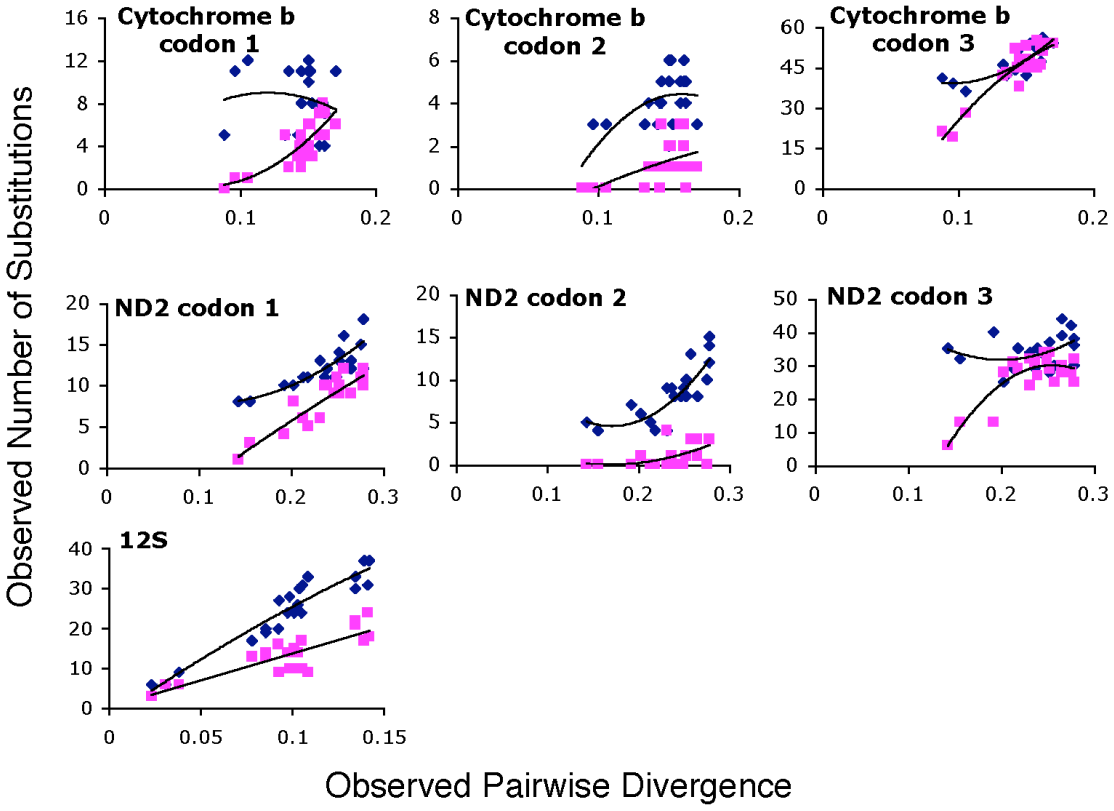


**Figure 2.** Saturation plots of transitions and transversions for mitochondrial genes. Codon positions of cytochrome b and ND2 are presented separately. Transitions are represented by blue diamond and transversions by pink squares. 2nd order polynomial regression lines have been plotted to illustrate trends.

although the relationships among these three species were unresolved (Figure 3). With these data there was strong support for the New Zealand wattlebirds nesting within the oscines (1.00 PP, 94 % BS ML, 99% BS MP). New Zealand wattlebirds were excluded from the Passerida, which was recovered with 0.98 PP in the Bayesian analysis. They were also excluded from Barker et al.'s (2004) 'core Corvoidea', although this was only recovered with weak support (0.71 PP).

*Molecular dating of the New Zealand wattlebirds*

For each calibration, r8s determined an optimal smoothing factor of 320 by cross-validation. Using a calibration of 82 mya for the divergence of the New Zealand wrens,

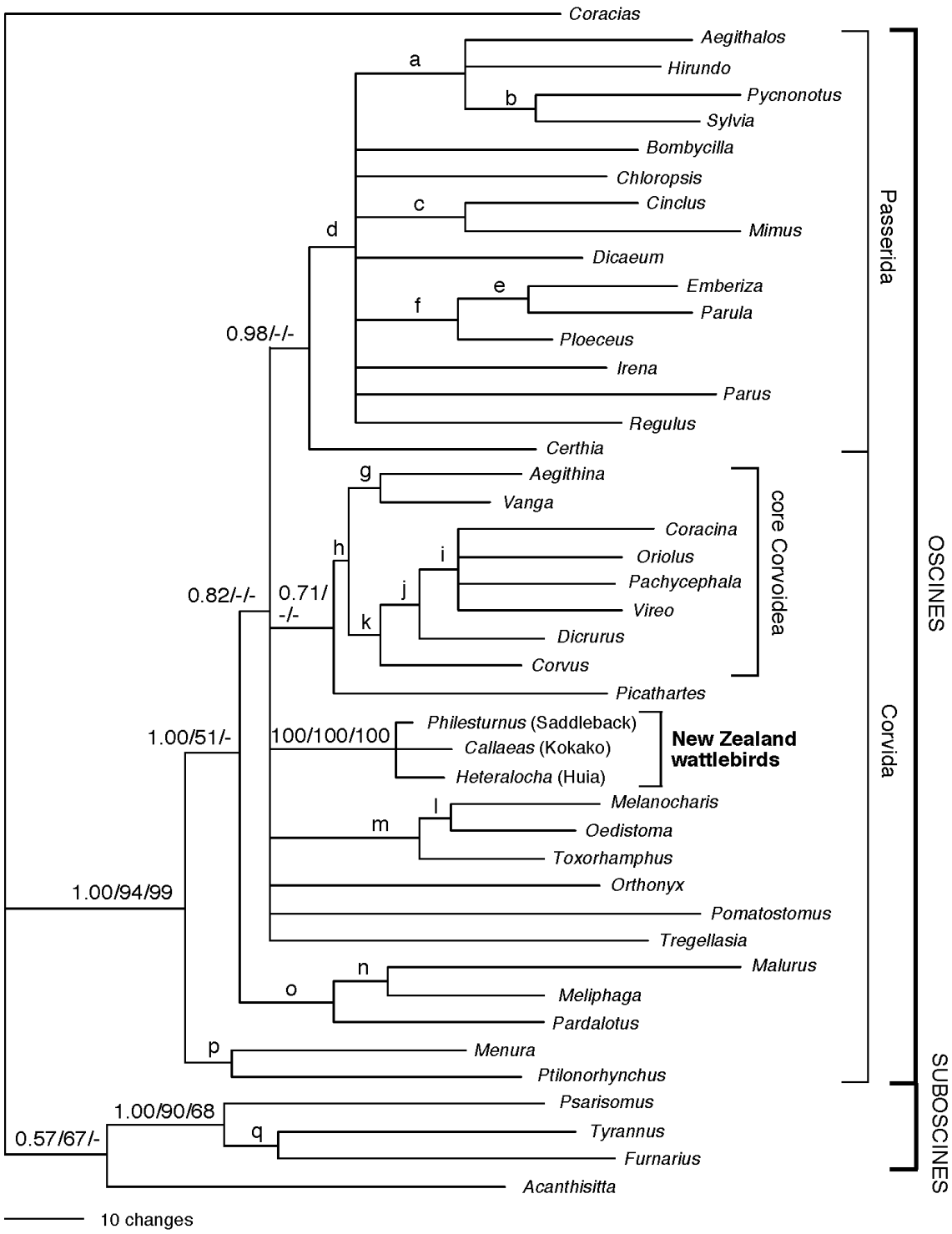


**Figure 3.** Bayesian phylogeny of 1424 bp of c-mos and RAG-1 DNA sequence data for the passerines. Support values for major nodes are given on the phylogeny in the following order: Bayesian posterior probabilities/ML bootstrap/MP bootstrap. Support for remaining nodes is given in Appendix 1.

r8s calculated a point estimate of 33.7 mya for the divergence of the New Zealand wattlebirds, represented by saddleback, from the cnemophiline birds of paradise (node C; Table 4 and Figure 4). Using this same calibration, r8s calculated a divergence date of 39.2 mya for the node, node B, preceding the poorly supported nodes grouping the New Zealand wattlebirds with the cnemophiline birds of paradise and berrypeckers (Table 4 and Figure 4). When the date of 82 mya was applied to node C, where the New Zealand wattlebirds diverged from the cnemophiline birds of paradise, thus assuming a vicariant origin for the family, r8s calculated a divergence for the New Zealand wrens of 226.9 mya (minimum-maximum age estimates 173.9-263.7 mya) (node A; Table 4 and Figure 4).

*Mitochondrial DNA sequence analyses*

The partition homogeneity test indicated that the mitochondrial sequence data from the three regions could be combined (P = 0.70). Phylogenies constructed from the different mitochondrial DNA sequences indicated that the outgroups attached to different branches depending on the sequence and method of analysis, although support values

**Table 4.** Divergence dates estimated in r8s by applying a calibration date of 82 mya to various nodes in the passerine phylogeny. The positions of nodes A, B and C are labelled in Figure 4. Point estimate refers to the divergence date calculated from the Bayesian consensus phylogeny. The mean, standard deviation, minimum and maximum date estimates were determined from the branch lengths of 100 trees bootstrapped on the Bayesian consensus phylogeny.

| Node where calibration was applied | Node for which divergence date was estimated | Point estimate (mya) | Mean ± standard deviation (mya) | Minimum – maximum age estimates (mya) |
|---|---|---|---|---|
| A | C | 33.7 | 35.2 ± 2.5 | 29.3 – 41.1 |
| A | B | 39.2 | 39.4 ± 2.3 | 33.8 – 47.2 |
| B | A | 198.5 | 193.9 ± 15.1 | 155.0 – 227.9 |
| C | A | 226.9 | 214.5 ± 19.8 | 173.9 – 263.7 |

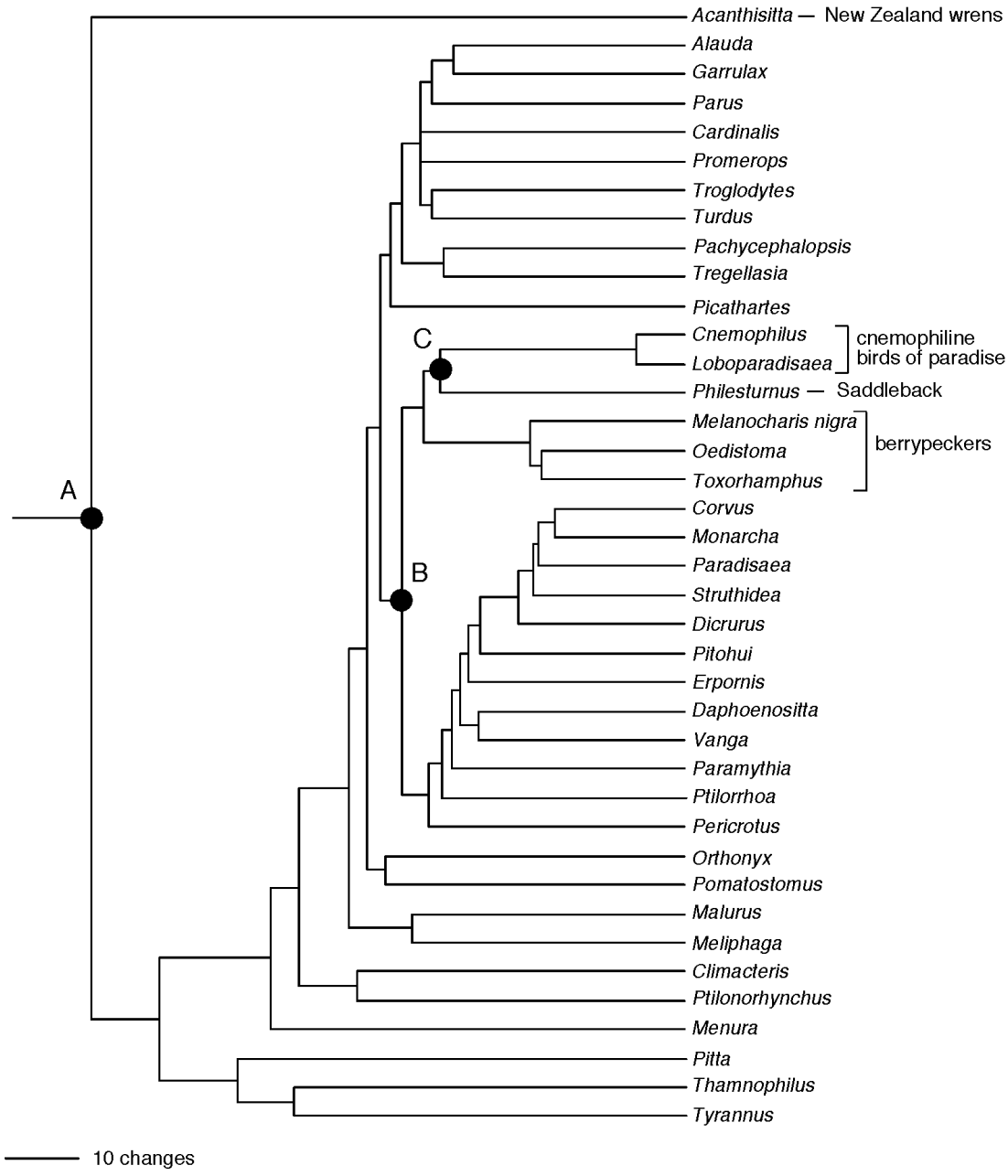


**Figure 4.** Chronogram from r8s of nuclear RAG-1 and RAG-2 sequences for forty representative passerine taxa. Labelled nodes correspond to those in Table 4.

were low in most cases (Table 5). RY coding, or removal, of saturated sites did not improve phylogenetic resolution. However, phylogenetic analyses of the separate and combined mitochondrial DNA sequences indicated that, like the nuclear data alone, there was strong support for the New Zealand wattlebirds constituting a monophyletic group (BS 94-100% for ML and MP analyses; PP 1.00).

**Table 5.** Results of phylogeny reconstruction from independent and combined mitochondrial DNA sequence data for the three New Zealand wattlebirds and four outgroup taxa. Topology refers to the four possible four-taxon trees illustrated in Figure 1. * denotes a dataset with saturated sites removed (MP and ML trees only). # denotes a dataset with RY coding of saturated sites.

| Topology | Basally-diverging taxon | Sequence | Tree-building method | Support value for internal node |
|---|---|---|---|---|
| Tree 1 | huia | ND2 | MP | 62% BS |
| | | Cytochrome b | MP | 76% BS |
| | | Cytochrome b | ML | 68% BS |
| | | Combined mtDNA | MP | 71% BS |
| | | Combined mtDNA | ML | <50% BS |
| | | Combined mtDNA | Bayesian | 0.76 PP |
| | | Combined mtDNA * | MP | 70% BS |
| | | Combined mtDNA * | ML | 52% BS |
| | | Combined mtDNA # | MP | 67% BS |
| | | Combined mtDNA # | ML | <50% BS |
| | | Combined mtDNA # | Bayesian | 0.74 PP |
| Tree 2 | kokako | 12S | MP | 61% BS |
| | | ND2 | ML | 54% BS |
| | | ND2 | Bayesian | 0.79 PP |
| Tree 3 | saddleback | 12S | ML | 52% BS |
| | | 12S | Bayesian | 0.95 PP |

Spectral analysis was used to examine support and conflict for each possible internal wattlebird branch (i.e. b1 to b3; Figure 1). Analysis of independent mitochondrial sequences (not shown) indicated that much of the phylogenetic signal was confined to terminal branches and that similar levels of support and conflict existed for all the possible internal New Zealand wattlebird branches. The number of characters supporting each possible internal branch is given in Table 6. All three possible internal branches received support from characters in at least two genes. Spectral analyses of

**Table 6.** Unambiguous synapomorphic sites supporting each of the possible New Zealand wattlebird internal branch topologies. Phylogeny refers to the four-taxon trees illustrated in Figure 1.

| Phylogeny | Basally-diverging taxon | Sequence | Number of supporting synapomorphies |
|---|---|---|---|
| Tree 1 | huia | Cytochrome b | 4 |
| | | ND2 | 2 |
| | | 12S | 0 |
| | | **Total** | **6** |
| Tree 2 | kokako | Cytochrome b | 2 |
| | | ND2 | 4 |
| | | 12S | 1 |
| | | **Total** | **7** |
| Tree 3 | saddleback | Cytochrome b | 1 |
| | | ND2 | 1 |
| | | 12S | 2 |
| | | **Total** | **4** |

the combined mitochondrial data recovered two contradictory splits {kokako, outgroup}{saddleback, huia} and {huia, outgroup} {saddleback, kokako} with similar levels of support and high levels of conflict for both splits. There was slightly higher support for the former split with the untransformed data (7 supporting synapomorphies versus 6 for the latter split; Table 6). Transforming the data with the Hadamard conjugation resulted in slightly higher support for a basally-diverging huia (i.e. internal branch b1; Figure 1) but did not significantly decrease the conflict (Figure 5).

The splits graph also indicated conflicting signal in the mitochondrial DNA sequence data (Figure 6). The presence of a box indicated conflict between the split placing kokako basally divergent (59% BS) and that placing huia as the first diverging lineage in the New Zealand wattlebirds (54% BS). There was no support in the splits graph for

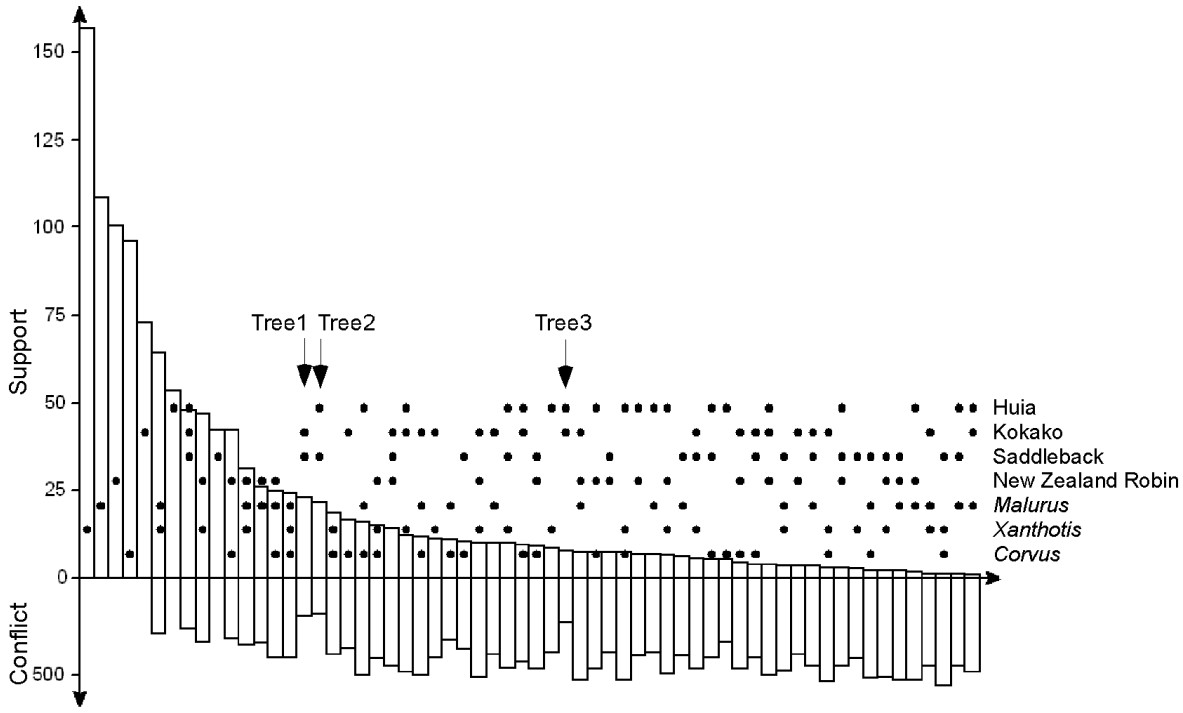


**Figure 5.** Lento plot of support and conflict for splits in the combined mitochondrial sequence data. Splits are identified by dots that indicate which samples are separated by the split. Support for a split is displayed as a bar above the x-axis and conflict against it is indicated below the x-axis. Conflict is normalised so that the sum of support and conflict is equal. Splits are ranked in order of decreasing support. The splits supporting Trees 1-3 in Figure 1 are indicated.

a split placing saddleback as basally-diverging. However, a weakly supported split (25% BS) partitioned huia with *Xanthotis flaviventer*.

None of the possible topologies (Figure 1), including the polytomy, were found to be significantly worse than any other with both the SH and KH tests ($p > 0.05$). Furthermore, the likelihood ratio test for a zero-length internal New Zealand wattlebird branch indicated that phylogenies constructed from the independent cytochrome b and ND2 sequences could be collapsed without significantly changing the likelihood compared to the optimal tree. In contrast, the null hypothesis was rejected for the 12Sdata, where saddleback was basally-diverging, and the combined mitochondrial data

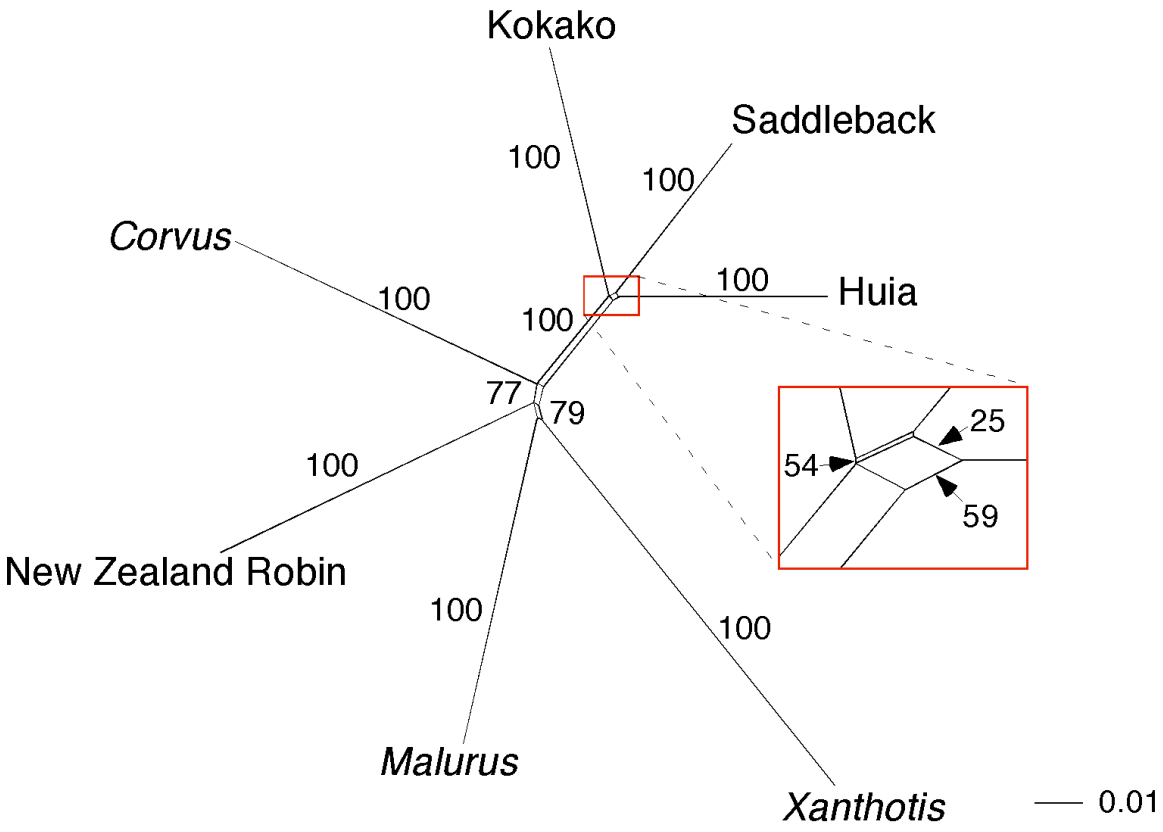


**Figure 6.** Splits graph for the combined mitochondrial sequence data constructed from distances computed under the GTR model of sequence evolution. The internal wattlebird branch is magnified and indicates considerable conflict is present in the data, as evidenced by the box in the graph. The fit of the graph was 84.99, indicating that not all splits were represented. Support for splits was estimated from 100 bootstrap replicates.

and with saturated sites retained, RY-coded or removed, where huia was basally-diverging.

**Discussion**

*The relationship of the New Zealand wattlebirds to other passerines.*

The New Zealand wattlebirds were strongly supported as a monophyletic group with both the nuclear and mitochondrial DNA sequence data. Furthermore, the position of the New Zealand wattlebird taxa within the passerines, as determined in this study (i.e.

nested within the oscines but excluded from the Passerida and core Corvoidea), was consistent with the placement of saddleback in a previous study (Barker et al., 2004).

*Timing the date of divergence of the New Zealand wattlebirds*

Using the calibration of 82 mya for the split of the New Zealand wrens, the divergence of the New Zealand wattlebird lineage is estimated at approximately 34-39 mya. There is considerable uncertainty regarding the accuracy of estimating divergence times from a single calibration (Graur and Martin, 2004; Sanderson et al., 2004). However, applying a date of 82 mya to the node where saddleback diverged resulted in a divergence date for the New Zealand wrens that is before when modern avian orders are thought to have evolved (reviewed in Ericson et al., 2003). Therefore, it does not appear plausible that the New Zealand wattlebirds have a vicariant origin. Instead, their ancestors are likely to have arrived in New Zealand via transoceanic dispersal, possibly via 'stepping-stone' islands on the Lord Howe Rise or Norfolk Ridge (Sanmartin and Ronquist, 2004). Dispersal may have been assisted by the prevailing westerly wind that was established with the opening of the Southern Ocean between Australia and Antarctica during the late Eocene, as has been suggested for the ancestors of the piopio (*Turnagra capensis*), an extinct endemic New Zealand bird (Christidis et al., 1996).

*Relationships amongst the New Zealand wattlebirds*

Analysis of the mitochondrial DNA sequence data did not allow complete discrimination between alternative hypotheses regarding the most basally-diverging New Zealand wattlebird. However, the data presented here provides most support for either a basally-diverging kokako or huia. Given the relatively large amount of conflict in the data for the internal New Zealand wattlebird branch, as evidenced by the spectral analyses and splitsgraph, it is not surprising that the tree building methods produced inconsistent results. ML consistently gave low support values. MP gave higher support values, but this tree-building method is susceptible to long-branch attraction (Hendy and Penny, 1989) and has a tendency to incorrectly resolve polytomies (Slowinski, 2001). Bayesian analyses resulted in low support values, except for the analysis of 12S sequences where a basally-diverging saddleback (0.95 PP) was supported. However, for this split in the 12S data spectral analysis indicated considerable conflict and phylogenetic analysis under the ML criterion indicated low support, while analysis under the MP criterion supported a basally-diverging kokako. Overall the phylogenetic

analyses indicated that the internal New Zealand wattlebird branch is relatively short or unresolved. Furthermore, neither a KH or SH test rejected a polytomy. In contrast, the likelihood ratio test indicated that the internal New Zealand wattlebird branch was significantly greater than zero for some data sets. However, caution has been suggested in interpreting the results of this test (Felsenstein, 2004) because it has a tendency to be overly significant.

Resolving short, deep branches has been a problem in many studies (e.g., Fishbein et al., 2001; Engstom et al., 2004; Poe and Chubb, 2005). A tree with short or unresolved internal branches may result from inadequate data (a 'soft' polytomy) or from rapid or simultaneous lineage diversification that leaves little opportunity for character fixation (a 'hard' polytomy). To resolve such nodes, if indeed they are resolvable, DNA sequence is required that is evolving at a sufficient rate to have changed on the short branch, but not to such an extent that the information is subsequently lost through multiple substitutions. Saturation of transitions at cytochrome b and third codon positions of ND2 suggests that these partitions may be evolving too rapidly to resolve this particular node; i.e. multiple substitutions are obscuring the relationships. In the unsaturated mitochondrial sequence data (i.e. 12S and the remaining sites of ND2 and cytochrome b) mutations may not be occurring fast enough to resolve an old, short branch like that observed in the New Zealand wattlebirds. If this node represents a soft polytomy then it can potentially be resolved with the addition of more sequence data and other studies have had success resolving old, short nodes with nuclear intron sequences (e.g., Engstom et al, 2004). Furthermore, DNA sequence from multiple nuclear loci would give a more accurate picture of species relationships in contrast to the linked mitochondrial sequences that represent a single lineage-sorting event, and which may or may not reflect species' histories (Nichols, 2001). However, a complication with obtaining more sequence data to further examine New Zealand wattlebird relationships is that only degraded DNA is available from the extinct huia. Short, overlapping DNA fragments must be obtained, thereby increasing the expense and time required to retrieve more sequence. Moreover, finding sufficient regions of conserved sequence in which to design the large number of primers necessary to amplify such short fragments may pose an additional problem, although this may be less difficult with more conserved nuclear markers.

*The evolution of sexual bill dimorphism in the huia*

In previous phylogenetic analyses the New Zealand wattlebirds did not have a close relationship to the birds of paradise, represented by *Paradisaea*, (Barker et al., 2002; Barker et al., 2004) as had been hypothesised by Frith (1997). Therefore, the sexually dimorphic beaks of these two families are not homologous. However, the cnemophiline birds of paradise, which are sister group to the New Zealand wattlebirds, albeit with weak support (Barker et al., 2004), do exhibit slight differences in bill length between male and female. This difference can be up to 9% but in only one of the three species examined by Frith and Frith (1997) did the length of the female bill exceed that of the male.

Analysis of the mitochondrial sequence data indicated similar levels of support for a basally-diverging huia or kokako. If additional sequence strongly supports a basally-diverging kokako (or saddleback), then the sexually dimorphic huia bill would be most parsimoniously explained as a derived character state on the lineage leading to huia. In contrast, if further analyses indicate that huia is the basally-diverging New Zealand wattlebird then, because of the lack of well-supported relatives, it will be difficult to ascertain the polarity of this character (i.e. whether it was ancestral with subsequent loss in the ancestor of kokako and saddleback, or was an autapomorphy for huia).

The sequences presented in this study confirm both the monophyly and genetic distinctiveness of the New Zealand wattlebirds. This family also appears to be one of a growing number of New Zealand taxa once thought to have had a vicariant origin, but which molecular dating now indicates arrived in New Zealand via transoceanic dispersal.

**Acknowledgements**

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**Appendix 1**. Support values for minor nodes from Figure 3. Values are given in the following order: Bayesian posterior probability/ML bootstrap/MP bootstrap.

a. 0.98/-/-
b. 0.89/57/59
c. 1.00/82/86
d. 0.89/-/-
e. 1.00/85/94
f. 1.00/82/70
g. 0.96/58/60
h. 0.57/-/-
i. 0.62/-/-
j. 0.63/-/-
k. 0.60/-/-
l. 0.61/-/-
m. 1.00/100/100
n. 0.63/66/-
o. 1.00/80/59
p. 0.75/54/70
q. 0.87/82/50

# CHAPTER EIGHT

## Genetic homogeneity of nuclear microsatellite DNA markers in the extinct New Zealand huia.[1]

**Abstract**

Population structure of the extinct New Zealand huia was examined by using ancient DNA methods to genotype six polymorphic microsatellite DNA markers for a large number of huia skins. Bayesian assignment methods detected no genetic structure in the huia samples examined. However, interpretation is complicated by the unknown provenance of many of the huia samples. Three huia (a male, female and immature female) caught together appear to comprise a family group thus supporting anecdotal observations that huia lived in family groups. A comparison of genetic diversity in huia with that of the extant North Island saddleback suggests that huia were more genetically variable than their extant relative.

### Introduction

Advances in ancient DNA techniques now permit investigation into the evolutionary histories of extinct organisms (e.g., Huynen et al., 2003). Information that was previously only available through contemporary observation can now be obtained via ancient DNA analyses, thus providing insight into the biology of extinct species. For example, the diet and movement patterns of extinct sloths have been elucidated from analyses of faecal DNA (Hofreitner et al., 2000).

In this study aspects of behaviour and ecology of the huia, an extinct New Zealand passerine, were indirectly examined. The huia (*Heteralocha acutirostris*) is one of the most internationally recognisable members of the New Zealand avifauna because it exhibits the most extreme sexual bill dimorphism known from any bird species. Male huia have a short, stout bill, whereas females have long curved bills about a third longer than those of the male. The huia is a member of the endemic passerine family

[1] The data in this chapter has been combined with huia sexing data and submitted to *Molecular Biology and Evolution*: Lambert, DM; Shepherd, LD; Huynen, L and Millar, C. The evolution of sexual dimorphism in the extinct Huia: A molecular test.

Callaeatidae or New Zealand wattlebirds. The other members of this family are the saddleback *(Philesturnus carunculatus*) and kokako (*Callaeas cinereus*). New Zealand wattlebirds are characterised by a pair of fleshy, colourful wattles at the gape of the mouth, strong feet and short, rounded wings (Heather and Robertson, 2000). All are vulnerable to introduced predators (Worthy and Holdaway, 2002) and consequently their distributions have been reduced with the arrival of humans and their associated commensals.

The pre-human distribution of huia bones in caves, dunes and middens indicates that they were once found throughout the North Island of New Zealand but were absent from the South Island (Worthy and Holdaway, 2002). However, following Maori settlement this range was restricted to the lower North Island. Huia were hunted by Maori for their white-tipped black tail feathers which were considered sacred. Prior to European arrival, only chiefs of high rank were permitted to wear the tail feathers in their hair. Following the arrival of European settlers hunting pressure increased. For example, in 1888 a group of Maori hunters collected 646 huia skins in a single month from the southern Hawke's Bay (Buller, 1888). Hunting reached a climax at the turn of the nineteenth century when huia tail feathers became fashionable with Europeans following a Maori guide placing a huia tail feather in the hatband of the visiting Duke of York. The contrast in huia bill morphology also led to mounted skins and beak brooches made from huia becoming popular in the late nineteenth century. Hunting pressure combined with the clearance of lowland forest and the introduction of predators led to the extinction of the huia with the last confirmed sighting in 1907 (Heather and Robertson, 2000).

Little is known about the behaviour and social structure of the huia, apart from limited observations made by early naturalists. Buller (1888) observed that huia inhabited thick forest and moved mainly on foot 'by a series of bounds or jumps'. Colenso (1887) recorded that huia were social birds and Buller (1888) noted that they were almost always observed in pairs or sometimes in groups of four or more. Potts (1885) observed huia young accompanying their parents for a considerable time after fledging and gave an account of four juveniles, barely distinguishable from adults, still being fed by their parents. Moorhouse (1996) suggested that huia were highly territorial based on Buller's (1888) observation that pairs were attracted by imitations of their call. In

addition, both the saddleback and kokako are territorial (Heather and Robertson, 2000). The social organisation and limited powers of flight of huia suggests that they may have demonstrated a high level of population genetic structuring.

These issues were investigated by attempting to amplify single copy microsatellite DNA loci from huia. Microsatellite DNA loci are popular genetic tools for investigating population structure. They are bi-parentally inherited and commonly exhibit high levels of polymorphism. The flanking sequences of microsatellite DNA loci are commonly highly conserved (e.g., FitzSimmons et al., 1995). Therefore, the primers designed for loci in one species can often be cross amplified in other species (e.g., Primmer et al., 1996). Previous studies have isolated microsatellite DNA markers from kokako (Hudson et al, 2000) and saddleback (Lambert et al., 2005) and these markers were trialled for cross amplification in huia.

Molecular studies involving extinct species such as huia are constrained by the degraded nature of DNA extracted from specimens. Firstly, all ancient DNA studies are potentially at risk from contamination by modern DNA. Contamination can largely be avoided through the use of a dedicated ancient DNA laboratory and appropriate negative controls (Cooper and Poiner, 2000). Secondly, there are two forms of error that can be especially problematic for microsatellite DNA loci amplified from degraded DNA: allelic dropout and false alleles (Taberlet et al., 1999). Allelic dropout occurs by chance when only one allele of a heterozygote is amplified and results in individuals being incorrectly scored as homozygotes. False alleles are generated by polymerase error during PCR amplification and can be recognised in heterozygotes by the presence of three alleles. Genotyping errors are commonly recognised by methods that have been designed largely by wildlife management researchers wanting to obtain reliable genotypes for individual identification from shed hair, sloughed skin and faeces (Taberlet et al., 1999).

In this study, variation at six microsatellite loci polymorphic in huia was used to investigate whether anecdotal observations of huia behaviour and social organisation were reflected in patterns of genetic structuring. Furthermore, diversity levels were compared between huia and North Island saddleback (*P. c. rufesater)*.

**Methods**

Twenty-five huia footpad samples were obtained from the Canterbury Museum and one sample was provided by Rhys Cullen (Hastings Boys' High School) (Table 1). No provenance was recorded for sixteen of these samples; the collecting locations of the remaining ten samples, including two labelled as 'possibly Pipiriki', are illustrated in Figure 1.

Extraction of ancient huia DNA was performed in a dedicated ancient DNA laboratory that was physically separated from where modern DNA and PCR products were handled. UV-irradiation and hypochloride treatment were regularly used to decontaminate the ancient laboratory. Negative extraction controls were performed to check for potential contamination with each batch of samples. Four $mm^2$ of huia footpad tissue was removed and cut into several pieces using a sterile razor blade. Huia

DNA was extracted by incubating footpad fragments overnight at 50°C in 2.5 ml extraction buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 10 mM EDTA), 250 µl of 10% SDS, 15 µl of 200 mg/ml Dithiothreitol (DTT), 25 µl of 50 mg/ml Proteinase-K. Samples were then extracted with Tris-saturated phenol followed by chloroform:isoamyl (24:1), and concentrated to 200 µl on a Vivaspin-30 (Viva Science, U.K.) membrane.

Seventeen dinucleotide microsatellite DNA loci isolated from a North Island saddleback genomic library (Lambert et al., 2005; T. King and D. Lambert, unpublished data), and eight loci isolated from a kokako microsatellite library (Hudson, 1999; Hudson et al., 2000) were screened for polymorphism in huia. PCR amplification was performed in 10 µl volumes containing 0.5 units Taq polymerase (Roche), 200 µM each dNTP, 0.8 pmol each primer, 1.5 mM $MgCl_2$, 1× PCR buffer (500 mM Tris pH 8.8, 200 mM $(NH4)_2SO_4$) and 1 µl extracted DNA. Samples were amplified at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealment at 50-55°C for 50 seconds and elongation at 72°C for one minute; and with a final extension of 5 minutes. For each locus, PCR products amplified from seven randomly selected huia DNA samples were size fractionated on a high percentage agarose gel (2% MS, 1% LE) to screen for polymorphism. Five of the North Island saddleback

**Table 1**. Huia samples used in this study with their museum numbers and, where known, their collection details and sex (as determined by morphology). HBH = specimen obtained from Hastings Boys' High School. The samples marked with * did not amplify across a sufficient number of loci during the initial PCR screen and were therefore excluded from the analyses. The four samples marked with $^{+}$ were independently extracted and amplified at the University of Auckland by L. Huynen.

| Museum Number | Location | Collection Information | Sex |
|---|---|---|---|
| AV1076 | Wairarapa | Buller, 1892 | Female |
| AV1078 | Makuri | Buller, 1892 | Male |
| AV1079$^{+}$ | Ngarara | Buller, 1891 | Male |
| AV1081 | - | - | Female |
| AV1082 | Wairarapa | Buller, 1892 | Male |
| AV1083 | - | - | Female |
| AV1085 | - | - | Male |
| AV1087 | - | Moorhouse | Male |
| AV1126 | - | - | Male |
| AV2244 | - | Parker | Female |
| AV2245$^{+}$ | - | Parker | Male |
| AV2283 | - | Parker | Male |
| AV2727 | - | O'Connor bought | Female |
| AV2729 | - | O'Connor bought | Male |
| AV2744 | Wellington | - | Male |
| AV2745$^{+}$ | Mangaroa Hill | Len Harris, 1885 | Male |
| AV2746 | Mangaroa Hill | Len Harris, 1885 | Female |
| AV2747 | Mangaroa Hill | Len Harris, 1885 | Female (juvenile) |
| AV21283 | - | - | Male |
| AV21289 | - | - | Female |
| AV36838 | - | F. Grimwood, 1870s Gifted by a North Island Maori Chief | Female |
| AV37493A | Possibly Pipiriki | - | Not measured |
| AV37493B | Possibly Pipiriki | - | Not measured |
| HBH$^{+}$ | - | - | Female |
| AV1070* | - | - | Female |
| AV1084* | - | - | Female |

microsatellite DNA loci (Pca01, Pca05, Pca12, Pca13, Pca16), including three loci that were monomorphic in North Island saddleback, and one kokako locus (K9/K10) were found to be polymorphic in huia. Fluorescent dyes were used to label the reverse

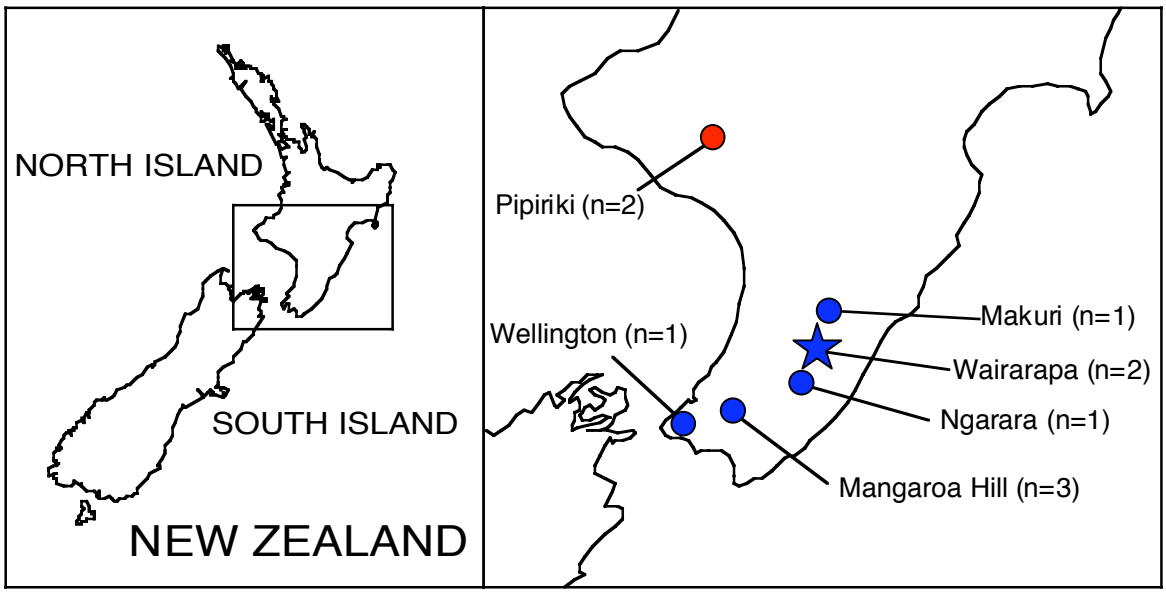


**Figure 1.** Provenance of labelled huia samples used in this study. Precise sample localities are indicated by circles; approximate localities by a star. Blue symbols represent samples with reliable provenance data; red symbols indicate samples with uncertain provenance.

primer of each primer pair. Specifically Pca05, Pca12, Pca13 and Pca16 were labelled with 6-FAM, Pca01 with VIC and K10 with HEX. Microsatellite DNA loci were amplified using the method described above, with a negative PCR control included with every set of reactions. Single-locus PCR reactions were pooled within samples where possible. Genotyping was conducted using an ABI Prism 3730 sequencer and visualised using Genescan. A standard sample was included with each genotyping batch to account for between-run variation.

Two techniques were used to avoid genotyping errors. Firstly, an initial screen of the quality of the samples was performed from the first round of PCR reactions (Paetkau, 2003). Samples that amplified at fewer than three of the six loci were omitted from the study. For the remaining samples, the 'multiple tubes' method (Navidi et al., 1992; Taberlet et al., 1996) was used. This approach involves multiple independent PCR amplifications of each locus to produce a consensus genotype. Homozygotes were obtained seven times in order to discount allelic dropout (ADO) and both alleles of heterozygotes were detected twice in order to rule out false alleles (FA). In addition, a

subset of four huia samples (Table 1) were independently extracted and amplified for three loci (Pca01, Pca05 and Pca12) at the University of Auckland ancient DNA facility by L. Huynen.

General measures of genetic variation, including observed and expected heterozygosities ($H_O$ and $H_E$, respectively) and number of alleles were calculated in Arlequin 2.001 (Schneider et al., 2000). GENEPOP version 3.4 was used to test for linkage disequilibrium and deviation from Hardy-Weinberg proportions (Raymond and Rousset, 1995). The sequential Bonferroni correction was applied to adjust the level of significance for multiple tests (Rice, 1989). The rate of allelic dropout ($ADO_\mu$) and occurrence of false alleles ($FA_\mu$) was calculated for each locus and across all loci using the following equations as recommended by Broquet and Petit (2004). These calculations included the genotype data repeated at the University of Auckland.

1. $ADO_\mu = D_j/A_{hetj}$

$D_j$ = number of amplifications of locus j where an ADO event is observed
$A_{hetj}$ = number of positive amplifications of heterozygotes

2. $FA_\mu = F_j/A_j$

$F_j$ = number of amplifications at locus j where a false allele is observed
$A_j$ = number of amplifications (both hetero- and homozygotes)

False alleles can occur in heterozygous and homozygous genotypes. Therefore, the rate of false allele formation is estimated across all positive amplifications. In contrast, allelic dropout can only be detected in heterozygotes. Therefore, the allelic dropout rate is calculated using only the positive amplifications of heterozygotes.

The probability of false homozygotes at each locus after repeated PCR reactions (*P*) was calculated using the following equation (Gagneux et al., 1997).

3. $P = (K) \times (K/2)^{n-1}$

*K* = the $ADO_\mu$ at each locus
*n* is the number of repeated amplifications, in this case seven.

Consensus genotypes obtained from the multiple tubes method were used to examine population structure in the huia. The lack of provenance for the majority of samples prevented the application of traditional population genetic analyses such as F-statistics. Instead, two Bayesian clustering methods that do not require prior population information to partition samples into genetic groups were used to investigate genetic structuring in the huia microsatellite dataset: STRUCTURE version 2.1 (Pritchard et al., 2000; Falush et al., 2003) and PARTITION (Dawson and Belkhir, 2001). Neither method requires the population of origin for individual samples, or even the number of sampled populations (K), to be known. Both methods identify clusters of individuals that are in Hardy-Weinberg and linkage equilibrium but differ in their treatment of admixed individuals (Pearse and Crandall, 2004).

STRUCTURE was run using no prior population information and assuming admixture. Allele frequencies among clusters were assumed to be independent to prevent overestimation of the number of clusters (Falush et al., 2003). Four independent runs of K = 1-5 were performed using $10^6$ MCMC repetitions with the first 50 000 discarded as 'burnin' following visual confirmation that equilibrium had been reached. The posterior probabilities of the data, P(X/K), were calculated from the mean estimate log likelihood of each K, lnP(X/K), in order to select the optimal K.

PARTITION was applied to only the seventeen huia samples that possessed a full complement of genotype data because missing data are not permitted in this software package. The parameter *u* (the prior probability distribution on K) was set at 1; i.e. equal probabilities of each K were assumed, and the parameter theta (the prior distribution on the allelic distribution of the ancestral population) was varied from 1 to 20. The maximum number of source populations was changed over different runs, from 4 to 8. Estimates of the posterior probabilities were made after 50 000 observations of the Markov chain, with the first 5000 observations omitted as 'burnin'.

The mean number of alleles ($N_A$) per locus and mean expected heterozysity ($H_E$) were compared between huia and North Island saddleback. Data from 41 individuals from the Hen Island population of North Island saddleback (Lambert et al., 2005) were used

in these comparisons because all contemporary North Island saddleback populations derive from translocations from this island.

**Results**

*General genetic diversity measures*

Twenty-four of the twenty-six huia samples amplified for three or more of the six polymorphic microsatellite DNA loci (Table 1), and were therefore included in further analyses. All loci were in Hardy-Weinberg equilibrium. No linkage was observed between loci following adjustment of the significance level for multiple comparisons with a Bonferroni correction (Rice, 1989). Loci exhibited moderate variation with 2-10 alleles per locus and expected heterozygosities ranged from 0.437-0.766, with a mean of 0.637 across all loci (Table 2).

*Genotyping errors in huia microsatellite data*

The mean number of loci amplified per sample was 5.7 out of the 6 loci. The $ADO_{\mu}$ of each locus varied from 3.2% to 20% (Table 2) with a mean of 12.9%. The dropout rates at each locus gave a negligible probability of false homozygotes after seven replications ($P < 0.0001$). Across all loci, the longer of the two alleles in a heterozygote was significantly more likely to not amplify (21 longer alleles verses 6 shorter alleles 'dropped out'; Chi squared test $\chi^2 = 8.33$, d.f. = 1, $p = 0.004$). Allelic dropout rates also differed significantly between samples (Chi squared test $\chi^2 = 36.11$, d.f. = 23, $p = 0.040$).

Four false alleles were observed in the dataset (rate of occurrence = 0.7%). Three of the false alleles were longer than the true allele: one was a repeat unit (two base pairs) more; one was one base pair longer; and one formed a third allele at a heterozygote which was longer than the two true alleles by one and two repeat units. The fourth false allele was one repeat unit smaller than the true allele.

*Population genetic structure in huia*

The most likely number of populations in the huia dataset was estimated using the Bayesian clustering approach implemented in STRUCTURE (Pritchard et al., 2000). The posterior probability of the data, P(K/X), was maximum with K=1 (Table 3),

**Table 2**. Genetic diversity measures and genotyping errors at six microsatellite DNA loci amplified from huia. The number of alleles ($N_A$), their size ranges, observed and expected heterozygosities and genotyping errors (allelic dropout rate: $ADO_\mu$ and rate of occurrence of false alleles: $FA_\mu$) are given.

| Locus | Pca01 | Pca05 | Pca12 | Pca13 | Pca16 | K9/K10 | Overall |
|---|---|---|---|---|---|---|---|
| Number of alleles ($N_A$) | 4 | 3 | 10 | 2 | 5 | 6 | 30 |
| Allele size range (bp) | 178-186 | 131-135 | 113-130 | 157-159 | 113-127 | 69-85 | - |
| $H_O$ | 0.714 | 0.583 | 0.750 | 0.348 | 0.762 | 0.818 | 0.662 |
| $H_E$ | 0.692 | 0.494 | 0.766 | 0.437 | 0.713 | 0.723 | 0.637 |
| Allelic dropout: | | | | | | | |
| Longer allele missing | 6 | 0 | 6 | 4 | 2 | 3 | 21 |
| Shorter allele missing | 1 | 1 | 0 | 0 | 1 | 3 | 6 |
| $ADO_\mu$ | 0.179 | 0.032 | 0.133 | 0.200 | 0.088 | 0.146 | 0.129 |
| $FA_\mu$ | 0.024 | 0.019 | 0 | 0 | 0 | 0 | 0.007 |

**Table 3.** Estimated posterior probabilities from STRUCTURE, P(K/X), of K, the number of huia populations. The estimated probability of the data, lnP(X/K), is averaged over four independent runs for each K.

| K | lnP(X/K) | P(K/X) |
|---|---|---|
| 1 | -335.2 | 0.925 |
| 2 | -338.1 | 0.051 |
| 3 | -339.9 | 0.009 |
| 4 | -339.4 | 0.014 |
| 5 | -343.8 | 0.002 |

indicating no evidence of population differentiation among the huia samples examined. In addition, each individual had a similar posterior assignment probability to each of the K putative source populations, approximately equal to 1/K, providing further support for a lack of genetic subdivision.

Population structure was also investigated using PARTITION (Dawson and Belkhir, 2001). However, inference of the number of populations was not straight-forward because the posterior probability always concentrated on the maximum possible number of populations specified in the settings. This result was maintained despite trials varying the value of the prior parameter theta. Simulations by Dawson and Belkhir (2001) indicated that upwards bias in the posterior distribution of K may occur with small sample sizes. They recommend that in such cases inference may be drawn from the probability level plot and tree plot of individuals. The tree plot of huia data (not shown) provided no evidence of population subdivision (i.e. there were no well-supported clusters separated by long branches). The plot of Bayesian probability level versus generation time (not shown) also indicated no evidence of structure (i.e. the probability level declined gradually down to a single cluster of individuals).

Three of the huia samples analysed, a male, female and immature female, were collected from the same location in 1885 (museum numbers AV2745-7). The microsatellite alleles detected from the immature female were consistent with it being the progeny of the adult huia; i.e. it had a subset of the putative parents' alleles.

*Genetic diversity in huia and saddleback*

Huia exhibited a greater mean number of alleles per locus than North Island saddleback when polymorphic loci were considered alone, i.e. loci polymorphic in huia, loci polymorphic in North Island saddleback (Table 4). Huia also had a higher mean number of alleles per locus when all loci that were polymorphic in either huia or North Island saddleback (i.e. all loci in Table 4) were measured. However, neither of these comparisons were significant (2-tailed t-tests, $p = 0.135$ and $p = 0.623$, respectively).

The mean expected heterozygosity was higher in huia (0.637) than in North Island saddleback (0.559; Lambert et al., 2005) although the difference was not statistically significant (2-tailed t-test, $p = 0.311$). The length distributions of alleles at three loci

**Table 4**. The number of alleles ($N_A$) at microsatellite DNA loci isolated from North Island saddleback that are polymorphic in North Island saddleback and/or huia. The allelic size range at each locus, where determined, is also shown. * exact allele size not determined.

| | Saddleback | | Huia | |
|---|---|---|---|---|
| Locus | $N_A$ | Allele size range (bp) | $N_A$ | Allele size range (bp) |
| Pca01 | 1 | 184 | 4 | 178-186 |
| Pca02 | 4 | 110-132 | 0 | N/A |
| Pca05 | 3 | 148-158 | 3 | 131-135 |
| Pca08 | 3 | 81-85 | 1 | * |
| Pca10 | 2 | 140-142 | 1 | 135 |
| Pca12 | 2 | 105-121 | 10 | 113-130 |
| Pca13 | 1 | 164 | 2 | 157-159 |
| Pca14 | 3 | 112-116 | 1 | 104 |
| Pca15 | 3 | 206-211 | 1 | * |
| Pca16 | 1 | 102 | 5 | 114-127 |
| Mean $N_A$/locus ± SE | 2.30 ± 0.33 | | 2.8 ± 0.94 | |
| Mean $N_A$ /polymorphic locus ± SE | 2.86 ± 0.26 | | 4.80 ± 1.39 | |

(Pca02, Pca05 and Pca12; Table 4) were discontinuous in North Island saddleback. In contrast, microsatellite allele length distributions of alleles in huia tended to be more continuous. For example, locus Pca12 in huia exhibited ten alleles differing in size by 1-4 bp, whereas only two alleles were detected at this locus in North Island saddleback and these differed by 16 bp.

**Discussion**

*Lack of population structuring in huia*

The data presented here provide no evidence for population subdivision in huia. Similar low levels of genetic structure have been observed at microsatellite DNA loci in

the New Zealand kaka from both the North and South Islands of New Zealand (Sainsbury, 2004). However, kaka, unlike huia, are highly mobile. More comparable to huia are two studies of genetic variation in the North Island kokako, which is in the same family as huia and may exhibit similar behaviour. Low levels of genetic structuring among populations of North Island kokako have been reported with both mtDNA sequences (Double and Murphy, 2000) and microsatellite DNA loci (Hudson et al., 2000). Although North Island kokako adults remain in the same territory for many years, juveniles may disperse several kilometres to find a mate and/or territory (Innes and Flux, 1999). Gene flow in huia may also have been facilitated by juvenile dispersal. However, the interpretation of the population structure data is complicated by the lack of provenance for the majority of the huia samples. All huia with reliable collection data were obtained from the southern North Island and, because huia were most abundant in this area during early European settlement (Phillipps, 1963), many of the unlabelled samples are also likely to have originated from this region. Therefore, the huia samples examined in this study may lack genetic structuring because they all derive from this reasonably small area; the maximum distance between samples of known provenance is approximately 150 km (Wellington to Makuri). Prior to European settlement this area had been covered in continuous lowland forest and may have supported a large huia population. Furthermore, in contrast to the northern and central North Island, the southern North Island has lacked significant barriers to gene flow (e.g., volcanic eruptions) following post-glacial reforestation.

The genotype data are consistent with the three huia collected by Len Harris from the same locality in 1885 (Museum numbers AV2745-7) comprising a family group, consisting of a female, male and their offspring, an immature female. This supports observations by early naturalists that huia, at least soon after fledging, remained in family groups

*Accurate genotype data from ancient samples*

Allelic dropout is thought to be a significant problem for genetic census data from degraded DNA. If this problem is ignored it can result in creating false individuals and hence cause overestimation of population size (Paetkau, 2003). In contrast, the effect of allelic dropout on estimations of population structure is probably less of a problem (Creel et al., 2003), although it has not been rigorously investigated (Manel et al.,

2005). The microsatellite genotyping error rates determined for the huia dataset are within the range of those encountered in other studies of low template samples (reviewed in Broquet and Petit, 2004). Allelic dropout was a more common form of genotyping error in this dataset than the formation of false alleles, which is also consistent with other studies. The probability of false homozygotes at each locus owing to allelic dropout was calculated to be negligible after the seven replicates, when averaged over all samples. However, this conclusion is based on the assumption that dropout rates are equal between individuals. This is probably violated in most datasets, including this one. Consequently, a few undetected dropouts may remain in the huia dataset. However, the number is likely to be minimal because low quality samples were identified and removed prior to analysis, with this approach found to be reliable in decreasing genotyping errors in other studies (e.g., Paetkau 2003; Hung et al., 2004).

*Comparison of genetic diversity in huia and North Island saddleback.*

The polymorphic loci in huia had more alleles than the loci that were polymorphic in saddleback. In addition, over all loci that were polymorphic in either species huia also had more alleles. Moreover, huia had higher levels of heterozygosity than those in the Hen Island population of North Island saddleback, from which all contemporary populations derive. These results are unexpected because microsatellite loci are commonly longer and more variable in the species from which they are sourced (Ellegren et al. 1995). This probably results from ascertainment bias during cloning and locus selection (Hutter et al. 1998; but see Cooper et al. 1998). Although it is possible that North Island saddleback have always had low levels of genetic variation, this seems unlikely considering that saddleback are one of the most common passerines in subfossil deposits around New Zealand (Worthy and Holdaway 2002). A more plausible explanation is that North Island saddleback have lost a considerable portion of their genetic variation through a population bottleneck, as previously suggested (Lambert et al., 2005). Further support for this hypothesis is provided by the length distributions of alleles at each locus. In huia the allele length distributions tended to be more continuous than in North Island saddleback. In contrast three loci of North Island saddleback, Pca02, Pca05 and Pca12, possessed alleles that differed by a large number of base pairs. Microsatellite DNA is widely believed to evolve by slippage during replication usually resulting in the addition or deletion of one or a small number of repeat units (Levinson and Gutman, 1987). This mode of evolution typically results in

a series of alleles at a given locus differing by only a small number of repeats units. Therefore, the discontinuous allele distributions some of the North Island saddleback loci suggest that the intermediate sized alleles at these loci may have been lost through a population bottleneck.

In summary, the multiple tubes method was used to obtain genotype data from the extinct New Zealand huia. Substantial levels of genetic variation were found in huia, exceeding that found in the extant North Island saddleback, from which the microsatellite loci were isolated. Bayesian assignment tests indicated no genetic structuring in the huia samples examined.

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**Appendix 1.** Huia microsatellite DNA genotypes obtained following the 'multiple tubes' approach. The consensus genotypes and data verified at the University of Auckland are also shown. Allelic dropout events are highlighted in blue. False alleles are shown in red. Samples with 'NO AMP' did not amplify.

| | Locus | | | | | |
|---|---|---|---|---|---|---|
| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
| **AV2727** | 121/119 | 182/182 | 135/133 | NO AMP | 157/157 | 81/72 |
| | 121/119 | 182/182 | 135/133 | | 157/157 | 81/81 |
| | | 182/182 | | | 157/157 | 81/72 |
| | | 182/182 | | | 157/157 | |
| | | 182/182 | | | 157/157 | |
| | | 182/182 | | | 157/157 | |
| | | 182/182 | | | 157/157 | |
| CONSENSUS | 121/119 | 182/182 | 135/133 | - | 157/157 | 81/72 |
| **AV37493A** | 121/119 | 184/182 | 135/133 | 119/119 | 157/157 | 72/72 |
| | 121/119 | 186/184/182 | 135/133 | 121/121 | 157/157 | 72/72 |
| | | 184/182 | | 121/121 | 157/157 | 72/72 |
| | | | | 121/119 | 157/157 | 72/72 |
| | | | | | 157/157 | 72/72 |
| | | | | | 157/157 | 72/72 |
| | | | | | 157/157 | 72/72 |
| CONSENSUS | 121/119 | 184/182 | 135/133 | 121/119 | 157/157 | 72/72 |
| **AV1083** | 115/115 | 186/186 | 135/133 | 119/113 | 157/157 | 72/72 |
| | 119/115 | 186/182 | 135/133 | 119/113 | 157/157 | 83/83 |
| | 119/115 | 186/182 | | | 157/157 | 72/83 |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| CONSENSUS | 119/115 | 186/182 | 135/133 | 119/113 | 157/157 | 83/72 |
| **AV21283** | 130/116 | 186/184 | 133/133 | NO AMP | 157/157 | 72/72 |
| | 130/116 | 184/184 | 133/133 | | 157/157 | 72/72 |
| | | 186/178 (inconsistent) | 133/133 | | 157/157 | 72/72 |
| | | | 133/133 | | 157/157 | 72/72 |
| | | | 133/133 | | 157/157 | 72/72 |
| | | | 133/133 | | 157/157 | 72/72 |
| | | | 133/133 | | 157/157 | 72/72 |
| CONSENSUS | 130/116 | - | 133/133 | - | 157/157 | 72/72 |

Appendix 1 continued.

| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
|---|---|---|---|---|---|---|
| **HBH** | 119/115 | 186/186 | 135/133 | 121/117 | 157/157 | 72/72 |
| | 119/115 | 186/186 | 135/133 | 121/117 | 157/157 | 72/72 |
| | | 186/186 | | | 157/157 | 72/72 |
| | | 186/186 | | | 157/157 | 72/72 |
| | | 186/186 | | | 157/157 | 72/72 |
| | | 186/186 | | | 157/157 | 72/72 |
| | | 186/186 | | | 157/157 | 72/72 |
| CONSENSUS | 119/115 | 186/186 | 135/133 | 121/117 | 157/157 | 72/72 |
| AUCKLAND | 119/115 | NO AMP | 135/133 | | | |
| | | | | | | |
| **AV374934B** | 121/115 | 184/182 | 135/133 | NO AMP | 159/157 | 81/72 |
| | 121/115 | 184/182 | 135/133 | | 159/157 | 81/72 |
| CONSENSUS | 121/115 | 184/182 | 135/133 | - | 159/157 | 81/72 |
| | | | | | | |
| **AV2745** | 121/119 | 184/184 | 133/133 | 119/117 | 157/157 | 85/72 |
| | 121/119 | 184/184 | 133/131 | 119/117 | 157/157 | 85/72 |
| | | 184/184 | 133/133 | | 157/157 | |
| | | 184/184 | 133/133 | | 157/157 | |
| | | 184/184 | 133/133 | | 157/157 | |
| | | 184/184 | 133/133 | | 157/157 | |
| | | 186/184 | 133/133 | | 157/157 | |
| | | 184/184 | 133/133 | | | |
| CONSENSUS | 121/119 | 184/184 | 133/133 | 119/117 | 157/157 | 85/72 |
| AUCKLAND | 121/119 | NO AMP | 133/133 | | | |
| | | | | | | |
| **AV2244** | 119/115 | NO AMP | 133/131 | 117/117 | NO AMP | 85/72 |
| | 115/115 | | 133/133 | 117/117 | | 85/72 |
| | 119/115 | | 133/131 | 117/117 | | |
| | | | | 117/117 | | |
| | | | | 117/117 | | |
| | | | | 117/117 | | |
| | | | | 117/117 | | |
| CONSENSUS | 119/115 | | 133/131 | 117/117 | | 85/72 |
| | | | | | | |
| **AV1081** | 115/115 | 184/184 | 133/133 | 119/117 | 157/157 | 85/72 |
| | 115/115 | 186/184 | 133/133 | 119/117 | 157/157 | 85/72 |
| | 115/115 | 186/184 | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 134/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | | | 133/133 | | | |
| CONSENSUS | 115/115 | 186/184 | 133/133 | 119/117 | 157/157 | 85/72 |

Appendix 1 continued.

| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
|---|---|---|---|---|---|---|
| **AV2747** | 121/119 | 182/182 | 135/133 | 119/119 | 159/157 | 85/72 |
| | 121/119 | 182/182 | 135/133 | 119/119 | 159/157 | 85/72 |
| | | 184/182 | | 119/119 | | |
| | | 184/182 | | 119/119 | | |
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| | | | | 119/119 | | |
| | | | | 119/119 | | |
| CONSENSUS | 121/119 | 184/182 | 135/133 | 119/119 | 159/157 | 85/72 |
| **AV2283** | 121/115 | 186/178 | 135/135 | 117/117 | 159/157 | 81/81 |
| | 121/115 | 186/178 | 135/135 | 117/117 | 157/157 | 81/81 |
| | | | 135/135 | 117/117 | 157/157 | 81/81 |
| | | | 135/135 | 117/117 | 159/157 | 81/81 |
| | | | 135/135 | 117/117 | | 81/81 |
| | | | 135/135 | 117/117 | | 81/81 |
| | | | 135/135 | 117/117 | | 81/81 |
| CONSENSUS | 121/115 | 186/178 | 135/135 | 117/117 | 159/157 | 81/81 |
| **AV2744** | 115/115 | 182/182 | 135/133 | 121/119 | 159/157 | 85/72 |
| | 115/115 | 184/182 | 135/133 | 121/119 | 159/157 | 72/72 |
| | 122/115 | 184/182 | | | | 85/72 |
| | 122/115 | | | | | |
| CONSENSUS | 122/115 | 184/182 | 135/133 | 121/119 | 158/157 | 85/72 |
| **AV2746** | 121/115 | 182/182 | 135/135 | 121/119 | 159/159 | 72/69 |
| | 121/115 | 186/182 | 135/135 | 121/119 | 159/159 | 72/69 |
| | | 186/182 | 135/135 | | 159/159 | |
| | | | 135/135 | | 159/159 | |
| | | | 135/135 | | 159/159 | |
| | | | 135/135 | | 159/159 | |
| | | | 135/135 | | 159/159 | |
| CONSENSUS | 121/115 | 186/182 | 135/135 | 121/119 | 159/159 | 72/69 |
| **AV1082** | 129/121 | 184/178 | 135/133 | 117/113 | 157/157 | 83/83 |
| | 129/121 | 184/178 | 135/133 | 117/113 | 157/157 | 83/72 |
| | | | | | 157/157 | 72/72 |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| | | | | | 157/157 | |
| CONSENSUS | 129/121 | 184/178 | 135/133 | 117/113 | 157/157 | 83/72 |

Appendix 1 continued.

| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
|---|---|---|---|---|---|---|
| **AV2245** | 119/117 | NO AMP | 133/133 | 121/119 | 159/159 | 74/69 |
| | 119/117 | | 133/133 | 121/119 | 159/159 | 69/69 |
| | | | 133/133 | | 159/157 | 74/69 |
| | | | 133/133 | | 159/157 | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| CONSENSUS | 119/117 | - | 133/133 | 121/119 | 159/157 | 74/69 |
| AUCKLAND | 119/117 | NO AMP | 133/133 | | | |
| **AV1078** | 115/113 | 184/182 | 135/133 | 121/117 | 157/157 | 81/74 |
| | 115/113 | 184/182 | 135/133 | 121/117 | 157/157 | 81/74 |
| | | | | | 157/157 | |
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| | | | | | 157/157 | |
| CONSENSUS | 115/113 | 184/182 | 135/133 | 121/117 | 157/157 | 81/74 |
| **AV1085** | 130/115 | 186/184 | 133/133 | 121/119 | 159/157 | NO AMP |
| | 130/115 | 186/184 | 133/133 | 121/119 | 159/157 | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| | | | 133/133 | | | |
| CONSENSUS | 130/115 | 186/184 | 133/133 | 121/119 | 159/157 | |
| **AV21289** | 115/115 | 186/184 | 133/133 | 121/117 | 157/157 | 85/72 |
| | 115/115 | 186/184 | 133/133 | 121/117 | 157/157 | 85/72 |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| | 115/115 | | 133/133 | | 157/157 | |
| CONSENSUS | 115/115 | 186/184 | 133/133 | 121/117 | 157/157 | 85/72 |
| **AV36838** | 121/121 | 184/182 | 133/133 | 127/119 | 157/157 | 81/72 |
| | 121/121 | 182/182 | 133/133 | 127/119 | 157/157 | 81/72 |
| | 126/121 | 184/182 | 133/133 | | 157/157 | |

Appendix 1 continued.

| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
|---|---|---|---|---|---|---|
| | 126/121 | | 133/133 | | 157/157 | |
| | | | 133/133 | | 157/157 | |
| | | | 133/133 | | 157/157 | |
| | | | 133/133 | | 157/157 | |
| CONSENSUS | 126/121 | 184/182 | 133/133 | 127/119 | 157/157 | 81/72 |
| **AV1076** | 115/115 | 186/184 | 133/133 | 119/117 | 159/157 | NO AMP |
| | 115/115 | 186/184 | 133/133 | 119/117 | 159/157 | |
| | 115/115 | | 133/133 | | | |
| | 115/115 | | 133/133 | | | |
| | 115/115 | | 133/133 | | | |
| | 115/115 | | 133/133 | | | |
| | 115/115 | | 133/133 | | | |
| CONSENSUS | 115/115 | 186/184 | 133/133 | 119/117 | 159/157 | - |
| **AV1087** | 115/115 | 184/184 | 135/133 | 117/117 | 157/157 | 74/72 |
| | 115/115 | 184/184 | 135/133 | 117/117 | 157/157 | 74/72 |
| | 115/115 | 184/184 | | 117/117 | 157/157 | |
| | 115/115 | 184/184 | | 117/117 | 157/157 | |
| | 115/115 | 184/184 | | 117/117 | 157/157 | |
| | 115/115 | 184/184 | | 117/117 | 157/157 | |
| | 115/115 | 184/184 | | 117/117 | 157/157 | |
| CONSENSUS | 115/115 | 184/184 | 135/133 | 117/117 | 157/157 | 74/72 |
| **AV2729** | 119/115 | 184/184 | 135/133 | 121/117 | 157/157 | 81/72 |
| | 119/115 | 184/184 | 135/133 | 121/117 | 157/157 | 81/72 |
| | | 184/184 | | | 157/157 | |
| | | 184/184 | | | 157/157 | |
| | | 184/184 | | | 157/157 | |
| | | 184/184 | | | 157/157 | |
| | | 184/184 | | | 157/157 | |
| CONSENSUS | 119/115 | 184/184 | 135/133 | 121/117 | 157/157 | 81/72 |
| **AV1079** | 115/115 | 184/178 | 135/133 | 119/119 | 159/159 | 85/70 |
| | 115/115 | 184/178 | 135/133 | 119/119 | 159/159 | 85/70 |
| | 115/115 | | | 119/119 | 159/159 | |
| | 115/115 | | | 119/119 | 159/159 | |
| | 115/115 | | | 119/119 | 159/159 | |
| | 115/115 | | | 119/119 | 159/159 | |
| | 115/115 | | | 119/119 | 159/159 | |
| CONSENSUS | 115/115 | 184/178 | 135/133 | 119/119 | 159/159 | 85/70 |

Appendix 1 continued.

| Sample Name | Pca12 | Pca01 | Pca05 | Pca16 | Pca13 | K9/K10 |
|---|---|---|---|---|---|---|
| AUCKLAND | 115/115 | 184/178 | 135/133 | | | |
| | | | | | | |
| **AV1126** | 129/129 | 184/184 | 135/133 | 119/117 | 159/157 | 74/72 |
| | 129/129 | 184/184 | 135/133 | 119/117 | 159/157 | 74/72 |
| | 129/129 | 184/184 | | | | |
| | 129/129 | 184/184 | | | | |
| | 129/129 | 184/184 | | | | |
| | 129/129 | 184/184 | | | | |
| | 129/129 | 184/184 | | | | |
| CONSENSUS | 129/129 | 184/184 | 135/133 | 119/117 | 159/157 | 74/72 |

# CHAPTER NINE

# Summary and Future Work.

### Summary of major findings

In this thesis, ancient and modern DNA sequences were isolated and analysed for members of two endemic New Zealand avian families in order to investigate a number of conservation and evolutionary questions. The major findings are summarised below.

Ancient mitochondrial DNA (mtDNA) sequences were isolated from kiwi and compared to previously published modern sequences to examine the distribution of genetic variation across time and space. Previous studies (Baker et al., 1995; Burbidge et al., 2003) had revealed an extremely high level of matrilineal structuring with almost every population possessing private haplotypes. Ancient brown kiwi mtDNA sequences indicated that considerably higher levels of genetic diversity and structuring also existed in the past and that the high level of genetic structuring detected in modern populations was not a result of human mediated character state fixation (Chapter Two). Both rowi and tokoeka contained a number of well-supported subgroups that, using previous methods that have been used to delimit species in kiwi (e.g., Baker et al., 1995; Burbidge et al., 2003), could be considered separate species (Chapter Two). This is not recommended here because only mtDNA sequences have been obtained from these subgroups and data from nuclear markers is required to verify the distinctiveness of these groups.

In contrast to the high level of genetic variation in rowi and tokoeka, North Island brown kiwi demonstrated less genetic structuring. A previous study had indicated three genetically distinct groups in the North Island (Burbidge et al., 2003). They suggested an 'aberrant' modern kiwi sample from Taranaki, whose haplotype was identical to a sample from Northland, was the result of a past translocation of kiwi between genetically different populations. However, analyses presented here suggest that samples from Wanganui and Waitomo, that predate the proposed translocations, also group with sequences from Northland (Chapter Two, Chapter Four, Chapter Five). Therefore, not all kiwi populations in the North Island have monophyletic

mitochondrial DNA lineages. However, an east/west genetic split in the North Island was supported with the additional samples.

The retrieval of ancient DNA sequences permitted classification of subfossil bones from great spotted and brown kiwi, whose bone morphologies are indistinguishable (Chapter Two). This information allowed the determination of the former ranges of these species, and now enables the most appropriate species to be used in programmes seeking to re-introduce kiwi to areas where they are locally extinct.

In contrast to brown kiwi, little spotted kiwi exhibited different phylogeographic patterns (Chapter Three). Most genetic variation in little spotted kiwi was found in the North Island and the majority of samples from the South Island possessed a single haplotype. The difference in phylogeography between brown and spotted kiwi is proposed to relate to differences between their dispersal patterns and/or population history.

The ancient DNA sequences obtained for kiwi, in combination with modern sequences, were used as a reference database to determine the provenance of six unlabelled museum specimens (Chapter Four). All specimens were assigned to species with high support, and in some cases, the likely geographic region of origin was also inferred. The same strategy was used to investigate provenance of brown kiwi feathers from Maori cloaks and baskets (Chapter Five). DNA was successfully amplified from kiwi feathers from fifteen artefacts. All artefacts examined were constructed from North Island brown kiwi feathers and DNA sequences from fourteen of the artefacts possessed haplotypes previously detected in kiwi from the Bay of Plenty and Hawke's Bay in the North Island.

Preliminary genotype data for all species of kiwi, using five microsatellite DNA loci, was presented (Chapter Six). A high level of divergence was detected amongst taxa. A Bayesian assignment test indicated that further subdivision might be present within some species.

The second avian family examined by molecular methods in this thesis was the New Zealand wattlebirds (Chapter Seven). Analyses of nuclear sequences showed kokako,

saddleback and huia to be a monophyletic group, with strong support. A divergence time estimate for the New Zealand wattlebirds suggested their ancestors were more likely to have arrived by transoceanic dispersal than have a Gondwanan origin. The relationships between the three genera were unable to be resolved, even with the addition of further mtDNA sequences from three genes.

Microsatellite DNA data from the extinct New Zealand huia revealed considerable levels of genetic variation, exceeding that found in extant North Island saddleback, from which the loci had been isolated (Chapter Eight). Assignment tests indicated no genetic structuring within huia, although interpretation was complicated by a lack of detailed provenance information for many of the skins. The multilocus genotypes of three huia (a male, female and immature female) caught together were consistent with them comprising a family group, thus supporting anecdotal observations that huia lived in family groups.

**Proposed future directions**

*Relationships among kiwi taxa*

There are a number of phylogenetic relationships within kiwi that warrant further investigation. Firstly, the relationship between ancient samples from the north-west of the South Island and the lower North Island with modern rowi was only weakly supported in analyses (Chapter Two). Secondly, little spotted kiwi was paraphyletic with respect to great spotted kiwi, although support was weak (Chapter Three). These relationships could be investigated using sequences such as ATPase6 and 8, which are already available for all modern kiwi species and appear better for resolving basal divergences in kiwi (Burbidge et al., 2003). However, such additional sequences would have to be amplified as short overlapping fragments from degraded ancient DNA, considerably increasing cost and effort.

Isolating nuclear markers, e.g., microsatellite DNA loci, from each of these groups would also be desirable to confirm that mitochondrial and nuclear markers show concordance. The microsatellite loci isolated here (Chapter Six) were trialled in some of the ancient kiwi samples, those with the best quality DNA (i.e. produced the brightest bands of mitochondrial DNA), but did not yield any product. Advances in ancient DNA extraction methods may, in future, allow the retrieval of better quality DNA.

Redesigning the primers to amplify shorter fragments may also increase the chance of success. However, designing the primers too close to the microsatellite repeat region should be avoided because of the higher mutation rate in this region (Brohede and Ellergren, 1999; Shepherd and Lambert, in press), which may lead to null alleles.

*Conservation of kiwi*

Accurate taxonomies are necessary for determining management strategies and priorities (Avise, 1989; Daugherty et al., 1990). Many previously widespread New Zealand species have become restricted to small, allopatric populations since human arrival. Such allopatric taxa commonly pose a problem in the determination of species boundaries because they cannot be directly assessed for evidence of interbreeding. Molecular markers have been used to infer a long history of geographic isolation and, by inference, likely reproductive isolation for some taxa (e.g., Daugherty et al., 1990; Baker et al., 1995; Burbidge et al., 2003; Bell et al., 1998). However, some of these species delimitations have been controversial (e.g., Holyoake et al., 2001). Small, isolated populations can quickly become genetically differentiated owing to genetic drift (e.g., Lambert et al., 2005). For kiwi, a number of options for determining the specific status of allopatric populations are possible and are discussed in Chapter Two.

Further analyses of nuclear microsatellite data with more samples and loci are also required to further understand the relationships between populations and the processes that have led to genetic differentiation. For example, the brown kiwi population at Haast is the only population of tokoeka to be conserved in a kiwi sanctuary, in part because of a fixed difference from other tokoeka at a single allozyme locus (Herbert and Daugherty, 2002). This population is listed as a species and has the highest priority for management action (DOC, 2004). However, despite this conservation management, Haast tokoeka have low productivity and poor recruitment (DOC, 2004). Analyses of the Haast population of tokoeka using further microsatellite DNA loci and larger sample sizes may allow determination of whether it is a relatively recently derived bottlenecked population or has had a long history of isolation. The result may raise a further issue: should conservation efforts be directed towards maintaining genetically distinct populations or genetically diverse populations (e.g., Holyoake et al., 2001; Berry and Gleeson, 2005)? If Haast tokoeka is actually only a small, inbred population, then conservation managers should consider protecting a more genetically diverse

population of tokoeka (e.g., from Fiordland), which possibly has greater long-term evolutionary potential.

Further work is also required to examine genetic structuring within North Island brown kiwi with nuclear markers such as microsatellite DNA loci. Three genetically distinct populations of North Island brown kiwi were recognised by Burbidge et al (2003): Northland; Taranaki, King Country and Wanganui; Hawke's Bay and East Coast-Bay of Plenty. These three groups, plus a forth population in Coromandel, are currently recognised as separate management units by the Department of Conservation (DOC, 2004). However, analyses of mtDNA presented here indicate that phylogeographic structure in North Island brown kiwi may be better explained by an east/west division. Nuclear markers could be used to examine whether this east/west partition in genetic variation or the four currently recognised management units are supported. If genetic variation is predominantly differentiated east/west, then the location of kiwi sanctuaries for North Island brown kiwi, which are all located in the west of the North Island, may need to be reconsidered.

*Molecular dating of kiwi*

Both of the methods previously used to date divergence times in kiwi have problems. The standard avian rate of 2% sequence evolution per million years was calculated from flying birds that are distantly related to kiwi. An alternative rate involved calibration of the clock with an emu fossil and extrapolation over tens of millions of years to kiwi. The use of these rates produced very different divergence times for kiwi lineages. Determining a rate of evolution specifically for kiwi by using DNA sequences from radiocarbon dated bones would allow a more accurate estimation of kiwi divergence dates. An ancient DNA rate would also provide a useful comparison with the previously determined rate from Adélie penguins (Lambert et al., 2002), which is considerably higher than other avian rate estimates (Garcia-Moreno, 2004). Twelve of the kiwi bones examined in this study had associated dates, all less than 3000 years BP. Bayesian phylogenetic analysis was used to calculate an evolutionary rate estimate for kiwi mitochondrial control region DNA sequences using BEAST version 1.2 (Drummond and Rambaut, 2003). The results indicated that more sequences with reliable dates would be required to obtain a rate. Indeed, Lambert et al. (2002)

examined DNA sequences from 96 bones up to 6000 years old in order to generate a rate for penguins and Shapiro et al. (2004) included 220 dated bison bones.

*DNA from cultural artefacts*

The retrieval of DNA from the kiwi feathers attached to Maori cloaks and kete indicated that DNA-based methods could potentially be an important method for identifying biological components of cultural artefacts. Obviously this method could be extended to other Maori kiwi feather artefacts, particularly those located in overseas museums that are targets for repatriation. This technique could also be applied to artefacts constructed from the feathers of other bird species, both from New Zealand and overseas. However, in many cases feathers can be assigned to species by morphology alone and this would be preferable to damaging priceless taonga (Maori treasures). Furthermore, the level of genetic structuring among populations of brown kiwi may prove to be exceptional since birds usually show low levels of phylogeographic structure (Avise, 2000). Therefore, determining precise geographic origin for artefacts from other bird species may not be possible. The verification of ancient DNA from feathered artefacts also needs consideration. Feather bases are small and each individual feather can only be sampled once. Therefore, an approach similar to that used to verify DNA sequences from sediment samples may be required (i.e. large numbers of amplifications performed in two separate DNA laboratories and the overall concordance between sequences examined; e.g., Willerslev et al., 2003).

*Phylogenetic relationships of the New Zealand wattlebirds*

The relationships among the three New Zealand wattlebird genera, which appear to have diverged in a relatively short period of time, could be examined with additional DNA sequence. Saturation was clearly a problem with the mitochondrial sequences (cytochrome b, ND2, 12S) used in this study (Chapter Seven). In contrast, the nuclear sequences, RAG-1 and *c-mos*, showed no saturation (data not shown), but also exhibited no parsimony informative sites between the three genera. Sequences are required that are evolving at an appropriate rate to recover the short internal branch between the three New Zealand wattlebird genera. Intron sequences have been shown to have a slower rate of evolution (Shapiro and Dumbacher, 2001; Driskell and Christidis, 2004) making them more appropriate for resolving deeper divergences owing to lower levels of homoplasy. However, such sequences are also likely to lack

variation over short time-scales, so a large amount of sequence would be required. This may prove difficult with the degraded DNA from the extinct huia.

The extinct New Zealand endemic piopio (*Turnagra* spp.), whose phylogenetic position is still controversial (Worthy and Holdaway, 2002), may have affinities to the New Zealand wattlebirds. Previous morphological studies suggested a close relationship with the cnemophiline birds of paradise (Olsen et al., 1983), with which New Zealand wattlebirds group in molecular studies. The relationship of piopio to the passerines has previously been examined with cytochrome b sequence (Christidis et al., 1997). However, few outgroups were included, and the use of cytochrome b to examine higher level systematic relationships in birds has been controversial (Edwards and Boles, 2002). Nuclear gene sequences (e.g., from *c-mos*, RAG-1 and RAG-2) may provide a better understanding of the relationships of this extinct family within the passerines, and may potentially provide a more suitable outgroup for the New Zealand wattlebirds.

*The effect of genotyping errors on the detection of population genetic structure*

Genotyping errors (allelic dropout: ADO; false alleles: FA) are particularly problematic for genetic identification because one error in a multilocus genotype creates a false individual and leads to overestimates of population size (Creel et al., 2003). Therefore, most studies of genotyping errors have focussed on this problem in relation to individual identification. The impact of genotyping errors on estimates of population structure has not been rigorously tested (Manel et al., 2005). Computer simulated genotype data with varying levels of ADO and FA would be desirable for assessing the impact of genotyping errors on population structure.

*Population genetics of huia*

Fast evolving mitochondrial control region DNA sequences may reveal matrilineal structuring in huia. Preliminary data from 200 bp section of domain III of the control region were amplified from five huia samples using primers designed from an unpublished huia sequence available from GenBank (accession number AY433205). Four of the generated huia sequences were identical and one sequence showed a 1 bp transition (Shepherd, unpublished data). However, the generated sequences differed extensively in one region from the huia sequence available on GenBank. Further study using longer sequences and/or different regions (e.g., domain I of the control region)

may exhibit higher levels of variation and thus give an indication of genetic structuring of mtDNA in huia.

*Studies of the New Zealand fauna with ancient DNA*

This study demonstrates that large-scale studies of New Zealand taxa with ancient DNA are possible. The temporal aspect provided by ancient DNA has offered considerable insight into the evolutionary histories of a number of New Zealand taxa. Therefore, ancient DNA methodology has the potential to increase and transform understanding of both extant and extinct New Zealand animals.

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APPENDIX A.

## Details of kiwi samples used in this study.

**Table 1.** Ancient samples used in this thesis that yielded DNA. Museum abbreviations: CM - Canterbury Museum, MNZ – Te Papa Tongarewa Museum of New Zealand, WM – Waitomo Museum. THW = held by Trevor Worthy.

| Species | Sample identifier in thesis | Sample Type | Musuem | Museum Number | Locality |
|---|---|---|---|---|---|
| North Island brown kiwi | 32 | Toe pad | MNZ | DM10276 | Waverley, coll. 1960 |
| | 33 | Toe pad | MNZ | DM9375 | Ohakune, coll. 1960 |
| | 34 | Toe pad | MNZ | DM13551 | Waitotara Valley, Wanganui, coll. 1968 |
| | 35 | Bone | WM | W080 | Maniapoto cave, Te Kuiti. |
| Rowi | 36 | Bone | MNZ | S.24355.1 | Perini Creek, Buller Gorge |
| | 37 | Bone | MNZ | S.24355.2 | Perini Creek, Buller Gorge |
| | 38 | Bone | THW | - | Takaka fossil cave, Takaka |
| | 39 | Bone | MNZ | S.23211 | Takaka Hill |
| | 40 | Bone | CM | AV16697 | Kiwi Hole, Caanan, Takaka |
| | 41 | Bone | MNZ | DM7869 | Martinborough |
| | 42 | Bone | MNZ | DM7896 | Martinborough |
| | 43 | Bone | MNZ | DM7900 | Martinborough |
| | 44 | Bone | MNZ | S.009401 | Poukawa |
| Tokoeka | 45 | Toe pad | CM | AV588 | Te Anau, coll. 1894 |
| | 46 | Toe pad | CM | AV589 | Te Anau, coll. 1894 |
| | 47 | Bone | MNZ | S.34531 | Castle Rock, Southland |
| | 48 | Bone | MNZ | DM6489 | Castle Rock, Southland |
| | 49 | Bone | MNZ | DM6555 | Castle Rock, Southland |
| | 50 | Bone | MNZ | DM6557 | Castle Rock, Southland |
| | 51 | Bone | MNZ | DM6498 | Castle Rock, Southland |
| | 52 | Bone | CM | AV12651 | Mt Somers Quarry, Sth Canterbury |
| | 53 | Bone | MNZ | S.33369.1 | Holocene Hole, Mt Cookson |
| | 54 | Bone | MNZ | S.33369.2 | Holocene Hole, Mt Cookson |
| | 55 | Bone | MNZ | S.33369.3 | Holocene Hole, Mt Cookson |
| | 56 | Bone | MNZ | S.33369.5 | Holocene Hole, Mt Cookson |
| | 57 | Bone | MNZ | S.33369.4 | Holocene Hole, Mt Cookson |

| | | | | | |
|---|---|---|---|---|---|
| Great spotted kiwi | 58 | Bone | MNZ | S.23187 | Imperial Jade Cave, Charleston |
| | 59 | Bone | CM | AV19163 | Charleston |
| | 60 | Bone | MNZ | S.34491 | Takahe Tomo, Mt Arthur |
| Little spotted kiwi | 61 | Bone | CM | AV22817 | Helectite Hole, north of Karamo, Raglan |
| | 62 | Bone | MNZ | S.24478 | Conoor |
| | 63 | Bone | WM | WO255 | Ann's Cavern, Stubb's farm |
| | 64 | Toe pad | MNZ | NM23036 | Banjo Creek, Westhaven Inlet, NW Nelson, coll. 1978 |
| | 65 | Bone | CM | AV16713 | Cave at Canaan, Takaka |
| | 66 | Bone | MNZ | S.27784.1 S.27784.2 | Earl Grey Cave, Takaka Hill |
| | 67 | Toe pad | MNZ | S.1174 | Otuhuhu or Rough River (Westland), coll. 1952 |
| | 68 | Toe pad | MNZ | S.23043 | Smyth River, South Westland, coll. 1978 |
| | 69 | Toe pad | MNZ | S. 22007 | Karangarua River, South Westland, coll. 1894 |
| | 70 | Toe pad | MNZ | S.2069 | Lake Manapouri, coll. 1888-96 |
| | 71 | Bone | CM | AV32392B | Cave at Springhill, Southland |
| | 72 | Bone | MNZ | DM6672 | Castle Rocks, Southland |
| | 73 | Bone | CM | AV25301 | King's Cave, South Canterbury |
| | 74 | Bone | CM | AV12648C | Limestone fissure, Mt Somers Quarry |
| | 75 | Bone | MNZ | S.33365 | Holocene Cave, Mt Cookson |
| | 76 | Bone | CM | AV25141 | West Coast, South Island |

**Table 2.** Modern kiwi samples sequenced and/or genotyped in this study and their collection details, where known.

| Species | Sample identifier in thesis | Sample type | Specimen code | Collection details |
|---|---|---|---|---|
| North Island brown kiwi | 77 | Feather | NTU75 | Opotiki, Bay of Plenty coll. R. Burns |
| | 78 | Footpad | 33806 | Opotiki, Bay of Plenty coll. R. Burns |
| | 79 | Blood | CD886 | Ex. Houpoto, Bay of Plenty (20.10.84) coll. B. Reid and A. Billing |
| | 80 | Blood | CD891 | Ex. Whakatane, Bay of Plenty (20.10.84) coll. B. Reid and A. Billing |
| | 81 | Blood | CD1212 | Ex. Whakatane, Bay of Plenty (3.7.85) coll. C. Daugherty |
| | 82 | Blood | CD890 | Ex. Te Puke, Bay of Plenty (20.10.84) coll. B. Reid and A. Billing |
| | 83 | Blood | CD887 | Ex. Whakatane, Bay of Plenty (20.10.84) coll. B. Reid and A. Billing |
| | 84 | Blood | CD889 | Ex. Gisborne, Hawke's Bay (20.10.84) coll. B. Reid and A. Billing |
| | 85 | Blood | CD1210 | Ex Wairoa, Hawke's Bay (15.6.85) coll. C. Daugherty and B. Reid |
| | 86 | Blood | CD963 | Ex. Wairoa, Hawke's Bay (7.11.84) coll. S. Triggs, S. Walker, B.Reid, M. Bell, I. Bryant. |
| | 87 | Blood | CD882 | Maungataniwha, Hawke's Bay (18.10.84) coll. B. Reid and A. Billing |
| | 88 | Blood | CD1295 | Urewera National Park, Hawke's Bay (6.2.86) J McLennan and C. Daugherty |
| | 89 | Blood | 74 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 90 | Blood | 52 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 91 | Blood | 51 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 92 | Blood | 82 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 93 | Blood | 53 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 94 | Blood | 56 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 95 | Blood | 58 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 96 | Blood | 59 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 97 | Blood | 64 | Tangiteroria, Northland 1986-7 coll. M. Potter |

| | | | | |
|---|---|---|---|---|
| | 98 | Blood | 65 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 99 | Blood | 83 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 100 | Blood | 79 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 101 | Blood | 78 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 102 | Blood | 86 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 103 | Blood | 81 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 104 | Blood | 71 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 105 | Blood | 73 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 106 | Blood | 84 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 107 | Blood | 68 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 108 | Blood | 72 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 109 | Blood | 75 | Tangiteroria, Northland 1986-7 coll. M. Potter |
| | 110 | Blood | R47372 | Coromandel |
| | 111 | Blood | - | Coromandel (10.5.94) |
| | 112 | Footpad | Inky | Tongariro coll. P. Morton |
| Rowi | 113 | Footpad | R57618 | Okarito coll. C. Rickard, R. MacCallum |
| | 114 | Footpad | R34152 | Okarito coll. S. Anderson |
| | 115 | Footpad | R55382 | Okarito coll. C. Rickard |
| | 116 | Footpad | R57626 | Okarito coll. S. Anderson |
| | 117 | Footpad | - | Okarito coll. S. Anderson |
| | 118 | Feather | R55111 | Okarito |
| | 119 | Feather | R55116 | Okarito |
| | 120 | Feather | R55126 | Okarito |
| | 121 | Feather | R55308 | Okarito |
| Tokoeka | 122 | Feather | - | Fiordland |
| | 123 | Feather | - | Fiordland |
| | 124 | Blood | CD1147 | Stewart Island (30.4.84) coll. S. Triggs, B. Reid, T. Reid, P. Garland, I. Adams, A. Richardson |
| | 125 | Feather | TR | Haast (21.11.02) coll. C. Wickes |
| | 126 | Feather | Slip | Haast (7.1.03) coll. C. Wickes |
| | 127 | Feather | Winding Glen | Haast (18.12.02) coll. C. Wickes |
| | 128 | Feather | LTF RVA | Haast (20.1.03) coll. C. Wickes |

| | | | | |
|---|---|---|---|---|
| | 129 | Feather | Bilbo | Haast coll. C. Wickes |
| | 130 | Feather | Kahu | Haast coll. C. Wickes |
| | 131 | Feather | Tahi | Haast coll. C. Wickes |
| | 132 | Footpad | - | Haast coll. C. Wickes |
| Great spotted kiwi | 133 | Blood | CD830 | Barrytown, Paparoas coll. B. Alexander, L. Alexander, L. Williams, R. Simpson |
| | 134 | Blood | CD1203 | No locality, coll. B. Reid, P. Walker, I. Bryant, M. Thomson, C. Daugherty? |
| | 135 | Blood | CD1205 | No locality, coll. B. Reid, P. Walker, I. Bryant, M. Thomson, C. Daugherty? |
| | 136 | Blood | GS13 | Ugly River (6.12.91) coll. J. McLennan |
| | 137 | Blood | M1 | Heaphy (April, 1987) coll. J. McLennan |
| | 138 | Blood | F3 | Heaphy (April, 1987) coll. J. McLennan |
| | 139 | Blood | MOP M | Gunner Downs (23.1.92) coll. J. McLennan |
| | 140 | Blood | GS12 | Ugly River (5.12.91) coll. J. McLennan |
| | 141 | Blood | GS17 | Ugly River (10.12.91) coll. J. McLennan |
| | 142 | Blood | GS14 | Ugly River coll. by J. McLennan. |
| | 143 | Blood | M3 | Heaphy, coll. April 1987 by J. McLennan. |
| | 144 | Blood | FT2921 | Kahurangi (10.10 90) coll. J. McLennan. |
| | 145 | Blood | GS21 | Taramakau River, Arthurs Pass coll. J. McLennan. |
| Little spotted kiwi | 146 | Blood | CD899 | Kapiti Island (27.10.84) coll. B. Reid and M. Finglan. |
| | 147 | Blood | CD1206 | Kapiti Island (28.5.85) |
| | 148 | Blood | WS1764 | D'Urville Island |

**Table 3.** Details of previously published kiwi sequences obtained from GenBank used in this study. Note that some sequences are found in more than one individual. However, frequencies of haplotypes were not reported in Baker et al. (1995) and Burbidge et al. (2003).

| Species | Haplotype identifier in thesis | Control region GenBank accession number | Cytochrome b GenBank accession number | Collection location |
|---|---|---|---|---|
| North Island brown kiwi | 1 | AY150609 | U28707 | Northland |
| | 2 | AY150610 | U28707 | Northland |
| | 3 | AY150611 | U28708 | Northland |
| | 4 | AY150611 | U28707 | Northland |
| | 5 | AY150617 | U28702 | Little Barrier Island |
| | 6 | AY150617 | U28701 | Little Barrier Island |
| | 7 | AY150618 | U28700 | Little Barrier Island |
| | 8 | AY150614 | U28710 | Bay of Plenty |
| | 9 | AY150612 | U28709 | Bay of Plenty |
| | 10 | AY150617 | U28702 | Taranaki |
| | 11 | AY150620 | U28700 | Taranaki |
| | 12 | AY150618 | U28698 | Taranaki |
| | 13 | AY150621 | U28700 | Taranaki |
| | 14 | AY150623 | U28707 | Taranaki |
| | 15 | AY150616 | U28712 | Hawke's Bay |
| | 16 | AY150615 | U28711 | Hawke's Bay |
| Rowi | 17 | AY150627 | U28697 | Okarito |
| | 18 | AY150624 | U28697 | Okarito |
| | 19 | AY150626 | U28697 | Okarito |

| | | | | |
|---|---|---|---|---|
| Tokoeka | 20 | AY150632 | U28703 | Haast |
| | 21 | AY150629 | U28703 | Haast |
| | 22 | AY150631 | U28703 | Haast |
| | 23 | AY150635 | U28705 | Fiordland |
| | 24 | AY150633 | U28706 | Fiordland |
| | 25 | AY150637 | U28706 | Fiordland |
| | 26 | AY150640 | U28713 | Stewart Island |
| | 27 | AY150638 | U28714 | Stewart Island |
| | 28 | AY150639 | U28715 | Stewart Island |
| | 29 | AY150639 | U28716 | Stewart Island |
| | 30 | AY150639 | U28717 | Stewart Island |
| | 31 | AY150638 | U28698 | Stewart Island |
| Great spotted kiwi | 149 | AY150641 | U28704 | - |
| Little spotted kiwi | 150 | AY150642 | U28699 | - |

APPENDIX B.

# Published manuscripts