Conservation Genetics of the Weka, *Gallirallus australis*

By
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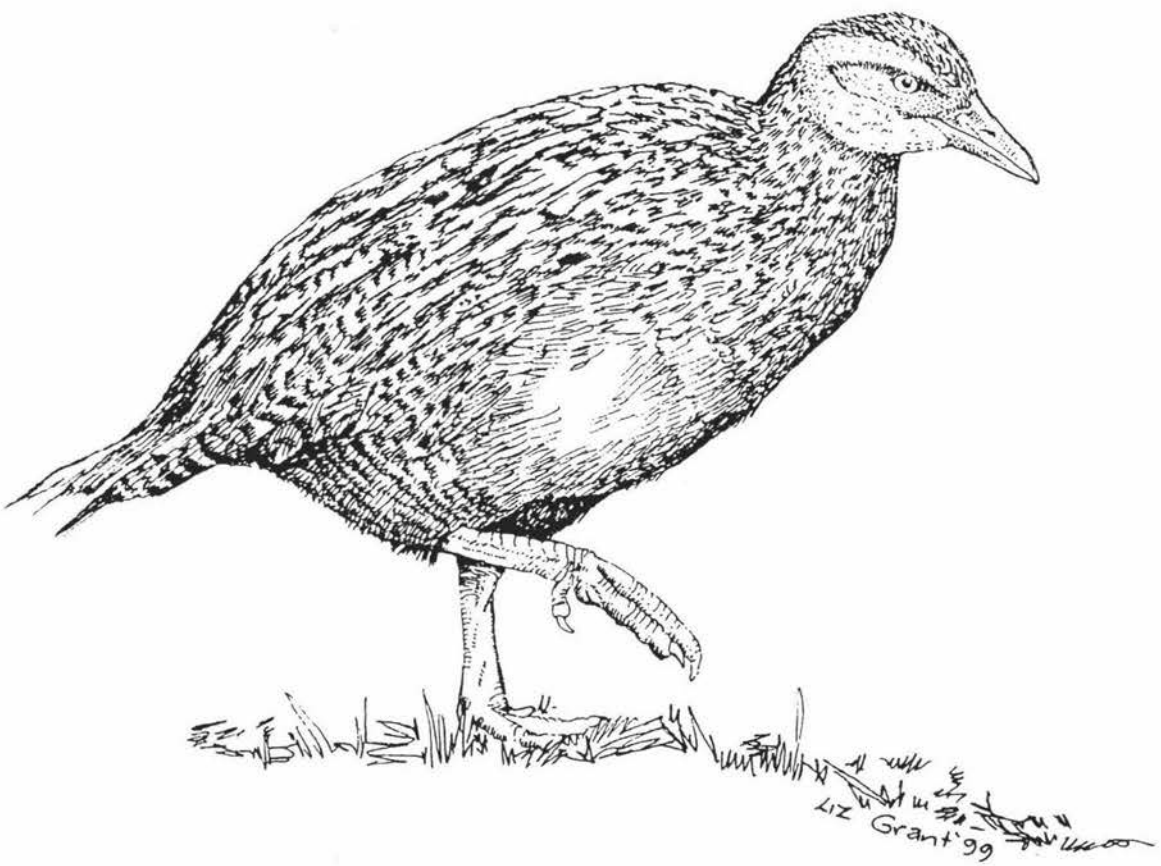

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Abstract

The weka (*Gallirallus australis*) is an endangered, flightless rail endemic to New Zealand. This ground-dwelling bird has four morphologically recognised subspecies and although was once distributed throughout New Zealand, its survival is now threatened.

Genetic methods were employed to investigate aspects of weka biology relating to their conservation in order to determine if the current taxonomy reflects the genetic diversity of weka and if the provenance of weka could be determined. These results are important as they will impact on the conservation management of weka in the future.

Weka genetic diversity was investigated by sequencing four mitochondrial DNA genes: cytochrome *b*, ATPase 6, 12S and the control region. The only region that was found to exhibit variation was the control region. Through phylogenetic analysis of a 216 base pair region, the subspecific status of some weka populations was determined. The provenance of weka of unknown origin was also able to be ascertained. Nuclear variation was investigated through a preliminary study of microsatellite variation.

Molecular sexing methods were trailed to sex this monomorphic species. These results are discussed and compared with the traditional methods of sexing weka through morphometrical techniques. It was found that molecular and morphological sexing techniques produced concordant results when sexing adult weka. Molecular sexing has an advantage over morphometric sexing as it better able to accurately sex individuals of all ages.

The relationship that the terminology of "subspecies" has on conservation management is also discussed with particular reference to the weka.

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Photo by Geoff Moon

Chapter 1

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1.1 INTRODUCTION

This chapter achieves seven tasks. Firstly, it provides a description of the weka and its ecology. Secondly, it reviews the relationship of weka to other Rallidae. Thirdly, it looks at the historical taxonomy of the weka. Fourthly, it examines the past and present distribution of the weka. Fifthly, it examines in detail the causes of decline of weka. Sixthly, it describes the significance of weka, both culturally and ecologically. Lastly, the aims of the entire study are highlighted.

The weka (*Gallirallus australis*) is a large brown flightless rail (Family Rallidae, in the Order Gruiformes) that is endemic to New Zealand. Weka can occupy a wide variety of habitats including rocky shores, sand dunes, sub-alpine grasslands, forests, scrublands and wetlands. (King et al., 1999). In some areas, weka utilise highly modified habitats, even semi-urban situations, where they depend on a diversity of vegetation types, particularly seral weeds with a high number of fruit bearing shrubs (Bramley, 1994).

The diet of weka consists mostly of invertebrates that dwell in soil and leaf litter, and fruit. They also take lizards, snails, rodents, and the eggs and young of ground nesting birds and carrion (King et al., 1999). Weka have been seen to take Fiordland Crested Penguin eggs and chicks (St Clair and St Clair, 1992) and snails (Beauchamp et al., 1993). Food scraps may also be an important diet item for birds at some sites, such as urban Gisborne and parts of Kawau Island (Beauchamp, 1997a).

The ultimate size of an individual weka is likely to depend on a variety of dietary factors including food types and availability during the growth period, the individual's behaviour during parental care (especially its responsiveness to its parents), the rate and timing of the start of food collection by the young bird, and the genetic and epigenetic characteristics of the individual (Beauchamp, 1987a). Consequently, the mean weights of weka vary with location. For example, in Westland, the mean weight for males was 1250 grams and for females 850 grams (Coleman et al., 1983). In contrast, on Kapiti Island, the mean weights were 813 grams and 590 grams for males and females respectively (Beauchamp, 1987a).

Weka have the ability to build up and store adequate fat reserves to live through two months of extreme food shortage and longer periods of low food availability. These reserves can be used for survival or to boost reproductive output (Beauchamp, 1987a). These fat storage levels are far higher in weka than the levels attained by migratory flighted birds prior to migration (Brackenbury, 1984).

The breeding success of weka is closely related to food supply and the associated ability of birds to put on weight beforehand. Breeding can occur year round if food is highly abundant. The productivity of pairs varies markedly from site to site and year to year with an average standard deviation of 0.3 to 3.45 young raised in a year (Beauchamp, 1987a). Up to six eggs may be laid in a clutch and one pair of weka has been recorded raising 14 young in a year (King et al., 1999). Weka are capable of breeding at a faster rate than other extant New Zealand flightless birds (Gorgas, 1968).

Typically weka display biparental care of young with both the male and female birds sharing the incubation and rearing (Miller and Moulette, 1985). Weka incubate eggs for 26-28 days and females tend to do a higher proportion of the daytime incubation (Beauchamp, 1987a). The young leave their parental territories when the parents are no longer interested in feeding them, or they have developed independent feeding methods, usually aged between 40 and 105 days. Immediately after leaving the parental territory the juvenile weka often lose weight as they move into unknown areas and run into conflict with other weka. A high proportion of these weka may die from starvation (King et al., 1999). Those young weka that survive are able to breed as young as five months old in areas of low population density (Graeme, 1994).

Weka breeding habits are predominantly monogamous (Beauchamp, 1987b). However, there have been indications of polygamous association in weka. These associations are frequent in some species of gallinules (Craig, 1974; Ripley, 1977; Goldzien et al., 1993), and polygamy, or helping, has been observed in other species, such as the pukeko (Craig, 1974). There have only been two conclusive reports of a non-monogamous association in weka, both reports having one male and two female weka. The first was by Guthrie-Smith (1914) and the second by Beauchamp (1986).

There is a male-biased sex ratio among weka in some areas (Coleman et al., 1983; Brothers and Skira, 1984; Beauchamp, 1997b). Brothers and Skira (1984) thought this bias was likely to be due to behavioural differences between the sexes but it is not immediately obvious why this should be. Coleman et al. (1983) postulated that disease may be responsible for the male-biased sex ratio. A male-biased sex ratio is common amongst other rails also (Craig, 1974; Ripley, 1977; Miller and Moulette, 1985 and Goldzien et al., 1993).

1.2 RELATIONSHIP OF WEKA TO OTHER RALLIDAE

Most authors consider the weka to have evolved from a common ancestor with the *philippensis* group, believing the weka lineage became flightless after dispersal throughout New Zealand (Olson 1973; Flemming, 1979). Olson (1973) grouped the weka and eleven other living or recently extinct South Western Pacific and

South East Asian rails in the genus *Gallirallus* based on its plumage colour, patterning, and skeletal features. Olson considered that the nearest members of the group are the Pro - *Rallus* of the Australasian, South Atlantic and Madagascan regions, for example, *Dryolimnas* and *Atlantisia.* Ripley (1977), in a monograph of the family, considered the wekas' distinctive size, general appearance, and neotonic characteristics to be sufficient to separate it into the genus *Gallirallus.* He placed all other rails that Olson (1973) placed in *Gallirallus* into the genus *Rallus,* and he split the Pro- *Rallus* complex into *Canirallus, Rallus* and *Atlantisia.*

The general view is that the *Gallirallus* group arrived in New Zealand from Australia more recently than 80 million years ago (Sibley et al., 1983). This view is based on three assumptions. Firstly, the similarity of the New Zealand *Gallirallus* species with *Gallirallus* species outside New Zealand suggests they have not been separated long (Sibley, 1982). Secondly, their late presence in the New Zealand fossil record. Thirdly, the late ages of separation and hence the arrival of other species perceived, by their uniqueness, to have been in New Zealand before *Gallirallus* (Beauchamp, 1997a). The oldest known weka bones have been dated at 25,000 years old using the C^{14} Accelerated Mass Spectrometry (AMS) (Milliner, 1982).

Three sub-fossil species of rails have been described in the genus *Gallirallus* in New Zealand as *Gallirallus minor* (Hamilton, 1893), *Gallirallus harteeri* (Scarlett, 1970) and *Gallirallus insignis* (Olson, 1977). Olson (1973) thought that the first two of these species represented stages in the increase of size of the weka from a *philippensis* ancestor. Olson (1977) later postulated that *Gallirallus minori* may represent the lower size limit of a past, more variable, morph of the weka. He also argued that the current size range of the weka is a result of preferential predation on the former smaller individuals in the range (Olson, 1977).

Beauchamp (1987a) disagreed with Olson's theory of preferential predation on *Gallirallus minor.* Instead he postulated that it is possible that mainland populations have gone through periods where small individuals were produced and even favoured. These periods occurred when populations went through times of restricted food availability or when food was periodically restricted during the

period of growth prior to ossification. Beauchamp (1987a) believed that this is a more likely reason for the smaller size of the sub-fossil form, *Gallirallus minor.*

Beauchamp's (1987a) theory was based on the fact that the Kapiti Island population produced smaller individuals in periods of lower food availability. Beauchamp presumed that these smaller individuals require less energy for maintenance and during good conditions are able to put on weight faster and utilise fat reserves at lower rates then larger wekas. However, he considers that they are disadvantaged in disputes with larger wekas and hypothesizes that within each population of weka, the size of the individuals appears to be set by a trade off between the energetic capacity of the individual to survive and reproduce, and the capacity of the territorial individuals to survive the conflict period before and during territorial establishment.

1.3 TAXONOMY OF WEKA

Historically, weka were split into six different taxa (Buller 1877; Oliver 1955). There are now four geographically separated sub species recognised (Kingsky, 1970; Turbott, 1990). The taxonomic distinction and distribution of each weka subspecies, except for the North Island form, has always been a matter of conjecture (Buller, 1877; Newton, 1896; Oliver, 1955; Marchant and Higgins, 1993).

Current taxonomy recognises one species of weka with four subspecies: North Island weka *Gallirallus australis greyi,* western weka *G. a. australis,* buff weka *G. a. hectori* and Stewart Island weka *G. a. scotti.* All weka subspecies are currently listed as Category B according to the Department of Conservations' ranking system designed by Molloy and Davis (1994). A ranking of Category B entitles weka to second priority in conservation action. However, the International Union for the Conservation of Nature uses a different ranking system, the IUCN Red List Categories. This system ranks the North Island weka as 'Critically Endangered', the western weka and buff weka as 'Endangered' and Stewart Island weka as 'Vulnerable'. The IUCN Red List rankings suggests a more drastic approach to the conservation of weka needs to be taken.

Below is a brief description of the four subspecies of weka. Table 1.1 displays a complete list of morphometric distinctions between the subspecies.

The North Island weka (*G. a. greyi*) is reddish on its flanks and rump, with its head, neck, back and wings blotched with black. It has black quills banded with reddish brown and the tail feathers are black with a reddish edge. It has a reddish brown band across its breast, and red to reddish brown irises. It also has a reddish brown bill and feet.

The western weka (*G. a. australis*) has three colour phases. The grey phase is similar to North Island weka. The chestnut phase is much like the North Island weka, but darker on the breast and lower abdomen. During its other phase, the black phase, the upper surfaces are brown black, with each feather edged with a blackish chestnut. The quills are brownish black with narrow chestnut bars. It has a grey throat with a streak above the eye and has a grey-brown patch on the upper abdomen.

The buff weka (*G. a. hectori*) has a yellowish buff upper surface, which is longitudinally spotted with brownish black. Its tail feathers are reddish with central and lateral black lines. It has a light grey throat and streak over the eye. The breast is a tawny colour with brown speckles.

The Stewart Island weka (*G. a. scotti*) also has three phases also. The first phase is grey and is similar to the North Island weka. The chestnut phase is similar to that of the western weka, but its irises are light chestnut and its bill and feet pinkish brown. The black phase is paler than the black phase of the western weka. It has large black centres and dark chestnut margins on its upper surface and breast feathers, and its throat and abdomen are dark brown.

There is substantial debate over whether or not these categories reveal subspecies, species or are indicative of environmentally induced colour variation (King et al., 1999; Beauchamp, 1987a; Oliver, 1955). Recent allozyme investigations of weka populations throughout the species' range have only identified one enzyme allele

difference between the North Island and the South Island/Stewart Island weka (Miskelly et al., 1993).

Table 1.1 Morphological variation of the four subspecies of *Gallirallus australis*. (From: King et al., 1999)

Feature	*G. a. greyi*	*G. a. hectori*	*G. a. australis*	*G. a scotti*
Colour	Grey	Yellowish Buff	Grey (G), Chestnut (C), Black (B)	Grey (G), Chestnut (C), Black (B)
Crown	Rufous-brown feather margin black centre	Narrow yellow brown or rufous brown feather margin	Brown rufous edges (G), red brown feather margin (C), dark reddish brown (B)	Brown rufous edges (G), red brown feather margin (C), dark reddish brown (B)
Supercilium	Base of upper mandible to side of nape. Light grey with pale buff anterior, broadening posteriorally.	Upper lores to side of nape. Light grey with faint light brown	Base of upper mandible to side of nape. Light grey with tinge pale buff lower margin in front of eye (G), narrower than G, pale rufous brown front to obscure behind (C), none (B)	Base of upper mandible to side of nape. Light grey with tinge pale buff lower margin in front of eye (G), narrower than G, pale rufous brown front to obscure behind (C), none (B)
Facial Stripe	Narrow in front than behind eye, extends over eye, generally extending to neck	Brown flecked black, less distinct posteriorally merging to neck	Broader under eye than *greyi* but not over it. May not extend beyond ear merging into neck	Broader under eye than *greyi* but not over it. May not extend beyond ear merging into neck
Front between wings and minor wing coverts	Rufous brown to rich brown	Narrow pale yellow and some olive or buff edges	Rufous brown to light brown edges (G), narrow rich brown to rufous brown edges (C), very narrow russet brown edges (B)	Rufous brown to light brown edges (G), narrow rich brown to rufous brown edges (C), very narrow russet brown edges (B)
Breast band	Dark reddish brown to brown, some with olive to yellowish brown flecks and darker brown centres to feathers	Brownish olive grading to straw yellow edges. Some with olive to buff flecks	Yellow brown and brown - dark reddish brown, some with darker brown indistinct centres (B). G and C olive buff and yellow brown flecks on feathers of 60-70% (G) and less (C)	Generally rich brown and rufous brown (G) and (C). Some with darker brown indistinct centres (G) and (C), lack marginal flecks (G) and (C)

Feature	*G. a. greyi*	*G. a. hectori*	*G. a. australis*	*G. a scotti*
Back band	Absent to broad olive brown to reddish brown band, many not clearly defined, can be mottled rich brown on edges and no spotting	Present, brown with olive tinge spotted with black, yellow brown on edges, some with scattered olive and buff spots	Present indistinct to pronounced. Brown grading rufous brown edges giving streaked appearance or brown with brown buff spots giving mottled appearance (G), rufous brown mottled black centre (C)	Always present and broad and pronounced in adults (G) and (C)
Lower breast	Grey to brown grey	Light grey to olive buff	Brown grey (G), red brown similar to upper parts (C), dark olive brown faintly grayer than rest of body (B)	Brown grey (G), red brown similar to upper parts (C), dark olive brown faintly grayer than rest of body (B)
Femoralis	Feathers never fully barred, occasionally with olive flecks	Boldly barred black brown and pale brown	Variable browns barred with black brown to dark brown (G), dark brown or rufous brown with black bars (C), black with russet to rufous brown fringes to feathers, no bars (B)	Variable browns barred with black brown to dark brown (G), dark brown or rufous brown with black bars (C), black with russet to rufous brown fringes to feathers, no bars (B)
Retrice	Brown margin, never barred	Black broadly edged and barred rufous brown at base and edged brown distally	Rufous brown with black centers barred black (G), rufous brown black barred (C), black with very narrow rufous or russet brown margin proximally (B)	Rufous brown with black centres barred black (G), rufous brown black barred (C), black with very narrow rufous or russet brown margin proximally (B)
Under tail coverts	Black rachis and notching, notched rufous brown brown, with distinct to no opposite or alternate baring in black brown	Rufous brown boldly barred black	Rich brown to rufous brown barred with black (G), rufous brown boldly barred black (C), black narrowly and indistinct bar rufous brown (B)	Rich brown to rufous brown barred with black (G), rufous brown boldly barred black (C), black narrowly and indistinct bar rufous brown (B)
Underwing greater and median coverts	Rufous brown to brown with or without grey black bars	Grey black with rufous brown barring at tips of feathers	Rufous brown with subdued black barring (G), grey black tipped and distally barred rufous brown (C), black tipped with russet brown (B)	Rufous brown with subdued black barring (G), grey black tipped and distally barred rufous brown (C), black tipped with russet brown (B)

1.4 PAST AND PRESENT DISTRIBUTION OF WEKA

Human colonisation of the Pacific has had a massive impact on its avifauna. This colonisation is believed to be associated with the probable loss of up to 1000 species of rails (McGlone et al., 1994). Three species of flightless rails of the *Gallirallus* group survived this process of which the weka is the only one that is still found in New Zealand (Olson, 1973).

The exact nature of the distribution and density of weka populations in most of New Zealand before and during European settlement is difficult to establish (Oliver, 1955). Turbott (1967) suggests at the time of European contact, weka were found over much of the North Island, South Island and Stewart Island, but at differing densities. Weka were also found on some offshore islands, less than a kilometre from the mainland, but were said to be absent from many islands futher than a kilometre offshore in 1840 (Atkinson and Bell, 1973).

Weka distribution changed dramatically after human colonisation of New Zealand. They were introduced to islands surrounding Stewart Island and in Foveaux Strait by Maori muttonbirders as a source of food (Miskelly, 1987). The Government Steamer *Hinemoa* also transported weka to other islands as a food source for shipwrecked sailors (Falla, 1937; Brothers and Skira, 1984).

By the end of the 1880s weka were still well established on the margin of European settlement but fluctuated wildly in numbers (Bramley, 1994). Now weka have disappeared from over 80% of their former mainland distribution (Miskelly et al., 1993) Translocations and local extinctions have produced a highly disjunct distribution, with over 20 populations that are now considered to be reproductively isolated (King et al., 1999).

There is evidence that some offshore islands (eg Kapiti Island) that presently hold reasonably large and seemingly stable populations of weka lacked them at the time of European arrival in New Zealand. As ancestors of the weka are known to have been in New Zealand for over 25 thousand years (Milliner, 1982), and all of these islands would have been connected to the mainland in the recent ice ages (Williams, 1981), it is expected that some of these islands in the past would have had

populations of weka. The reasons for their disappearance from these islands is not well understood, although on Kapiti there is evidence that the island habitat was almost entirely burnt by Maori (MacMorran, 1977). Futhermore, Beauchamp (1987a) postulated that Kapiti Island may not have been in the climatic zone that allowed the existence of wekas throughout some of the past 25,000 years.

1.4.1 North Island Weka

The North Island weka, once distributed throughout the North Island, are now confined to a few small populations on the mainland in Opua, Rawhiti and Poverty Bay (Beauchamp, 1987a). The North Island weka has been introduced to several islands, Rakitu, Kawau, Pakatoa, Mokoia, and Kapiti Islands, where they are reproducing and surviving at a higher rate than on mainland New Zealand (Beauchamp, 1987b).

Originally, the North Island weka appeared to benefit from European settlement with the conversion of original forests to mixed forest and grassland, producing good habitat for weka (Buller, 1876; Moncrieff, 1928). However by the 1920s, weka had declined to extinction in all but Poverty Bay and Northland. Between 1939 and 1953 the Northland weka population bacame extinct, and North Island weka became restricted to Poverty Bay. The Poverty Bay population expanded dramatically between 1853 and 1981, but then, went into a sharp decline, and the numbers are still very low today (Bramley, 1996).

In urban Gisborne, between the 1960s and 1970s, there were so many weka that they caused problems, for example, they became a pest at rubbish dumps and rubbish bins. This resulted in some of the weka being shifted away (Bramley, 1994). In the 1980s, the Gisborne population declined to only fifteen pairs of birds. Although the resident birds were breeding and producing chicks at a higher rate than the required recruitment, their young were not surviving beyond 3-4 weeks of age (King et al., 1999).

During the period from 1950 to the 1970s there were over 100 liberations of weka in the North Island. Most of these were unsuccessful. Unfortunately, only limited information describing these failures was gathered. Two of these translocations

(Rawhiti and Opua) were initially successful, but their numbers have been in decline since 1990-1994 (Beauchamp, 1997a). For example, the Rawhiti population has declined from approximately 400 birds in 1987 to just two birds in 1999 (King et al., 1999). Recent translocations of weka to sites in the North Island are still having difficulty in establishing a stable population. For example, a recent translocation of captive bred birds to Karangahake failed due to high levels of predation (Graeme, 1994).

An important factor implicated in the failures of attempts to re-establish weka in the North Island is the remarkable homing instinct of the adult weka. A weka taken from Gisborne to Hawkes Bay walked 130 km home (Graeme, 1991). Captured Gisborne weka were banded and released in the Waitakere Ranges, west of Auckland. Three weeks later, one of these banded birds was killed on the road at Tanetua, more then 300 km south of Auckland, on course for Gisborne (Graeme, 1994).

Translocations of North Island weka to offshore islands proved to be much more successful in establishing stable and reproducing populations. Translocated populations currently exist on Kawau (unknown number transferred in 1976), Rakitu (13 weka in 1951), Mokoia (13 in 1958), Pakatoa (10 in 1997) and Kapiti Islands (unknown date and number) (King et al., 1999). Despite water barriers the homing instinct has continued to be a problem to their sucessful relocation (Wright, 1981). For example, weka transferred from Maud Island to the mainland "swam" a kilometre back to the island (Graeme, 1991). There have also been reports of weka translocated from Codfish Island to Stewart Island, surviving the stormy seas and tide rip and "swimming" three kilometres back to Codfish Island (Graeme, 1991).

1.4.2 Western Weka

The western weka was historically distributed over the western part of the South Island from the Marlborough Sounds to Southland. They died out in South Westland in the 1930s and 1940s leaving only a small population that survived in the Copland Valley (Beauchamp, 1987a). Today, the western weka is the most abundant form of all weka subspecies, both numerically and in distribution, occuring in the Marlborough Sounds (including D'Urville and other islands),

Golden Bay, Northwest Nelson, Buller, North Westland south to Ross, Copland Valley and in Fiordland. Western weka have been introduced to islands in the Marlborough Sounds and also possibly to the Open Bay Islands off Haast, Solander Island and islands off Stewart Island (Oliver, 1955).

Although the western weka is fairing better then the North Island weka they are still vulnerable. For example, in the mid 1980s the northern Golden Bay weka experienced a 'catastrophic' decline (King et al., 1999). The reason for this decline is not known and it is likely to be multifactorial.

Presently, as with the North Island weka, the western weka island populations such as on the islands in the Chalky, Milford and Marlborough Sounds, are doing much better than the mainland populations, with the exception of the isolated mainland population of Copland Valley (King et al., 1999).

1.4.3 Stewart Island Weka

Stewart Island had a viable population of weka up until the 1960s. Today, weka are very scarce on Stewart Island due to predation, comprising no more then fifteen pairs (King et al., 1999). Stewart Island weka are present on many of the islands surrounding Stewart Island, such as on the islands in Paterson Inlet and Port Pegasus. They have also been introduced to at least twelve muttonbird islands, plus Codfish and MacQuarie Islands (Veitch and Bell, 1990).

In 1980, one thousand Stewart Island weka were liberated from Codfish Island to Stewart Island, but the release did not establish a viable population. The failure of this translocation is thought to be the result of high predation rates on the weka. Despite the weka's threatened status, the Department of Conservation has removed Stewart Island weka from Awaiti, Codfish, Herekopare and Kundy Islands, although weka have been reintroduced to Herekopare by muttonbirders (Veitch and Bell, 1990). Weka were removed due to suspected predation of chicks and young of other bird species by weka (Ward et al., 1992).

1.4.4 Buff Weka

The buff weka formerly occurred in the eastern districts of the South Island (Bell, 1992). However, the buff weka suffered badly in the conversion of tussock grasslands to European pasture in Canterbury and Otago and population numbers dropped. The main decline of buff weka was in 1918 and coincided with a major snowfall (Stead, 1927). Buff weka persisted on the mainland until 1927 (King et al., 1999).

Fortunately, 12 buff weka were released at Te One, Chatham Island in 1905 where they flourished (Ward et al., 1992). In 1961, buff weka were sucessfully released from the Chathams onto Pitt Island (Bell, 1992). The population on Pitt Island still exists, however this is a much smaller population than the Chatham Island population. The Department of Conservation has plans to eradicate this Pitt Island population of weka in the future (King et al., 1999). Three attempted transfers from Chatham Island back to Arthur's Pass, Mackenzie Basin and Peraki Bay have been unsuccessful (Bell, 1992).

The Chatham Island still has a large population of buff weka which appears to sustain a loss of approximately 5000 indivduals a year. It is estimated that 2000-3000 weka are eaten, 1000-2000 are killed through dog/sport kills, 500 weka are killed on roads and 800 are trapped by the Department of Conservation in the Tuku Valley during localised predator control to benefit taiko and parea (Ward et al., 1992).

1.5 CAUSES OF DECLINE OF THE WEKA

The majority of the recently extant flightless birds lived on islands throughout the world (Williams, 1981). Most of these species have been found to be very vulnerable to the introduction of predators, and have become extinct soon after the arrival of humans and other mammals (Diamond, 1981). Consequently, there has been a dramatic reduction in the formerly extensive world-wide flightless bird fauna, including ratites and some rails, geese, swans, ducks, pigeons, grebes, cormorants, loons, parrots, ibises and owls (Beauchamp, 1987a). During the past 500 years, at least 53 species of flightless rail have existed, representing over a quarter of the species that have been described in the family Rallidae (Diamond,

1981). These 53 species have now been reduced to 16 island forms and one continental form over this period (Milliner, 1982).

Weka are one of the few flightless terrestrial rails that has survived in the presence of introduced predators, however their numbers are dropping rapidly (Milliner, 1982). There are several factors that have led to the significant decline of weka. The main factors include predation by introduced mammals, competition for food sources by introduced mammals, changes in land use, disease and parasites, weather extremes, road kills, and poison drops to control introduced predators. These factors are discussed in detail below.

Predators are known to have threatened several rail populations. Predation by rats (*Rattus* spp.) has been blamed for the extinction of the Laysan rail (*Porzana palmeri*) (Ripley, 1977). Together with cats (*Felis catus*), the introduction of rats to the Chatham Islands is thought to have spelt the end for the Chatham Island Banded rail (*Rallus modestus*) (Ripley, 1977). Predators are also apparently responsible for the restricted distribution of other rails, such as the Lord Howe Island rail (*Gallirallus sylvestris*) (Ripley, 1977). The Adabran rail (*Canirallus cuvieri*) which now only exists on cat-free islands, and the Guam rail (*Rallus owstoni*) are suffering in the presence of cats, rats and the introduced tree snake (*Boiga irregularis*) (Haig et al., 1994).

Predation by introduced predators such as cats (*Felis catus*) (King, 1995), dogs (*Canis familiaris*) (King, 1995), ferrets (*Mustela furo)* (King, 1984), stoats (*Mustela erminea*) (Philpott, 1918), and harrier hawks (*Circus approximans)* (Bramley, 1994) are known to impact on weka (Bramley, 1996). As weka are ground-dwelling and have evolved without mammalian predators, they do not have well developed predator defences. Weka often stop their attempt to escape predators once they reach cover and take no further steps to protect themselves, thus they become easy prey (Bramley, 1994).

A recent study (1992-1994) in the Gisborne area showed predation by ferrets to be a major cause of death of adult weka. Ferrets and cats have had the most important impact on chick survival (Bramley, 1996). An experimental removal of cats and

ferrets from several weka territories was associated with an increase in weka productivity (Bramley, 1996). Predation by wild cats, domestic cats and dogs is thought to have caused the decline of weka on Stewart Island, as there are no mustelids present (Ward et al., 1992).

There have been reports of significant predation on weka nests. The identity of the predator involved has eluded researchers (Bramley, submitted to Journal of Avian Biology). It is thought that a combination of hedgehogs *(Erinaceus europaeus occidentalis)* and rats is most likely to be responsible for egg losses (Bramley, submitted to the Journal of Avian Biology).

While predation continues to be a major problem, competition with introduced mammals is also thought to have a significant effect on weka populations (Bramley, 1994). Potential 'new' direct competitors with weka include browsing mammals (deer, possum and wallaby) feeding on shoots and fruits, rodents competing for the fruits and invertebrates, while introduced birds add futher strain on the fruit supply, and wasps on the invertebrates (Ward et al., 1992). It is not only such direct impacts that pose a problem to the weka, mammals impact on forest composition and regeneration and this results in a flow on impact to the weka habitat and food source. For example, on Kawau Island, wallabies (*Wallabia bicolor*) tend to reduce soil accumulation, leaf litter, ground cover and understorey diversity, leading to a reduced food supply for weka and increasing the susceptibility of weka to drought (King et al., 1999).

Land use changes have also had direct impacts on weka (Bramley, 1994). Initially the introduction of high fruit producing weed species and introduced invertebrates were thought to benefit weka (King et al., 1999). However, the removal of thickets and weed communities (gorse and blackberry) from riparian strips and forest remnants, the under-grazing of those remnants, the burning of logs, and spraying of weed species on farmland and roadside verges, has turned much of the New Zealand landscape into unsuitable habitat for weka (Bramley, 1994; Ward et al., 1992).

Disease and parasites have also been implicated as playing an integral part in the decline of weka populations (Beauchamp, 1997b). Occurrences of fungal infections, avian pox (especially in captivity) and tick infestations have all been recorded. Recently, disease has been associated with the loss of weka in good condition on Kawau Island. However, the exact cause of death was not identified, as some potential mortality factors, like viral infection, were not investigated (Beauchamp, 1997a). Aspergillosis and coccidiosis have been important aviary diseases of weka chicks, and young were also lost to avian pox in the captive North Island Breeding recovery programme (Beauchamp, 1997b).

Droughts are also a factor associated with the loss or decline of some populations of weka. Periods of low rainfall, or other weather extremes, will always have been an occasional factor affecting weka, but their impacts are potentially much greater now because of changes in habitat caused by humans and introduced mammals (Beauchamp, 1997a). Droughts reduce the number and distribution of soil and litter dwelling invertebrates and wet weather reduces the availability of litter dwelling invertebrates. Also, frost kills open pasture invertebrates. These factors can dramatically change food availability from a feast to a famine in days. As a result of these factors, weka may be placed under sufficient stress, resulting in mass death encompassing all age groups (Ward et al., 1992).

The effect of future climate change on weka will depend on the degree of any limiting resources, and the effects on the various weka populations may be density dependent. The most important factors appear to be food and water availability, and changes in the ecological balance that may allow density independent factors, like predator switching by mustelids to bird prey, to dominate (King et al., 1999). King et al (1999) believe that it is probably too early to say if droughts are increasing as a result of human-induced changes to climates (such as the greenhouse effect).

Road kills of weka is also another major cause of death (Bramley, 1994). In a study in the Rakauroa/Matawai area, vehicles ranked with ferrets as a major cause of death in weka. Road kills also occur throughout other parts of New Zealand where weka survive, however this number is decreasing concurrently with the decrease in weka surviving on the mainland (Bramley, 1994).

Pest control operations have also had a negative and positive impact on weka. The ground foraging habit of weka makes them vulnerable as a non-target species for pest animal control operations (Stephenson, 1998). During sodium monofluoroacetate (1080) drops targetting possum, the direct effect on weka is death by poisoning, however, the rate of this occurring is believed to be low (King et al., 1999). The 1080 drops also result in a reduction of the numbers of invertebrates on which weka feed. These effects are thought to be temporary, and it is believed that the long term benefits of these poison drops for weka will be substantial, as the forest condition is gradually improved due to the reduction in possum numbers (Ward et al., 1992).

Aerial broadifacoum (3-{3-(4'-bromobiphenyl-4-yl)-1,2,3,4-tetrahydro-1-naphthyl}-4-hydroxycoumarin) operations to eradicate rodents from islands have significant direct impacts on weka, so much so that broadifacoum has been used to assist in the intentional eradication of weka populations (Ward et al., 1999). For example, on Nukuwaita island all weka were killed as the result of a broadifacoum poison drop (King et al., 1999). The susceptibility of weka to poison drop operations has resulted in their removal to captivity during the poisoning programme, and returned later. The long term benefits to weka populations in the eradication of rodents, is thought to be reduced competition (King et al., 1999).

While the benefits of such pest control operations are in their long term potential, there is a problematic by-product. Pest control operations at Rawhiti-Puekura Bay has resulted in an increase of mustelids. Mustelids have been known to affect weka populations greatly by predation on weka chicks and their young (Bramley, 1996). Other poisons (e.g. pindone, slug pellets) and traps are also considered to pose some threat to weka (Bramley, 1994).

1.6 THE CULTURAL AND BIOLOGICAL SIGNIFICANCE OF WEKA

Weka are of particular importance to some Maori iwi (Ward et al., 1992). It is believed that Maori admired the weka for their incurable and feisty personalities. Unfortunately, it is these characteristics that enabled them to be easily caught as they were not shy of humans, consequently, they were used as a source of food,

perfume, oil to treat inflammations, feathers in clothing, and for lures to catch dogs (Beattie, 1995, as cited in King et al., 1999). South Island Maori made capes by sewing together weka skins (Keating, 1977). The legal cultural harvest of weka continues to be a significant activity on the Chatham Islands today (King et al., 1999).

Weka were so integral in the life of Maori that proverbs were written about them: *Ka motu te weka i te mahanga, e kore a muri e hokia.* Meaning that when a weka breaks from a snare, it won't be back (Bateman, 1996). This highlights the curious yet intelligent nature of these birds. Weka were also taken by dogs, sometimes by hand and by *tawhiti* traps, which comprises a noose at the end of a rod (Keating, 1977). Figure 1.1 depicits an attempt to catch a weka via a *tawhiti* trap.

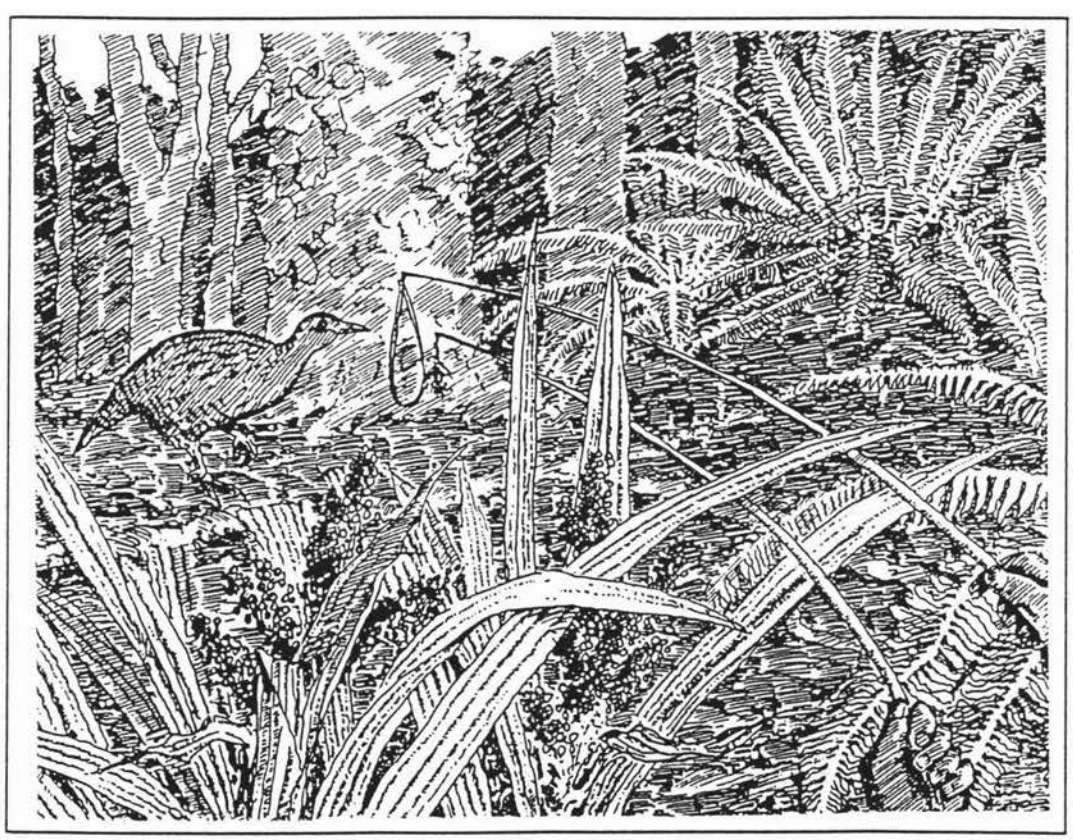

Figure 1.1 Drawing of a weka snare (From Oliver, 1974).

Weka were also frequently encountered and utilised by the early European explorers and settlers who gave them the name 'bush hen' (King et al., 1999). Weka were well known for their cheeky and curious nature by the European explorers. They were described by Captain Cook as "... so very tame or foolish as to stand and stare at us till we knocked them down with a stick. Most of them are of a dirty black or a dark brown colour, and eat very well in a pie or fricassee." (as cited in Oliver, 1974)

The weka were also a welcome article of food to the European explorers, settlers, gold diggers and prospectors of New Zealand. They caught weka with their dogs and after eating the meat they used the oil for cooking, as an embrocation for bruises and rheumatism, and for other purposes where grease was required (Oliver, 1974).

Weka also benefit New Zealand's biodiversity. Weka are one of the few remaining large birds that distribute the seeds of plants so are particularly significant as facilitators of forest regeneration (Ward et al., 1992). These benefits will vary in the habitats in which the weka are established. For example, weka in open farmland eat grass grub (*Costelytraa* spp.), black cricket (*Teleogryllus commodus*) and other invertebrate pests. They are also capable of distributing seeds of native and introduced plants which add to the diversity of farmland habitats. This is desirable for New Zealand's native seed eating birds and reptiles. The brushlands benefit from the spread of a substantial amount of the heavier seeds like hinau, toru, tawa and taraire in brushlands, as well as numerous small seed producing trees, such as *Coprosma* spp., *Pseudopanax* spp., mahoe and kawakawa (King et al., 1999).

Weka themselves are also effective predators. There are many cases where weka have been implicated in the decline of indigenous animals on islands where weka have been introduced. There are suspected and proven impacts of weka on species of sea birds, land birds, reptiles and invertebrates which have led to conservation agencies eradicating them on at least five islands (Veitch and Bell, 1990). Eradication has also been discussed for weka from at least ten other islands (Miskelly et al., 1993).

Weka have occasionally been seen killing lizards (Beauchamp, 1987a) as well as fallen nestling and ground-active young of the New Zealand Robin (*Petroica australis*), Whitehead (*Mohutta aibicila*), Saddleback (*Philesturnus caruculatus*), Californian Quail (*Callipepla californica*), Blue Penguin (*Eudyptula minor*), and Fiordland Crested Penguin (*Eudyptes pachyrhynchus*) (Beauchamp, 1996). There have been reports of weka killing adult birds, including Mottled Petrel (*Pterodroma inexpectata*), House Sparrow (*Passer domesticus*), Song Thrush (*Turdus philomelos*) and small mammals including stoats (*Mustela erminea*), and kiore (*Rattus exelans*) (Beauchamp, 1996). Weka have been reported to take eggs of many ground nesting birds (Jolly, 1989) and frogs (Beauchamp, 1996). However, most of the records of weka feeding on vertebrates are based on gizzard and faecal analysis and therefore it can not be determined whether the vertebrates were killed by the weka or eaten as carrion (Beauchamp, 1996).

Weka have been removed from Codfish Island because they were killing Mottled Petrel, a species threatened with extinction (Beauchamp, 1996). In the early 1980s western weka were removed from Maud Island, Marlborough Sounds, by the Wildlife Service to protect the Stephens Island frogs (*Leiopelma hamiltoni*) (Green, 1988). This was despite no relationship between weka foraging behaviour or predation on frog demography being established, and to date, this relationship still has not been established (Beauchamp, 1996). More recently, weka were removed from the Chetwode Islands, in the Marlborough Sounds, in preparation for the re-introduction of Stephens Islands frogs and other species. These weka eradications are controversial, especially as the concern over the ability of weka to survive long-term on the New Zealand mainland is very realistic.

1.7 AIMS OF THIS STUDY

There are four main aims of this study. The first aim is to use genetic methods to investigate aspects of weka biology related to their conservation in order to determine if the current taxonomy reflects the genetic diversity of weka. This is important as the results will impact on what level of protection weka are given. The second aim is to determine if the provenance of weka introduced to islands such as Kapiti, Chetwode, Open Bay, Solander and Codfish Islands can be established. This is important as these populations may be able to replenish dwindling mainland populations and also to provide information for ongoing eradications of weka on islands. The third aim is to determine whether weka are able to be genetically sexed and to compare these results with morpometric sexing results. Sexing weka from an early age is important to assist in captive rearing, as pair bonds are formed at an early age. The fourth aim is to examine how the commonly used terminology "subspecies" impacts on the conservation management of endangered species in general, and in relation to weka.

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Chapter 2

MtDNA and Avian Conservation Genetics

2.1 INTRODUCTION

This chapter achieves six goals. The first goal is to provide an overview of mtDNA. Second, to look at the novel gene order of avian mtDNA. Third, to explore the inheritance of mtDNA. Fourth, to review size differences of mtDNA in avian species. Fifth, to look at the evolution of mtDNA. Lastly, to look in detail at the two main regions of the mitochondrial DNA genome used in conservation genetics, the cytochrome *b* gene and the control region.

One of the main challenges in conservation genetics is being able to identify potential conservation units or evolutionary significant units (Woodruff, 1989). A number of powerful molecular methods have been developed that are able to quantify the amount and distribution of genetic variation within and between populations of plants and animals. The appropriate method to use depends on the level of resolution required, the related extent of financial expenditure and the biology of the organism under investigation. For a detailed discussion of the range of molecular markers available for use in conservation management of New Zealand's threatened flora and fauna see Lambert and Millar (1995).

The analysis of animal mtDNA polymorphisms represent the most commonly used means for revealing potential conservation units among closely related species, and among populations of the same species (Avise and Lansman, 1983; Moritz et al., 1987; Avise et al., 1987; Wilson et al., 1985; Amos and Hoelzel, 1992; Barrowclough, 1982). This is because mtDNA often exhibits considerable variation among individuals, both within and between populations, therefore providing an effective marker for intra-specific geographic variation. Examples of studies include the kiwi (Baker et al., 1995), humpback whales (Baker et al., 1990), wolves (Wayne and Jenkins, 1991), ducks (Avise and Ball, 1990), geese (Shields and Wilson, 1987), sparrows (Avise and Neilson, 1989), tits (Mack et al., 1986), white eyes (Degnan and Mortiz, 1992), dunlin (Wenick et al., 1994) and auks (Moum et al., 1994). As mtDNA variants are often population specific they can be particularly useful for determining the provenance of individuals or populations of unknown origin.

MtDNA is generally considered to be inherited maternally (Giles et al., 1980), with females transmitting many copies to each offspring. Giles et al. (1980) estimated that an egg contains approximately 100,000 mitochondria and the spermatozoon cell contains only about 50 mitochondria. The mitochondrial genome is maintained in a high copy number in every cell in the body, including germ cells. The cytoplasmic inheritance of the mitochondria means that usually the sperm does not contribute any mtDNA to the zygote (Giles et al., 1980).

Mitochondria are cytoplasmic organelles which provide energy for eukaryotic cells. Within each mitochondrion is a double-stranded molecule of DNA which is a covalently closed, circular molecule between 15,000-20,000 base pairs long. It has remarkable gene conservation across animals and contains 2 ribosomal RNA (rRNA), 22 transfer RNA (tRNA), 13 protein coding genes and a noncoding segment about 1000 base pairs long called the control region (Avise, 1987) (refer figure 2.1). The thirteen proteins are cytochrome *b*, two of the subunits of ATPase synthetase, three subunits of cytochrome c oxidase, and six subunits of NADH

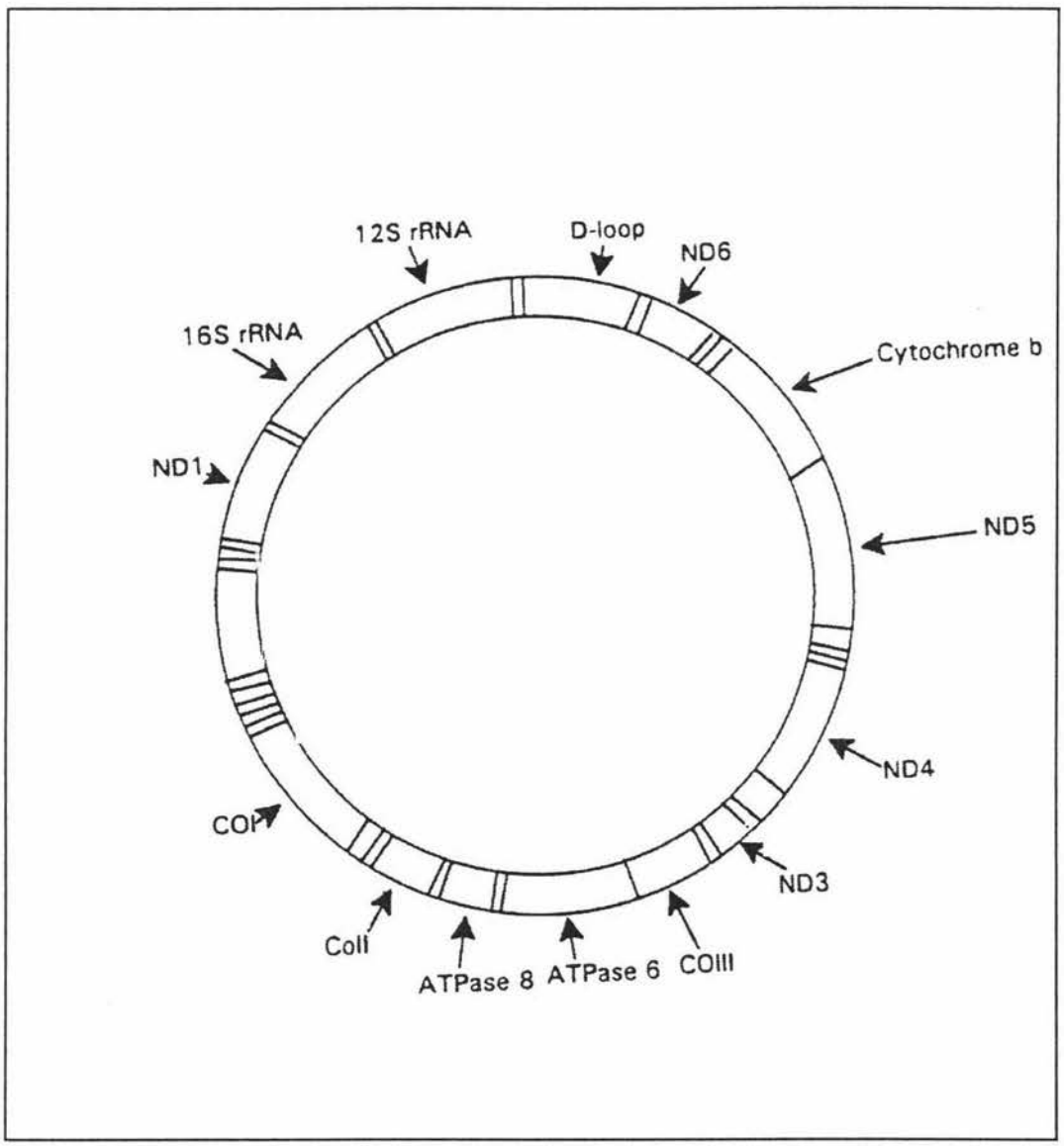

Figure 2.1 Map of the avian mitochondrial DNA genome

dehydrogenase and there is one unassigned reading frame (URF). The mtDNA gene structure is simple in comparison to nuclear genes due to the absence of introns, repetitive DNA, pseudogenes or transposable elements (Avise et al., 1987).

In order to synthesise proteins, mitochondria require ribosomes and a full complement of tRNAs to synthesise proteins. To use all of the codons, making

allowance for wobble in the third position of the codon, it was expected that 32 different tRNAs would be required. However, the mitochondrial translation apparatus has a slightly different codon usage to the cytoplasmic system and uses a more extensive wobble ("superwobble"). This superwobble enables them to incorporate all the amino acids with only 22 tRNAs (10 fewer tRNAs then expected) (Irwin et al., 1991).

Since DNA-sequencing methods had been developed in the mid-1970s, the small size of the mitochondrial DNA has made them attractive subjects for sequencing. The first complete sequence to be resolved was that of the human mitochondrial genome (Anderson et al., 1981). Since then many vertebrate mitochondrial genomes have been sequenced, including that of the domestic fowl (Desjardins and Morais, 1990) and much of the quail (Desjardins and Morais, 1991) and the duck (Ramirez et al., 1992).

2.2 NOVEL GENE ORDER OF AVIAN MtDNA

In 1990, Desjardins and Morais reported that chicken mtDNA has a novel gene order among vertebrates. The contiguous tRNAglu and ND6 genes are located immediately upstream of the control region, instead of the contiguous tRNApro, tRNAthr, and cytochrome *b* genes in other vertebrate mitochondria (Anderson et al., 1981) (refer figure 2.2). This gene rearrangement is presumed to be indicative of the mitochondrial genome in birds (Ramirez et al., 1992).

Quinn and Wilson (1993) believed that this altered gene arrangement, in comparison to other vertebrates, provided the opportunity to gain new insights on (1) the effect that gene order has on evolutionary constraints in a tightly packed genome where the tRNA sequences can act as punctuation marks during processing of the primary transcript, (2) the mechanisms involved in gene rearrangements, and (3) phylogenetic relationships across widely divergent taxa. Possible interpretations of the altered gene order in birds is that it has resulted from intergenic recombination, or that gene duplication to subsequent deletions generated new arrangements (Quinn and Wilson, 1993).

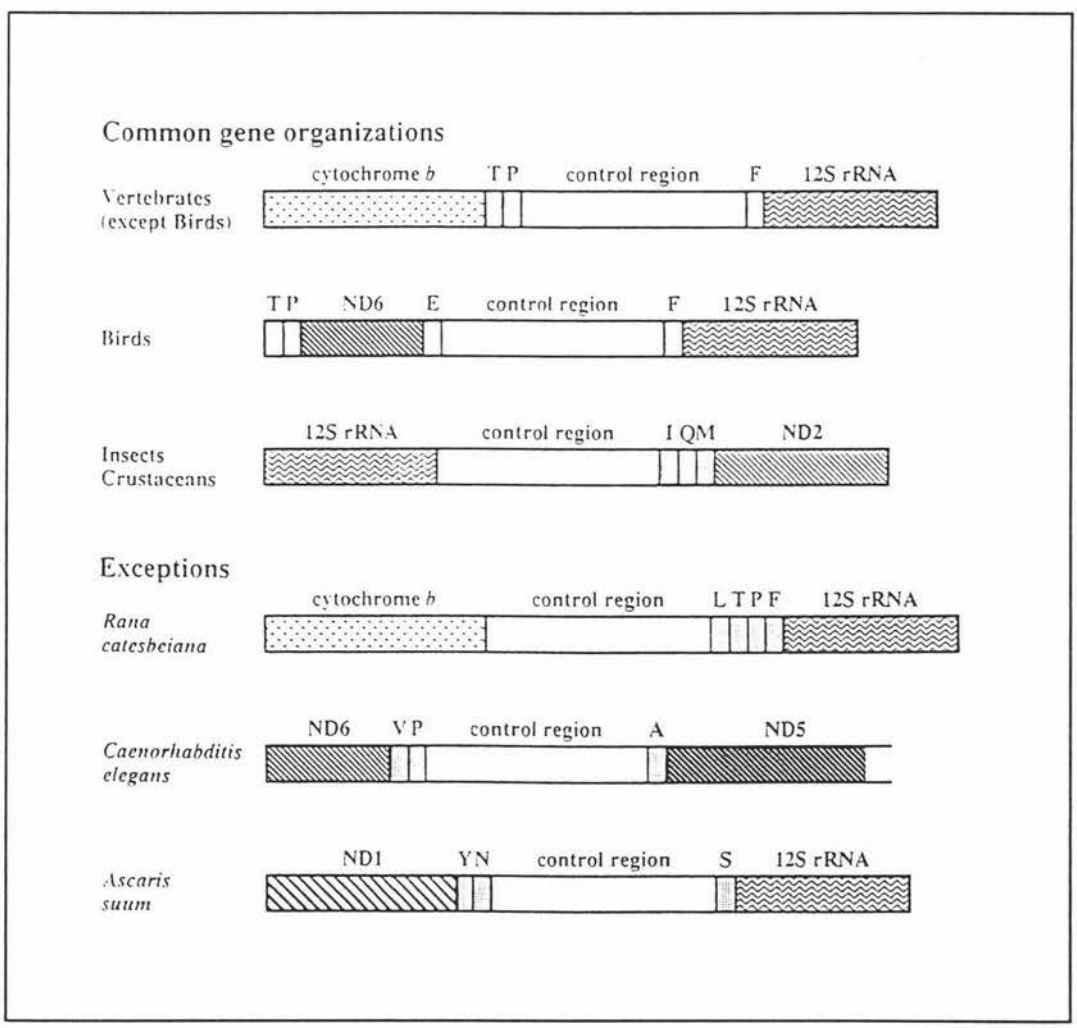

Figure 2.2 Different gene organizations found around the control region in animal mtDNA. The three commonly observed gene organisations are given, as well as three exceptions. Knowledge of the gene order is necessary to design new primers when no sequence data are available for the considered species. (From: Taberlet, 1996).

2.3 INHERITANCE OF MtDNA

MtDNA is thought to be inherited without recombination and is therefore able to be interpreted as an historical record of molecular changes within a lineage (Avise et al., 1987). Changes within a mitochondrial lineage arise when a single molecule within a single cell lineage mutates. If fixation of the variant occurs in the population of molecules and in the population of organisms, a new mtDNA variant arises (Avise et al., 1987).

It is possible for an individual to contain two or more different variants of mtDNA. When this occurs it is called heteroplasmy and it can result from three different situations. Firstly, heteroplasmy may occur during fixation of a new mtDNA variant, where an individual contains copies of mtDNA from both cell lineages (i.e. the original and the variant). This, however, is rare. The second and more common occurrence of heteroplasmy is due to length variations. More often than not this length variation is located within the control region in vertebrates or the A-T rich control region equivalent in invertebrates (Harrison, 1989; Wilson et al., 1985). The third method of heteroplasmy is due to a phenomenon called paternal leakage. This is when paternal mtDNA is inherited at varying frequencies. Gyllenstein et al. (1991) reported low frequencies of paternal inheritance in mice and Zouros et al. (1992) have found extensive paternal inheritance of mtDNA in marine mussels, *Mylilus*. Heteroplasmy resulting from paternal leakage of mtDNA is more likely to be based on base pair differences than on length variation (Kondo et al., 1992). Generally, paternal leakage is rare.

There are mechanisms in some species that ensure that paternal inheritance does not occur. For example, paternal mitochondria failing to enter the sperm in crayfish, being destroyed in the zygote in sea urchins, being diluted out in xenopus and being discarded from the sperm before it enters the egg as in ascisians (Birky, 1991).

Mitochondrial heteroplasmy has also been identified in birds. This was identified through the use of RFLP analyses in rails (*Rallus elegans* and *R. longirostris*) (Avise and Zink, 1988) and the Mourning Dove (Ball and Avise, 1992). The rails

and the Mourning dove were heteroplasmic for two to three discrete length classes, which differed in size by more than 100 base pairs.

For heteroplasmy to persist through generations or become fixed in a population, it must be contained in the mtDNA of the germ cells, not only in the somatic cells which may result from events in the individual's life. It is important to keep in mind the possibility of paternal leakage when applying mtDNA variation to population studies, particularly when heteroplasmy is observed.

2.4 SIZE DIFFERENCES IN MtDNA

Large size differences occur in the mitochondrial genome between species, however they mostly code for the same genes. The difference between the larger and smaller genomes is mainly in the amount of non-coding DNA present. The vertebrate mitochondrial genomes, which are among the smallest, contain only a very small proportion of non-coding sequences. The human mitochondrial genome contains 16 569 base pairs, of which 15 368 base pairs code for RNA or proteins. Of the remaining 1201 base pairs, a substantial proportion is the specific sequences of the replication origin, and only about 90 base pairs are apparently genetically unimportant material. The genetic material in mtDNA genomes is used very economically, containing no introns, leader sequences or intergeneic sequences (Avise et al., 1987).

The majority of sequence changes that occur in mtDNA can be attributed to point mutations, with transitions occurring more often than transversions. This is due to a mutation bias towards transitions (Brown et al., 1982). Other sequence changes in mtDNA do occur in the form of small additions and deletions. Large length differences do sometimes occur, but these are mainly confined to within the control region (Moritz et al., 1987).

2.5 EVOLUTION OF MtDNA

Evolution of the mtDNA nucleotide sequence is very rapid, up to ten times faster than that of single-copy nuclear DNA (Brown et al., 1979; Taberlet, 1996). This rapid evolution is due to a high rate of mutation, a high fixation rate or a combination of both (Brown et al., 1979). A number of factors result in the elevated rate of mutation in the mitochondrial genome. Firstly, mtDNA turnover is independent of cell division which results in a high turnover rate in mtDNA in comparison to nuclear DNA. That higher turnover rate leads to increased opportunities for replication errors. Secondly, mtDNA is believed to have an inefficient editing function, which could substantially increase the mutation rate even further (Brown et al., 1979). Amos and Hoelzel (1992) claim that mtDNA lacks an error detection mechanism that is present in the nucleus. Thirdly, oxygen radicals, which are highly reactive molecules, are known to damage DNA. As 90% of the oxygen in cells is used by the mitochondria, mtDNA is exposed to large quantities of damage causing oxygen radicals. Martin and Palumbi (1993) believe that in the process of repairing the damage, the rate of nucleotide substitutions could increase the rate of mutation even further.

The rate of fixation in populations is higher in mtDNA than in nuclear genes because mtDNA has an effective population size as little as one quarter of that for nuclear genes (Birky et al., 1983). This is because an individual will inherit mtDNA from only one of its grandparents, but nuclear DNA from all four (Brown et al., 1979). This is also dependent on other factors such as a skewed sex ratio.

Analysis of mtDNA where there have been multiple mutations at individual nucleotide sites can be complicated (Moritz et al., 1987; Avise et al., 1987). Nucleotide sites which evolve rapidly are likely to have undergone back-mutations at some stage due to the transition bias and the limited number of character states that are available (A, C, G or T). Due to the mutation levels, Avise et al. (1987) suggests treating the entire mtDNA molecule as a character, rather than a specific portion within the molecule.

2.6 COMMONLY USED MtDNA SEGMENTS USED FOR CONSERVATION GENETICS

2.6.1 Cytochrome *b*

The cytochrome *b* region of the mitochondrial genome is often used for phylogeographic, population structure and systematic studies of closely related species. Examples include shearwaters (Austin, 1996), kiwis (Baker et al., 1995) and pinnepids (Lento et al., 1995). Cytochrome *b* has also been used to study questions of deep phylogeny such as the origin of tetrapods (Meyer and Wilson, 1990). However, Edwards and Wilson (1990) warn that although informative, the number of silent substitutions displayed by cytochrome *b* alone is too small to be of rigorous use in studying within region or within population variation, and longer or more variable sequences, such as the control region, are likely to prove more valuable for this purpose.

Cytochrome *b* is particularly appropriate for studying phylogenetic relationships between vertebrates because it is a protein coding gene. It is one of the best known of the 9 or 10 proteins that make up complex III of the mitochondrial oxidative phosphorylation system, and the only one coded for by the mitochondrial genome. As a result its structural and functional relationships are well characterised (Hatefi et al., 1985). Complex III transfers electrons from the dihydroubiquitone to cytochrome c. This reaction is coupled with translocations of proteins across the mitochondrial inner membrane (Hatefi et al., 1985).

Being a protein coding gene means that cytochrome *b* is under reasonably tight structural and functional constraints, and thus variation in the base pair sequence is limited. Some areas of cytochrome *b* are subjected to more structural and functional constraints than others. In particular, part of the outer surface including the Q_o redox centre is more constrained than the transmembrane sequences and the surface on the mitochondrial matrix side (Irwin et al., 1991).

Most of the variation within cytochrome *b* is found to occur in the third position of codons. For example, 77% of variation occurs in third position codons in Australian song birds (Edwards and Wilson, 1990) and 88% in flightless rails (Trewick, 1997). The majority of variation occuring in the third position codon is typical for mitochondrial protein-coding genes (Brown et al., 1982; Thomas and Beckenbach, 1989).

Trewick (1997) found that the overall nucleotide composition of cytochrome *b* sequences are similar among rail taxa, with a light-strand bias towards cytosine derived principally from an excess at third codon positions. A strong bias against guanine and thiamine at third codon positions is also evident.

2.6.2 The control region

The control region is also called the "A-T rich region" for invertebrates and the "D-loop region" for vertebrates (although the D-loop does not encompass all of the control region). The control region is the major noncoding region of vertebrate mtDNA and known to be relatively variable in species, both in length and primary sequence (Cann and Wilson, 1983). The majority of variation in mtDNA is assigned to the control region (Avise et al., 1987; Harrison, 1989; Wilson et al., 1985; Kocher et al., 1989).

The control region has proven useful in revealing previously undetectable genetic structure within and among such closely related taxa as subspecies of dunlins (Wenick et al., 1994), populations and subspecies of grey-crowned babblers (*Pomatostomus temporalis*) (Edwards, 1993), and populations of lesser snow geese (Quinn, 1992).

In vertebrates, the control region varies in length from 0.73 kb (white sturgeon) to 2.1 kb (*Xenopus*) (Marshall and Baker, 1997). Within the avian control region there is a high frequency of length mutations. For example, the size difference observed in the control region of the chicken (1228bp) and Japanese quail (1153bp) is due to many small (1-3 bp) addition/deletion events and to a large 57 bp deletion that occurs at the 5' end of the Japanese quail control region.

Although the control region of the mitochondria is not a coding gene, it does contain a number of important elements including the origin of heavy strand (H-strand) replication, and initiation points for transcription. As it is a non-coding gene, it is not subject to the strict functional constraints that are evident in other areas of the genome, where sequence mutations may be fatal to the individual (Moritz and Brown, 1986). This lack of coding constraints means that the control region evolves up to ten times faster than the remainder of the mitochondrial genome (Wenick et al., 1994; Bibb et al., 1981; Greenberg et al., 1983; Brown et al., 1993).

Even though the control region is not subjected to the strict functional constraints of coding regions of the genome, it is the target site for numerous proteins and enzymes such as DNA and RNA polymerases (Irwin et al., 1991). It is also the target site for transcription and regulatory factors, and hence is subjected to evolutionary pressures. These factors however are all nuclear in origin, so the control region may be an interesting area to study the processes involved in nuclear/mitochondria coevolution (Saccone et al., 1991).

The control region can be split into three domains (refer figure 2.3). The left domain (or 5' end) containing one or more termination-associated sequences (TAS). Where the synthesis of the nascent heavy strand pauses, it contains a conserved sequence block (CSB-1) and the light and heavy strand promoters (Marshall and Baker, 1997). It is characterised by high adenine and low guanine content. The central conserved region, C-G rich, has been implicated in the regulation of the heavy strand replication. It is low in L-strand adenine and is responsible for the formation of the three-strand displacement loop structure (Clayton, 1991). The right domain (or 3' end) contains the site of initiation for heavy-strand replication (O_H), the promotors for both heavy and light strand replication and two or three short conserved sequence blocks (CSBs), (Brown et al., 1986; Southern et al., 1988; Saccone et al., 1991). The right domain is high in adenine, and tends to be the most variable domain and the one in which length variation due to repeat structure is found (Brown et al., 1986). The CSBs are thought to act as processing signals for the enzymes involved in the generation of RNA primers for heavy strand replication (Saccone et al., 1991).

The displacement loop (D-loop) represents the part of the control region ranging from the site of initiation for heavy strand replication (O_H) to the termination associated sequence (TAS). The D-loop corresponds to a three stranded structure during replication, made up of the two original strands and the nascent heavy strand (Clayton, 1982).

Brown et al. (1986) found this region in *Rattus* to be one of the most conserved regions of the mitochondria. This region may be functionally constrained, as it has a higher G content than any other part of the molecule (Saccone et al., 1991)

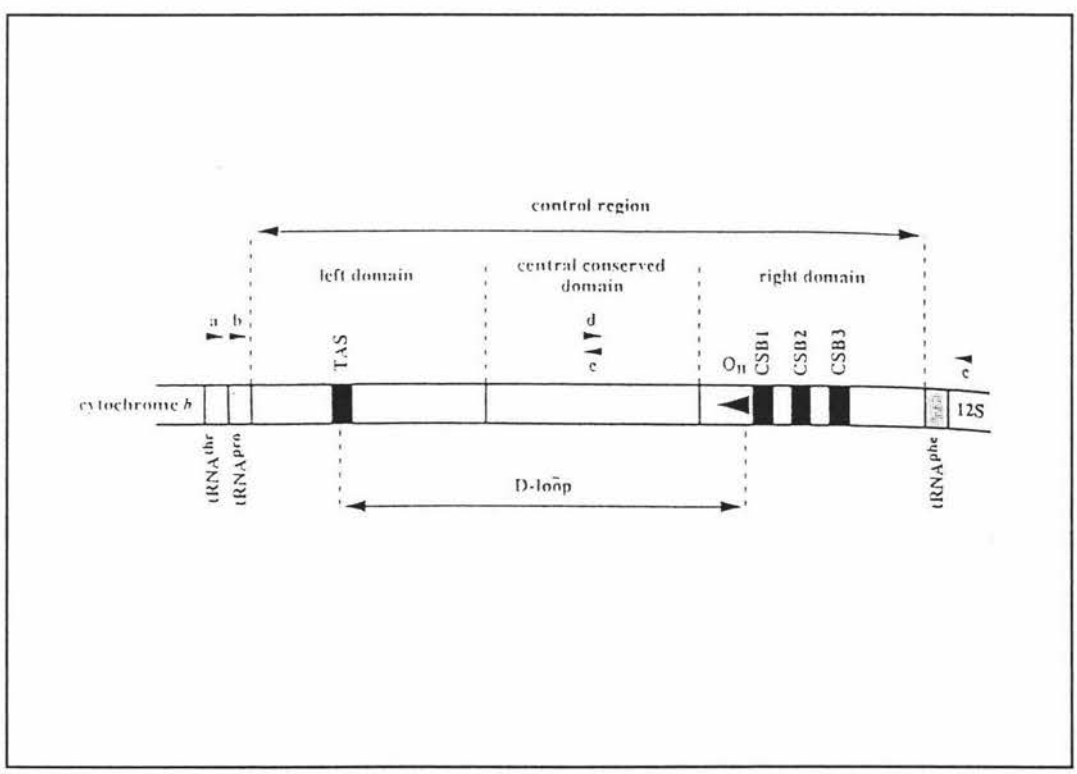

Figure 2.3 Schematic diagram of the mammaliam mtDNA control region, showing the central conserved region and the more variable left and right domains. Orientation is 5' and 3' relative to the light strand. (From: Taberlet, 1996).

Wenick et al. (1994) found that in Turnstone (*Arenaria interpres*) and Dunlins (*Calidris alpina*) most differences between species were concentrated at the beginning and end of the control region, outside the bipartite conserved central part. For example, the sequence divergence between turnstone and dunlin, was 21.8% for part 1 and 29.5% for part III, yet only 7.5 for part II. Therefore region I and III are the most useful regions for variation analysis.

The number of transitions that occur in the control region is approximately equal to the number of transversions for parts I and parts III, whereas there is a 3:1 bias for transitions over transversions for part II (Wenick et al., 1994). As transitions are far more likely to occur over transversions, the low ratios in parts I and III may indicate extensive back mutation between transitional states once transversions have occurred. In a phylogenetic study, these multiple hits will cause an underestimation of genetic distance. Wenick et al. (1994) believe that domains I and III are therefore less suitable for between-species evolutionary studies.

Also within the control region are several conserved sequence blocks. These were initially discovered in the mammal control region, and four of these have also been found in the avian control region. These include the F, D, and C boxes (Southern et al., 1988) and CSB-1 (Brown et al., 1986). As in mammals, CSB-1 partially overlaps with a region capable of assuming a cloverleaf structure. Also as in mammals, there is a section capable of assuming secondary structural configurations near the tRNAglu end of the control region, although the likely conformation is a hairpin rather then a cloverleaf. The mammalian cloverleafs flank the initiation and termination sites for D-loop synthesis and therefore their involvement in synthesis and termination has been proposed (Brown et al., 1986). Thus far, it is unknown where the D-loop strand maps relative to such structures in birds (Quinn and Wilson, 1993).

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Chapter 3

Investigation of DNA Variation in Weka: Methods and Results

3.1 INTRODUCTION

The two aims of this chapter were firstly, to obtain informative DNA data so that it would be possible to determine whether the current taxonomy of the four subspecies reflects the genetic diversity of weka, and secondly, to determine the provenance of weka of unknown origin that were introduced to islands. These aims were achieved by direct DNA sequencing of four different regions of the mtDNA genome: cytochrome *b,* ATPase 6, 12S and the control region. The results for each section are discussed. This chapter includes a description of the trialing of chicken and Tasmanian Native Hen microsatellite primers and the preliminary results discussed.

3.2 METHODS

3.2.1 Sampling sites

Weka samples collected from 16 populations throughout New Zealand were available for study (refer figure 3.1). Nine of these populations represent the current range and morphological diversity of weka: Gisbourne, Marlborough Sounds, Golden Bay, Westport, Copland Valley, Doubtful Sound, Chalky Inlet, Chatham Island and Bravo Sounds (Stewart Island). Chatham Island weka are included here as the subspecies *Gallirallus australis hectori* is extinct within its natural range (East Coast of the South Island). The remaining seven populations (Mokoia Island, Kapiti Island, Te Kakaho, Nukuwaiata, Open Bay Islands, Solander Island, Codfish Island) are claimed to have resulted from introductions (King et al., 1999) and these are referred to as introduced populations.

3.2.2 Collection of blood samples

Blood samples were collected by the Department of Conservation from a total of 180 weka (refer table 3.1). The majority of weka were located by playback of territorial calls and/or chick distress calls, with the exception of 30 weka from Mokoia Island that were already in a holding pen due to a poison drop on the island. Once located, the weka were caught with a handnet (n=87), in cage traps (n=55), with a dog (n=6 on Chatham Island) or shot (n=2 on Chatham Island) (Miskelly et al., 1996).

Live weka were restrained in a cotton bag with their legs extended through the bag opening. Up to 1.5 ml of blood was taken from the medial saphenous vein in the inside of the tarsus immediately distal to the tibio-tarsal joint, after the area had been swabbed clean with alcohol. The syringes had previously been flushed with heparin to prevent blood clotting. Blood was stored on ice in labelled 1.8 ml cryotubes. Blood samples from the eight dead Chatham Island weka were taken from the abdominal cavity and stored in the same manner as the live weka samples.

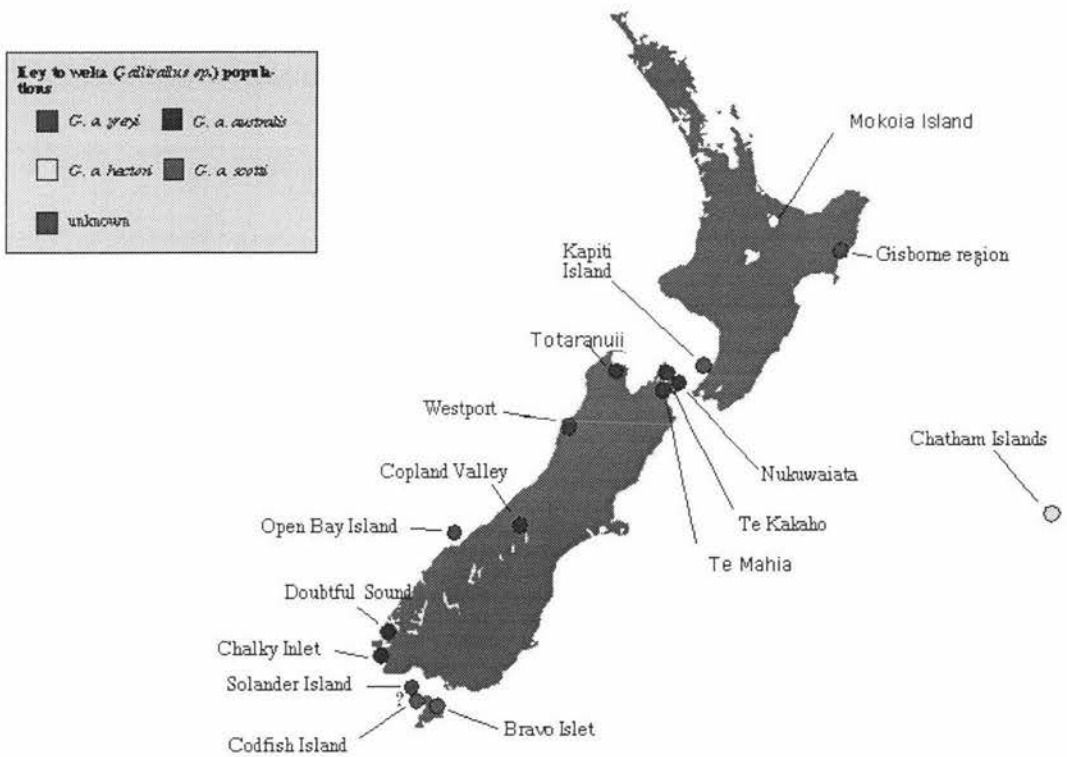

Figure 3.1 Map of New Zealand showing the location of the sampling sites of *Gallirallus australis*.

Table 3.1 Information regarding the 180 *Gallirallus australis* samples, including the location of collection, the presumed subspecies, the number of samples, the date collected and the fieldworker(s). (?) represents populations whose subspecific status is not known.

LOCALITY	*SUB-SPECIES*	NO. OF SAMPLES	DATE	FIELD-WORKER
Gisborne region	*greyi*	11	Dec. 1992, June 1993	C. Miskelly
Kapiti Island	*(?)*	30	June 1993	C. Miskelly
Te Kakaho	*australis*	10	May 1986, April 1993	C. Miskelly
Nukuwaiata	*australis*	14	April 1993	C. Miskelly
Te Mahia	*australis*	10	January 1993	C. Miskelly
Totaranui	*australis*	2	May 1993	C. Miskelly
Westport	*australis*	10	January 1993	C. Miskelly
Copland Valley	*australis*	8	January 1993	C. Miskelly
Solander Island	*(?)*	5	June 1993	P. McClelland
Doubtful Sound	*australis*	12	June-July 1993	C. Miskelly
Chalkey Inlet	*australis*	4	June 1993	C. Miskelly
Chatham Island	*hectori*	9	June 1993	S. Harris
Open Bay Islands	*(?)*	14	February 1991, January 1993	C. Miskelly
Bravo Islets	*scotti*	10	January 1993	C. Miskelly
Codfish Island	*scotti (?)*	1	January 1993	C. Miskelly
Mokoia Island	*greyi*	30	November 1996	B. Stephenson T. Poldmaa

The blood was originally collected for isozyme analysis, so the samples were centrifuged for four minutes at 16,000g to separate red blood cells from the plasma. The plasma fraction was transferred by pipette into labelled 1.0 ml cryotubes. The red blood cell fraction was mixed with an equal quantity of distilled water, and all samples were stored in liquid nitrogen until placed in the ultrafreezer in the Genetics Laboratory, School of Biological Sciences, Victoria University. In late December 1996, these samples were transferred to Massey University, Palmerston North, where they were stored in the Molecular Ecology Laboratory -80 °C freezer.

3.2.3 DNA extraction

Total genomic DNA was extracted from the red blood cell samples following Sambrook et al. (1989). Approximately 5µl of red blood cells were suspended in 500µl of extraction buffer (0.1 M Tris pH 8.0, 0.1 NaCl, 1 mM EDTA, 2% SDS) containing 0.5 mg/ml proteinase K and incubated at 55-60°C with gentle rotation in a hybridisation oven overnight. After incubation the blood sample was completely digested with no visable residual fragments. The digested blood cells were purified by standard methods of phenol-chloroform extraction as follows:

Proteins were removed from the samples with a series of phenol/chloroform washes. Briefly, 400 µl of Tris-buffered phenol was added and rocked gently for 30 minutes. Following centrifugation at 13,000g for five minutes, the bottom layer was removed using a 200µl pipette. Four hundred microlitres of phenol/chloroform/isoamyl (25: 24: 1) was added to the aqueous layer and rocked for 30 minutes, then spun at 13,000g for five minutes. The bottom layer was removed and another phenol/chloroform/isoamyl wash was performed. Finally, the bottom layer was removed as above. Samples were centrifuged again at 13,000g for five minutes, and the last of the remaining bottom layer was removed.

To precipitate the DNA, the molecules were dehydrated by the addition of 40 µl 3 M NaOAc pH 5.2 and 1 ml of 100% ethanol (4 °C), followed by vigorous shaking, and then rocked for 15 minutes. The samples were placed at -80 °C for one hour to allow the DNA to precipitate fully and then spun at 13,000g for five minutes to pellet the DNA. The supernatant was removed and the pellet washed twice with 70% ethanol by gentle inversion. Ethanol was decanted off and the DNA pellet was

left to desiccate at room temperature for approximately 0.5-1 hour. All DNA pellets were resuspended in milli-Q water and stored at 4 °C. Resuspension volumes varied proportionately to the size of the DNA pellet (i.e. 40-100 µl).

To assess the efficiency of each extraction, 2 µl of the isolated resuspended genomic DNA was electrophoresised in an 0.8 % agarose gel in TBE buffer on a BRL minigel chamber and visualised with ethidium bromide (0.5 μgml^{-1}) using a 312 nm UV transilluminator. Alternatively the DNA quantity was evaluated with a Hoefer DyNA Quant 200 flurometer using Hoefer dye, or a Pharmacia GeneQuant spectrophotometer at a wave length of 260 nm.

3.2.4 Polymerase Chain Reaction

PCR is a fast alternative to conventional cloning (Kocher et al., 1989). PCR is relatively simple and is composed of three major steps: (1) denaturing or melting of template DNA to make single-stranded DNA, (2) annealing or hybridisation of single-stranded DNA and the PCR primer, (3) extension of the primer by a DNA polymerase (refer figure 3.2). PCR allows unique sequences to be amplified *in vitro* in a matter of hours. Furthermore, the procedure is easily automated, so that hundreds of samples can be amplified each day. The enzymatic amplification of a specific DNA segment is made possible by the highly specific binding of oligonucleotide primers to sequences flanking the segment. These primers allow the binding of a DNA polymerase that then copies the segment. Because each newly made copy can serve as a template for further duplication, the number of copies of the target segment grows exponentially (Kocher et al., 1989).

All PCR amplifications in this study were double stranded and were carried out in an Hybaid Omni Thermal Cycler. Mineral oil was added to prevent evaporation during thermal cycling. Negative amplification controls (in which the template is replaced by water only), were also performed as an indicator of contamination by foreign template in the PCR reactions. Unless otherwise stated, PCR products were visualised on 0.8 - 1.2% agarose gels run in 89 mM Tris-borate, 1 mM EDTA (TBE) buffer (Sambrook et al., 1989). PCR products were electrophorised at 12.5 $volts.cm^{-1}$ for 40 minutes and visualised by ethidium bromide staining (0.5 μgml^{-1}) using a 312 nm UV transilluminator.

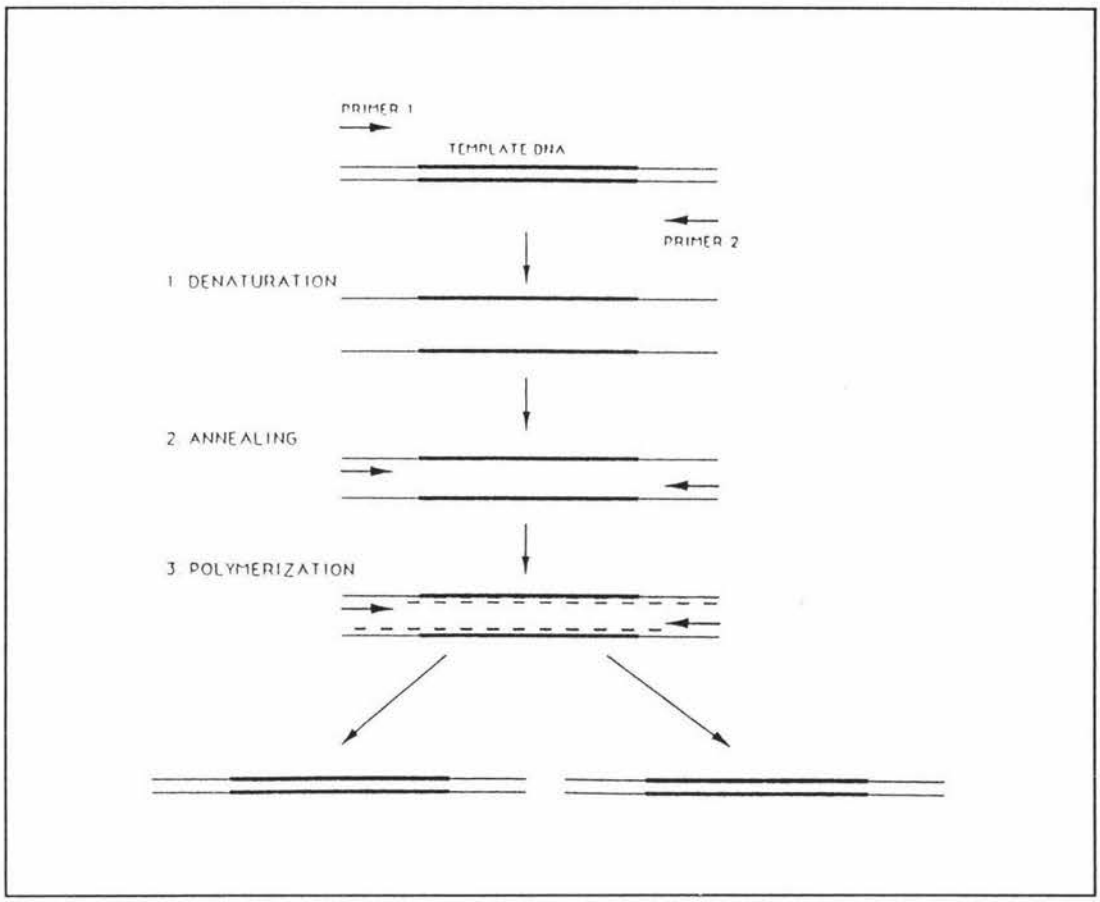

Figure 3.2 Schematic representation of PCR (Hoezel and Green, 1992)

In order to optimise the PCR, amplifications were performed using a range of DNA dilutions (1:25, 1:50, 1:75 and 1:100) so as to increase yield without background smearing of high molecular weight DNA. The annealing temperatures were varied (T_A = 45°C, 50°C, 55°C, 56°C, 57°C, 58°C and 60°C) in order to increase specificity. The numbers of thermal cycles used (25, 30 and 35 cycles) was varied in order to reduce background bands. Different brands of polymerases (Perkin Elmer *Amplitaq*, Boehringher Manheim *Taq* and Perkin Elmer Gold *Taq*) were used depending on the size of the fragment to be amplified. Various primer dilutions (2.5, 2.0, 1.5, 1.25, 1.0 µl (10pmol/µl) of the final 25 µl reaction) were used to reduce primer dimers. Differing $MgCl_2$ concentrations (0.8, 1, 1.5, 2, 2.5 and 3µl of 25mM of the final 25 µl reaction) were used to decrease non-specific binding.

The standard reaction contained: 1 µl of DNA, 2.5 µl of Perkin Elmer 10X PCR buffer II, 2.5 µl of $MgCl_2$ (25 mM), 2.5 µl of each primer (10 pmol/µl), 0.25 µl of dNTPs (20 mM of each deoxynucleotide), 0.1 µl Perkin Elmer *Ampli-Taq* Polymerase (5U/µl), and Milli-Q H_2O to make up 25 µl. Refer table 3.2 for the optimal conditions of all primer combinations used. Every PCR had an initial denaturation of 94°C for two minutes and ended with a final extension of 74°C for ten minutes. Figure 3.3 shows the location of all primers used in this research.

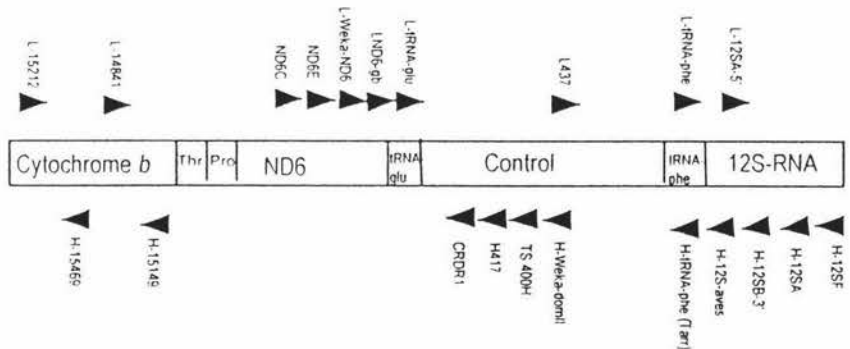

Figure 3.3 Location of the cytochrome *b*, ND6, tRNA-glu, tRNA-glu, control region and 12S-RNA primers used in this research.

Table 3.2 Conditions used for optimal PCR conditions for amplifying cytochrome *b*, 12S, ATPase 6 and the control region ofthe weka mitochondrial genome when clean amplified product was obtained and used.

Primers used in mtDNA analysis	10X PCR buffer II (in µl)	PCR primers (10 pmol/µl) (in µl)	dNTPs (20 mM od each dNTP)	$MgCl^{2+}$ (in mM)	*Taq* Polymerase (5U/µl)	Temperature Profiles (in °C)
L15212 & H15649	2.0	2.5	0.25	2.5	0.1	92: 1 min, 55: 1 min, 72: 1 min
L14841 & H15149	2.0	2.0	0.125	2.0	0.2	94: 1 min, 56: 1 min, 74: 1 min
A1 & A5-1	2.5	1.25	0.125	1.0	0.1	94: 1 min, 56: 1 min, 74: 90 sec
12SA & 12SB2	2.5	2.0	0.125	2.5	0.125	94: 40 sec, 55: 40 sec, 72 1 min
CRDR1 & ND6E	2.0	2.5	0.25	1.0	0.1	94: 45 sec, 50: 75 sec, 72: 2 min
H417 & ND6E	2.5	2.5	0.25	2.0	0.1	94: 1 min, 50: 1 min, 72: 1 min
H417 & ND6C	2.5	2.5	0.25	2.0	0.1	94: 1 min, 55: 1 min, 72: 1 min
H-12S-aves&L-ND6-gb	2.5	2.5	0.25	2.0	0.1	94: 1 min, 55: 1 min, 72: 1 min
Ts 400H & L-ND6-gb	2.5	2.5	0.63	2.0	0.1	95: 1 min, 55: 1 min, 72: 1 min
tRNA-glu & tRNA-phe	2.0	2.0	0.25	2.5	0.2	92: 1 min, 55: 1 min, 72: 1 min
L-Weka-ND6 & H-Weka-domII	2.5	2.0	0.1	2.0	0.1	94: 1 min, 62: 1 min, 72: 1 min

3.3 AMPLIFICATION OF CYTOCHROME *b*

The investigation of variation in weka DNA began by using the protein coding cytochrome *b* gene. The first cytochrome *b* primers used were L15212 and H15469 (Baker et al., 1995). Refer table 3.3 for all primer sequence information. Optimisation was achieved and clean bands of the appropriate size, approximately 370 bp were obtained (refer figure 3.4).

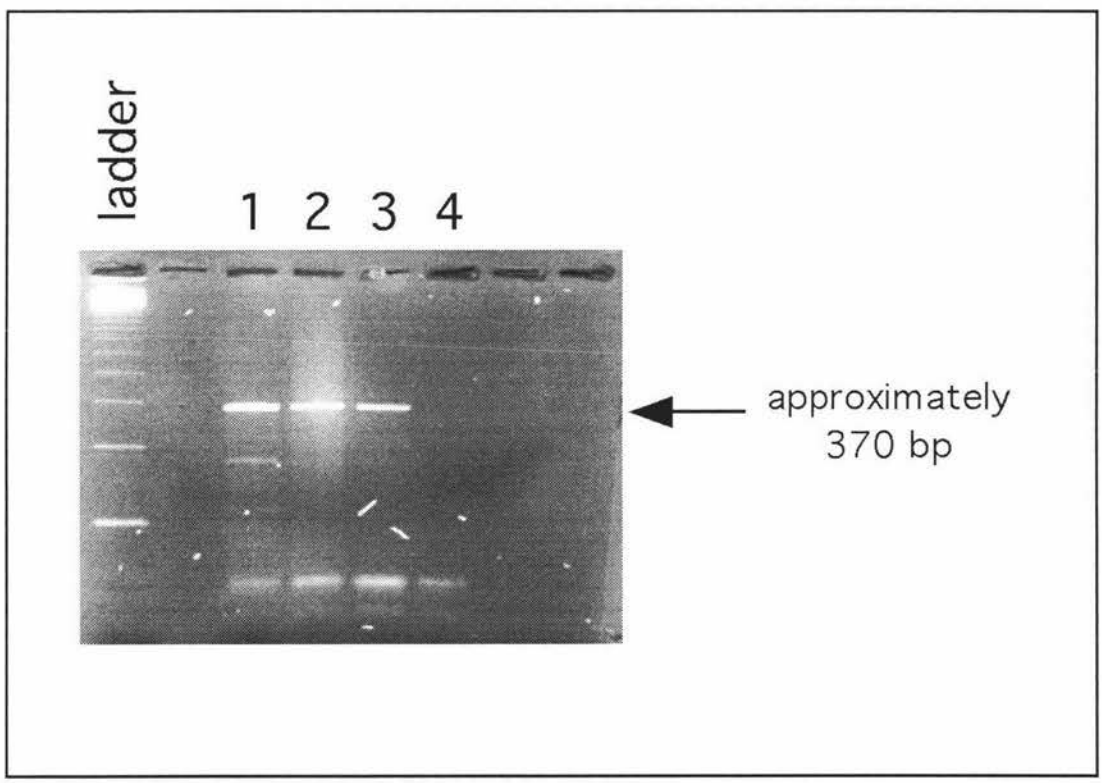

Figure 3.4 2% Nusieve gel of PCR product of cytochrome *b* primers L15212 and H15649. Lanes 1-3 contain PCR product from weka (1: Mokoia Island, 2: Chatham Island, 3: Bravo Islet). Lane 4 is the negative control. The ladder was a 123 bp ladder.

The excised bands were removed from the gel by taking a gel core. The excised bands were purified using a High Pure PCR Product Purification Kit (Boehringer Mannheim) following the manufacturer's instructions. The elutate (100 µl) was dried down to a volume of approximately 10 µl. To check the effective recovery of this purification, 1 µl of the recovered PCR product was electrophorised on a 1% agarose gel at 12.5 volts.cm^{-1} for 40 minutes and visualised by ethidium bromide staining.

Table 3.3 Primer sequence information for PCR primers used in this research.

PRIMER NAME	PRIMER SEQUENCE (5' TO 3')	DESIGNED BY
L15212	GGACGAGGCTTTTACTACGGCTC	Baker et al. (1995)
H15649	TTGCTGGGGTGAAGTTTTCTGGGTC	Baker et al. (1995)
L14841	AAAAAGCTTCCATCCAACATCTCAGCA TGATGAAA	Kocher et al. (1989)
H15149	AAACTGCAGCCCCTCAGAATGATATTT GTCCTCA	Kocher et al. (1989)
A1	ATGAACCTAAGCTTCTTCGACCAATT	Alan Baker & Oliver. Haddraith
A5	TAGGAGTGTGCTTGGTGTGCCATT	Alan Baker & Oliver Haddraith
A5-1	GTTTTCTTGTAAGTACAGGC	Martin Kennedy
12SA	CAAACTGGGATTAGATACCCCACTAT	Cooper (1994)
$12SB_2$	GAGGGTGACGGGCGGTATGTACGT	Cooper (1994)
CRDR1	CCAGTGGCGCAAAAGAGCAAGTT	Sandie Degnan
ND6E	CCATAACCAACAACCTGTCAAT	Edwards (1993)
12SB	GGGTGACGGGCGGTGTGTGC	Cooper (1994)
12SF	AAAATGTAGGCCATTTCCTCC	Cooper (1994)
H417	AGTAGCTCGGTTCTCGTGAG	Tarr (1995)
L437	CTCACGAGAACCGAGCTACT	Tarr (1995)
ND6C	CCGAGACAACCCACGCACAAG	Edwards (1993)
HD4	CCCGACCAGCTGCATCTGTG	Edwards (1993)
H-12S-Aves	AGT ACC CGT GGG GGT GTG GC	Peter Ritchie
L-ND6-gb	CCATAATACGGCGAAGGATTAGACGC	Bruce Robertson
L-Weka-ND6	CCCAAGACCTGCGACCTGAAAA	Bruce Robertson
H-Weka-domII	CCGTCAGAAAGGAGGCAGTTA	Bruce Robertson

A Lambda/*Hin*d III molecular weight marker of known concentration was loaded onto each agarose gel alongside the recovered PCR product to estimate their concentration before DNA sequencing. The average resultant concentration was 20 pmol/µl.

Sixteen PCR products, comprising four individuals from each of the four subspecies, were sent to the Applied Biosystems 377 Automated Sequencer for sequencing using both primers. These samples were run according to the instructions of the manufacturer. The sequence was aligned by eye in SeqEd. This region of cytochrome *b* in weka was found to be almost identical across all four subspecies (refer figure 3.5).

	10	20	30	40	50
Mokoia 11	ACCCCATCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Mokoia 12	--CCCATCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Mokoia 13	-ACCCCTCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Mokoia 14	-----CACCA	ACATCTCAGC	ATGATGAAAC	TTCGGTTCAC	TCTTAGGAAT
Te Kakaho 1	-ACCCATCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Te Kakaho 2	--ACCCTCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Nukuwaiata 1	--CCCCTCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Nukuwaiata 2	-ACCCCACCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Bravo Islet 3	---CCCCCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Bravo Islet 4	---NCNC--A	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Bravo Islet 5	---CCCCCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Bravo Islet 6	---CCCCNCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Chatham Island 3	-CCCCCC---	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Chatham Island 4	--CCCCCCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Chatham Island 5	---CCCCCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
Chatham Island 6	---CCCCCCA	ACATCTCAGC	ATGATGAAAC	TTCGGCTCAC	TCTTAGGAAT
	60	70	80	90	100
Mokoia 11	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Mokoia 12	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Mokoia 13	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Mokoia 14	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATNCATTANA
Te Kakaho 1	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Te Kakaho 2	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Nukuwaiata 1	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Nukuwaiata 2	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Bravo Islet 3	CTGTTTAATC	ACACAAATCC	TTACAGGCNT	ACTACTAGCC	ATGCATTATA
Bravo Islet 4	CTGTTTAATC	ACACAAATCC	TTACAGGCNT	ACTACTAGCC	ATGCATTATA
Bravo Islet 5	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Bravo Islet 6	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTANTAGCC	ATGCATTATA
Chatham Island 3	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Chatham Island 4	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Chatham Island 5	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
Chatham Island 6	CTGTTTAATC	ACACAAATCC	TTACAGGCCT	ACTACTAGCC	ATGCATTATA
	110	120	130	140	150
Mokoia 11	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Mokoia 12	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Mokoia 13	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Mokoia 14	CTGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGTCGCAAC
Te Kakaho 1	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Te Kakaho 2	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Nukuwaiata 1	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Nukuwaiata 2	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Bravo Islet 3	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Bravo Islet 4	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Bravo Islet 5	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Bravo Islet 6	CCGCAGANAC	AACCATGGCC	TTTTCCTNTG	TTGCCCAAAA	ATNCCGCAAA
Chatham Island 3	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Chatham Island 4	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Chatham Island 5	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC
Chatham Island 6	CCGCAGACAC	AACCATGGCC	TTCTCCTCTG	TTGCCCACAC	ATGCCGCAAC

Figure 3.5 The first 300 based pairs of aligned sequence from 16 indidviduals (4 from each subspecies)of the cytochrome *b* gene using the primers L15212 and H15649. (*G. a. greyi* Mokoia 11, 12, 13, 14; *G. a. australis* Te Kakaho 1, 2, Nukuwaiata 1, 2; *G. a. scotti* Bravo Islet 3, 5, 6, 7; *G.a.hectori* Chatham Island 3, 4, 5, 6).

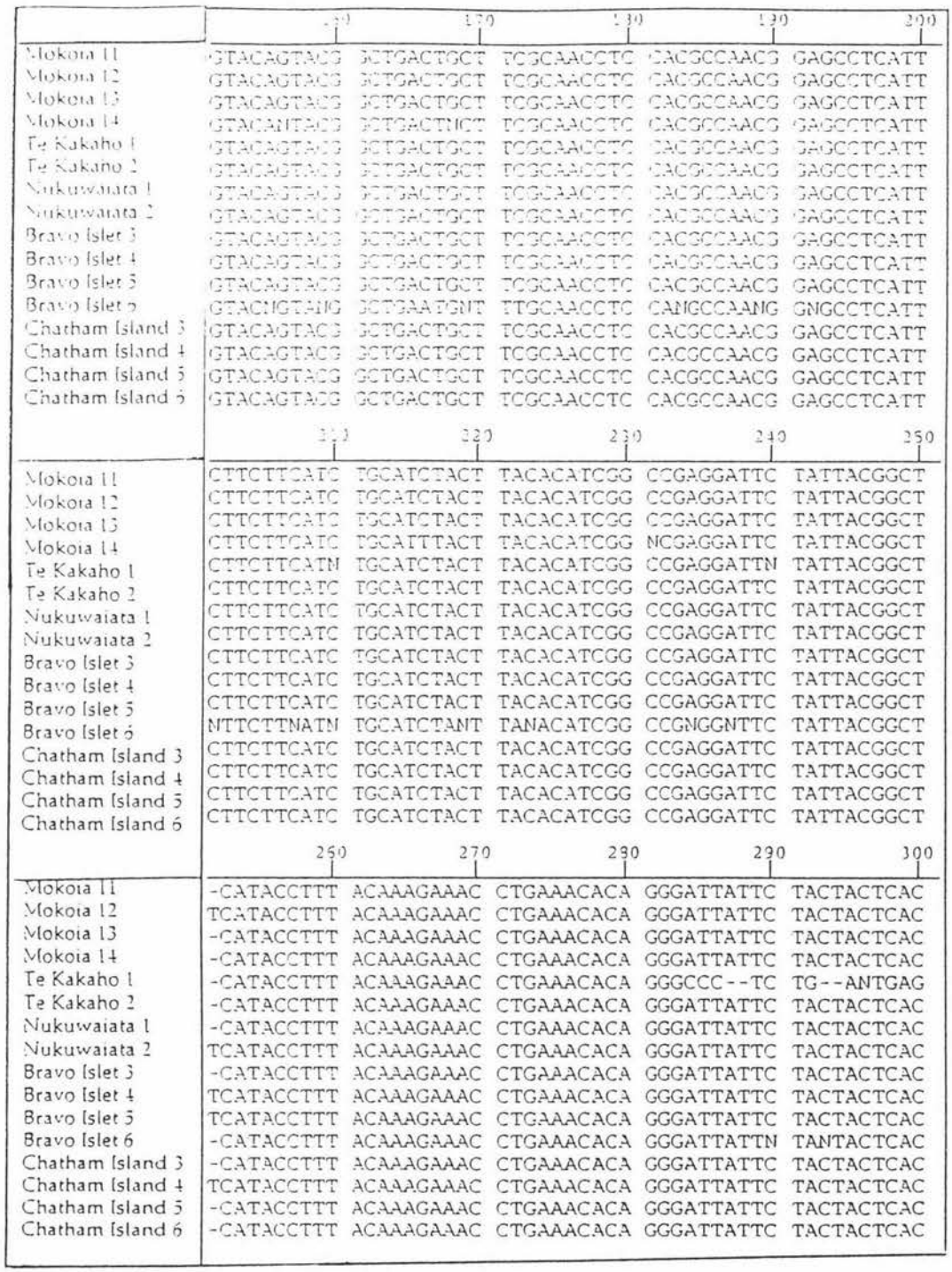

Figure 3.5 Continued

The next region of the mtDNA genome to be amplified was another portion of cytochrome *b*. The primers L14841 and H15149 were used. These primers were designed by Kocher et al. (1989) by comparing published sequences for mammal, frog and *drosophila* mtDNAs and finding highly conserved regions. These primers are contiguous to the first set of cytochrome b primers used (Baker et al., 1995). After optimisation, PCR products of the appropriate size, approximately 400 bp were obtained (refer figure 3.6).

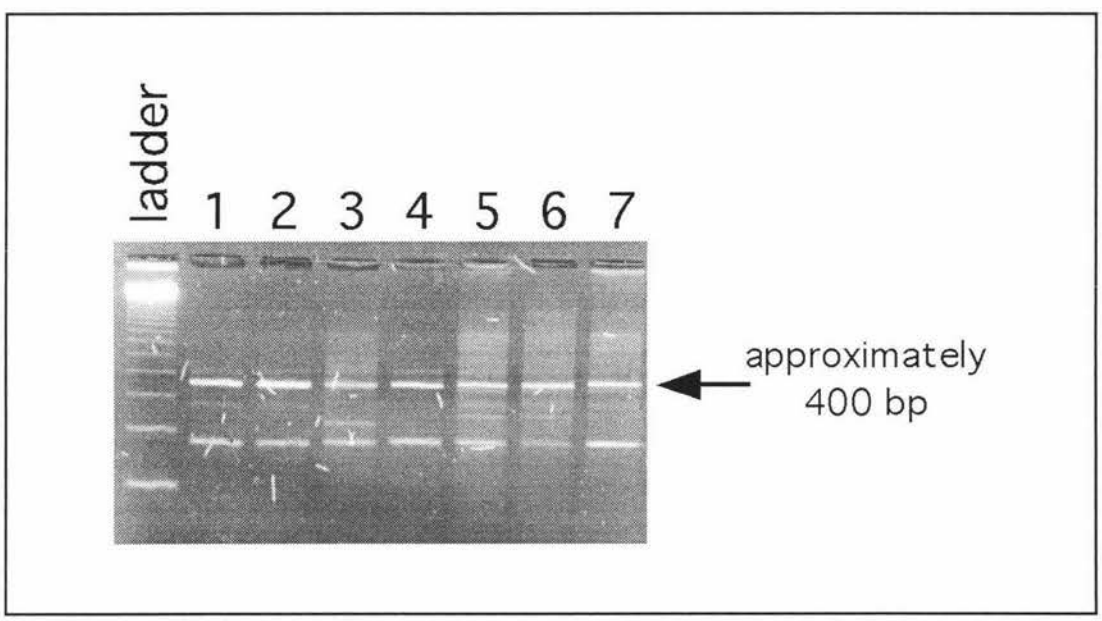

Figure 3.6 1% Agarose gel of PCR product obtained using primers L14149 and H151149. Lane 1-3 contained *G. a. greyi*, lanes 4 and 5 contained *G. a. scotti* and lanes 6 and 7 contained *G. a. hectori.* A 123 bp ladder was used.

Eight PCR products (20 pmol/µl), comprising of 2 individuals from each subspecies, were sent to the Applied Biosystems 377 Automated Sequencer for sequencing. These samples were run according to the instructions of the manufacturer. The sequence was aligned by eye in SeqEd. This region of cytochrome *b* in weka was also found to be invariant across all four subspecies. The cytochrome *b* gene proved unsuccessful in obtaining nucleotide variation between weka subspecies.

3.4 AMPLIFICATION OF ATPase 6

The next gene used in an attempt to establish the levels of variation in weka populations was ATPase 6. Three primers were used : A1, A5 and A5-1, which is an internal primer to A5. The primer combination of A1 and A5-1 amplified the brightest product of approximately 500 base pairs with weka DNA (refer figure 3.7).

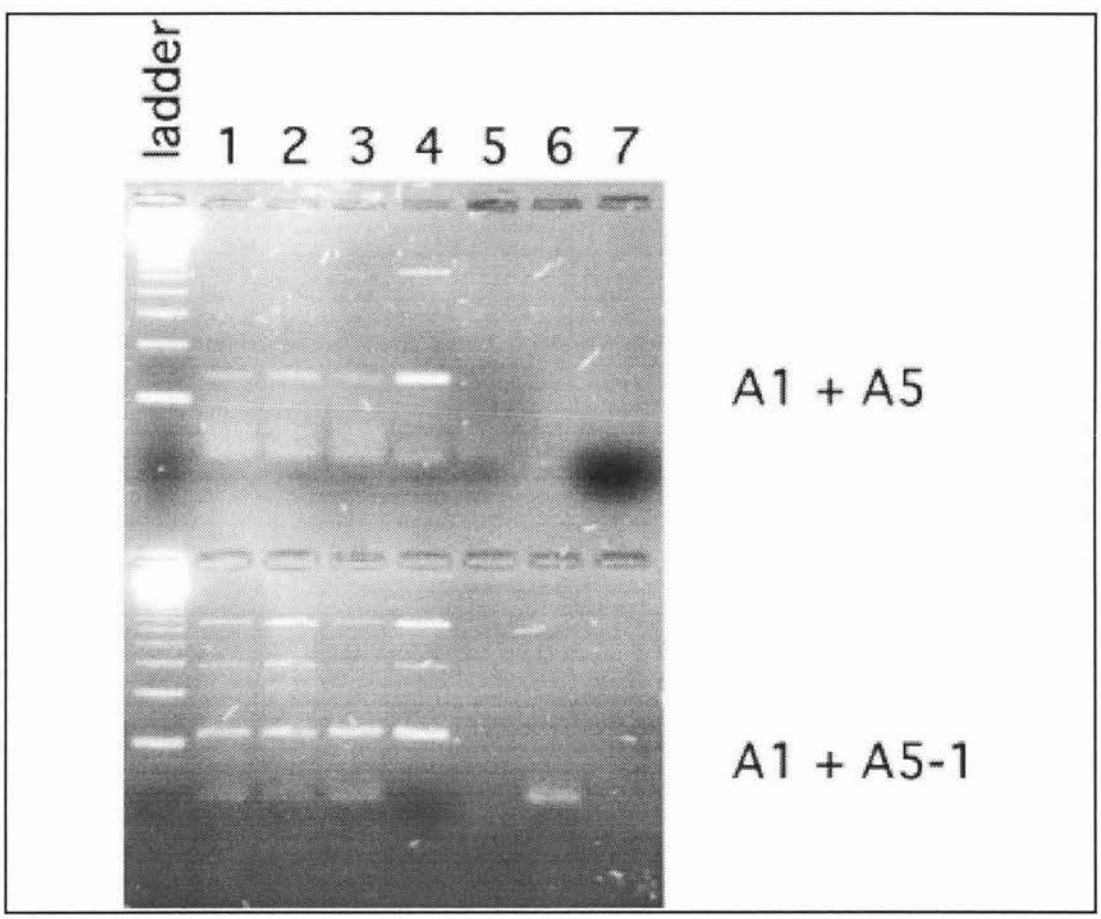

Figure 3.7 2% agarose gel of PCR product of two PCR primer combinations, A1 + A5 and A1+ A5-1. Lanes 1-6 contain ATPase-6 amplifications (Lanes 1 & 2: *G. a. greyi*, 3 & 4: *G. a. australis*, 5: *G. a. scotti*, 6: *G. a. hectori*). Lane contained the negative control. A 123 base pair ladder was used.

This A1 and A5-1 primer pair combination consistently yielded brighteramplification of bands so this combination was used for sequencing. The excised bands were removed from the gel by taking a gel core. The excised bands were purified using a High Pure PCR Product Purification Kit (Boehringer

Mannheim) following the manufacturer's instructions. The average resultant concentration was 20 pmol/ µl.

A representative individual from each morphometrically distinguished subspecies was manually sequenced using an AmpliCycle™ Sequencing Kit (Perkin Elmer) (refer figure 3.8). Sequencing primers A1 / A5-1 were end-labelled with [$-^{33}$ P] ATP using T4 Polynucleotide Kinase, following the 'routine sensitivity' protocol described in the kit. AmpliCycle™ sequencing reactions of the purified PCR products were subjected to 30 cycles of 60°C for 30 seconds, 72°C for 30 seconds and 94°C for 30 seconds. The reactions were stopped using a 'STOP' loading dye containing formamide, bromophenol blue and xylene cynol. The sequencing reactions were denatured at 90°C and then loaded onto a 6% denaturing polyacrylamide gel and electrophorised at 55 W. Two set of lanes were run per sample, one short (1 1/2 hours, until the bromophenol blue had run off the gel) and one long (at least 3 hours, until the xylene cynol was close to the bottom of the gel). This enabled the sequence to be read close to the primer and also further into the gene. The acrylamide gels then were dried and exposed to Kodak Bio-Max MR film for 4-7 days, depending on the signal, with longer exposures used if the signal was weak.

The sequence was manually read and aligned by eye using SeqEd. The only variation that was seen was at two sites that differed between the North Island and South Island subspecies of weka (Refer figure 3.8).

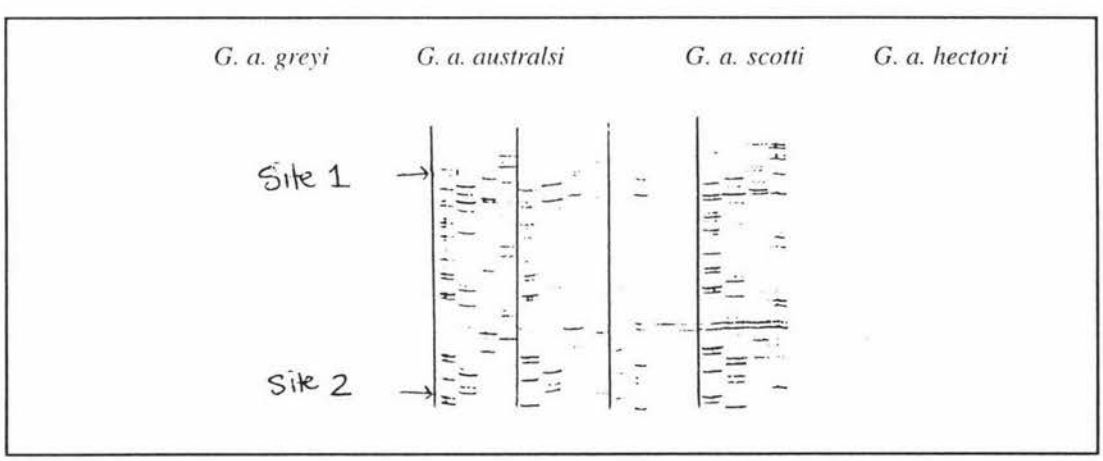

Figure 3.8 Portion of the autorad of ATPase 6 that showed the only 2 areas of variability that differed between the North Island subspecies and South Island subspecies in weka.

3.5 AMPLIFICATION OF 12S

The next gene trialed in the investigation for variation in weka mtDNA was 12S. A portion of the 12S gene was amplified using the primers 12SA and 12SB.(Cooper, 1994). Eight samples were visualised and clean bands resulted (refer figure 3.9). The excised bands were purified using a High Pure PCR Product Purification Kit (Boehringer Mannheim) following the manufacturer's instructions. The average resultant concentration was 20 pmol/µl. Two samples, a North Island and South Island sample, were sequenced using an Applied Biosystems 377 Automated Sequencer, run according to the instructions of the manufacturer. The sequences were aligned in SeqEd by eye. It was found that the 12S gene in weka was invariant in the two sequenced individuals. These sequences were found to be identical to weka sequence in Trewick (1997). This region was found to be very conserved across rails (refer figure 3.10).

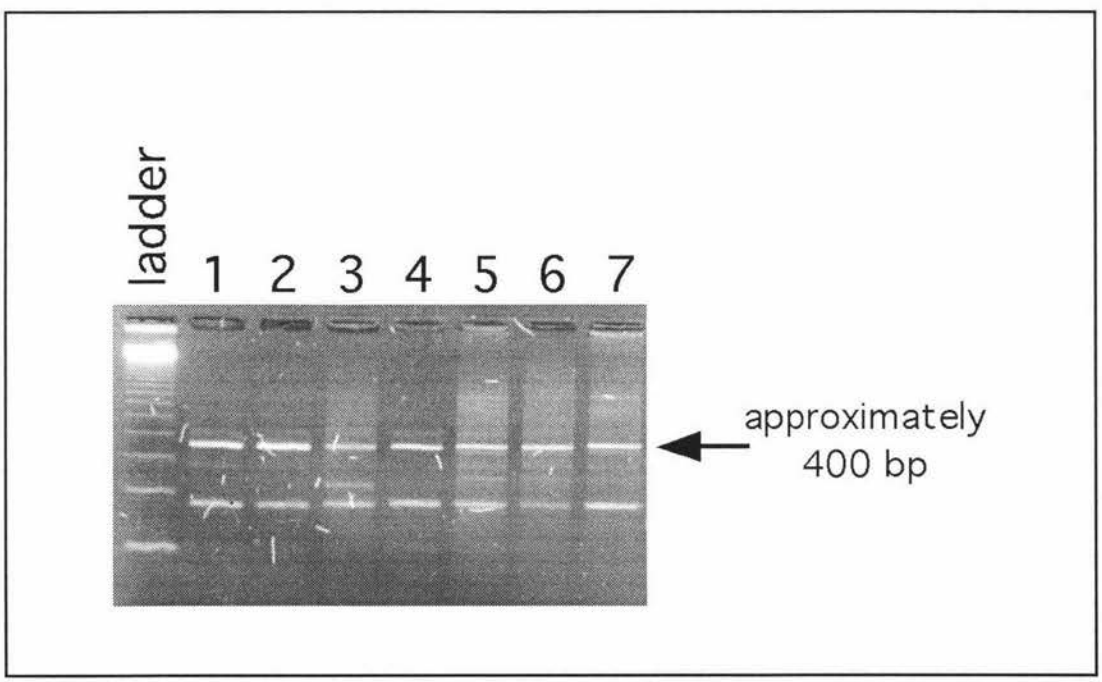

Figure 3.9 PCR amplified product with 12S primers visualised on a1.0% agarose gel. A 123 bp ladder was used. Lanes 1-7 contained 2 representative individuals for *G. a. greyi, G. a. australis, G. a. hectori* and 1 representative indidvidual from *G. a scotti.*

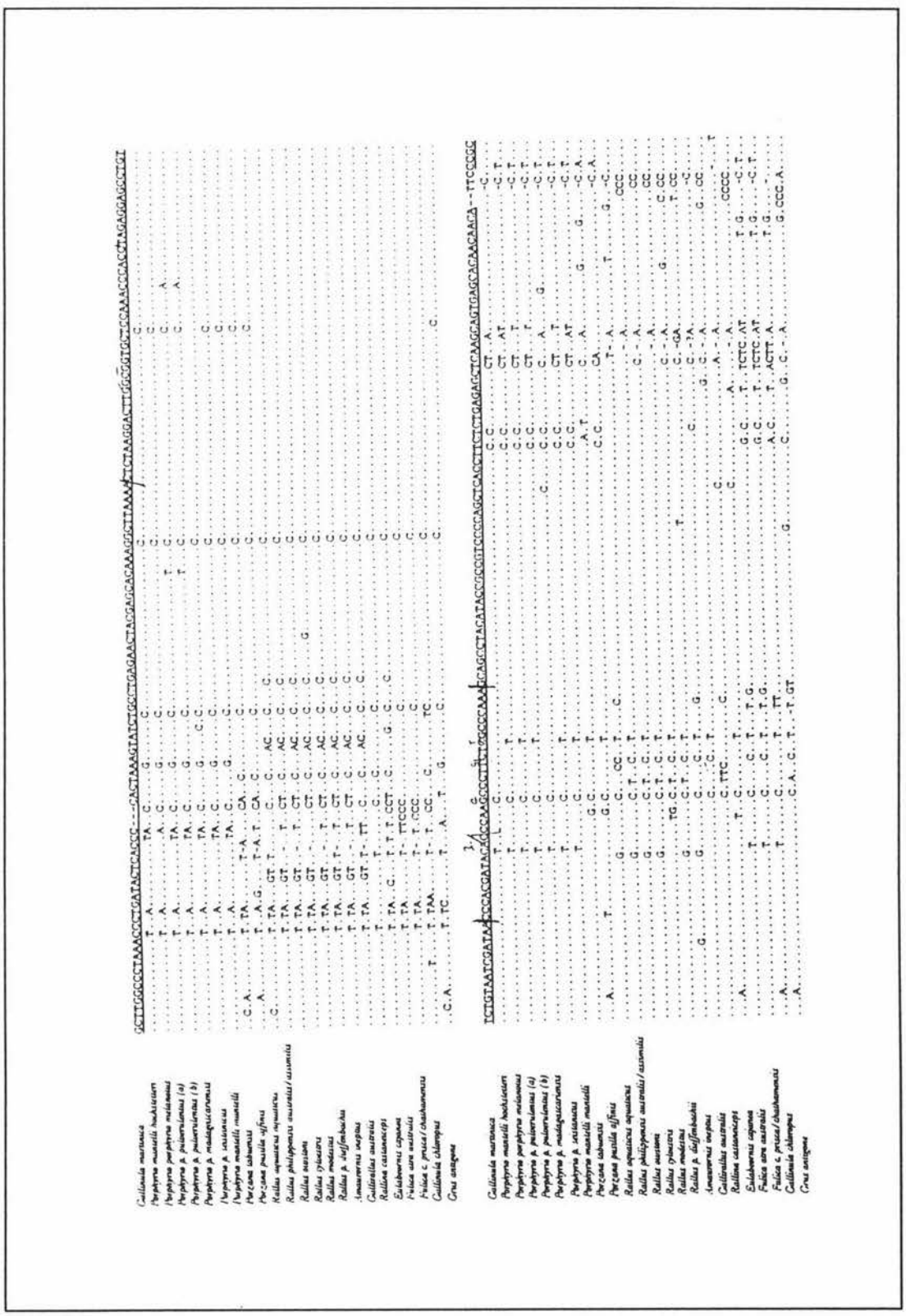

Figure 3.10 Portion of the 12S sequences of rails aligned (From Trewick, 1997)

3.6 AMPLIFICATION OF THE CONTROL REGION

The control region of the weka mitochondrial genome was the last area investigated for variation between the subspecies of weka utilising the mitochondrial genome. The hypervariable nature of the control region between taxa (Saccone et al., 1991) made these PCR amplifications much more challenging. Initial amplifications used primers CRDR1 and ND6E. This region encompasses a 800 bp region, including approximately 350 bp of the control region and the remaining 450 bp in the ND6 gene. These primers had been successful with a number of New Zealand avian species, such as the Pukeko (*Porphyrio porphyrio*) and the Saddleback (*Philestnus carunculatus*) (pers comm. Dr Bruce Robertson).

This primer combination was tested under various conditions but only produced multiple bands ranging between 400 and 1350 bp (refer figure 3.11). The PCR products were visualised on a gel. The band of the appropriate size was band stabbed. This was used as a template for reamplification PCR in anticipation of a cleaner product. However, after several attempts it was apparent that this set of primers was not going to specifically amplify the control region in weka due to low signals and a lot of background noise.

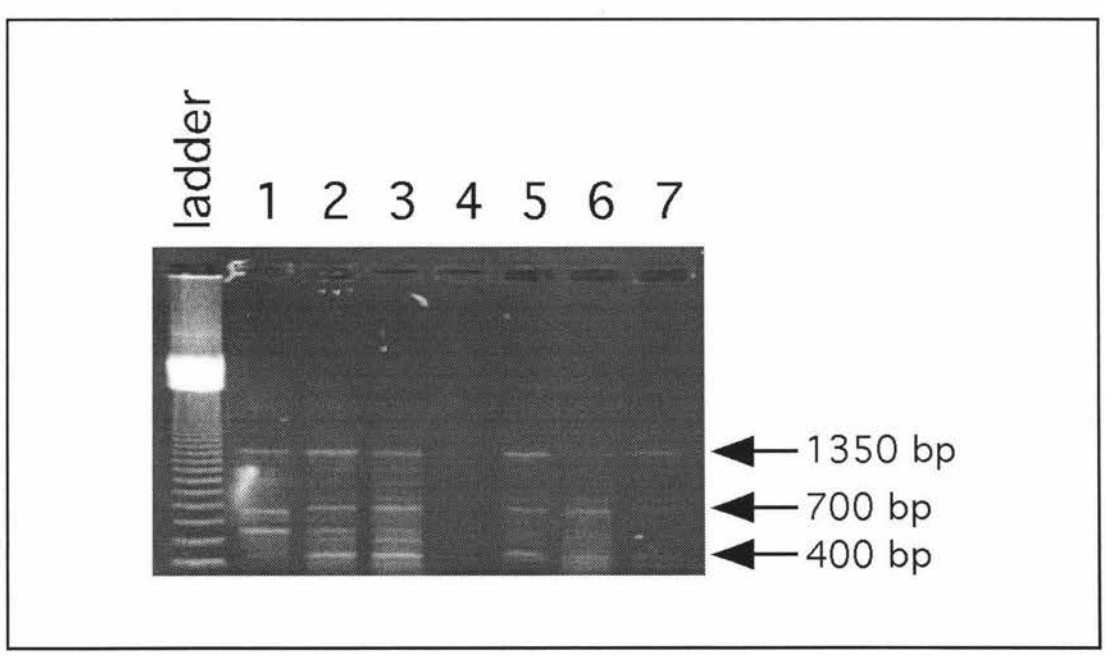

Figure 13.11 1% Agarose gel containing the amplified PCR products of H417 and ND6C. The 700 bp band was band stabbed. Lanes 1-3 and 5-7 contain amplified weka DNA. Lane 4 was the negative control and a 123 bp ladder was used.

The next primer combination trialed was ND6E and 12SB. It was anticipated that this primer combination would be successful as it encompassed the whole of the control region. This would allow weka specific primers to be developed at the edge of the control region. After several optimisation attempts, only smeary multiple products could be obtained. Further attempts with this primer combination were abandoned.

A similar primer combination comprising of ND6E and 12SF was trialed. The only difference between this primer combination and the previous pair was the different 12S primer. This too had to be abandoned due to the inability of the primer combinations to produce 'clean' PCR products, instead it resulted in smeary multiple bands.

Two primers (H417 and L437) from Tarr (1995) were synthesised at Dr Tarrs recommendation. The first primer H417 was synthesised as this primer was designed in the F box of the control region, which is a very highly conserved sequence block. The second primer L437 was synthesised as it is said to work well with 12S primers (C. Tarr, pers. comm.), in order to obtain the 3' end of the control region.

Initially two primer combinations were trialed: H417 with ND6E and L437 with 12SB. Both sets of primers produced amplifications. However, the L437 and 12SB primer combination was abandoned in favour of the H417 and ND6E combination which produced fewer background bands. Despite optimisation attempts these PCR amplifications produced multiple products. Consequently, the PCR product of the approximate size was band stabbed and used as a template for 50 µl PCR reactions. A gel core was taken and purified using an Agarose Gel DNA Extraction Kit (Boehringer Mannheim) following the manufacturer's instructions. DNA concentrations obtained ranged from 11 - 36 ng/µl.

Three of these samples, one from each of the subspecies *G. a . greyi, G. a. australis* and *G. a. hectori* were sequenced on an Applied Biosystems 377 automated sequencer. Low signals with a lot of background noise was produced

suggesting that maybe primers did not anneal well with the sequence or that there was multiple template. This primer combination was abandoned.

Another pair of control region primers were trialed. These were ND6C and HD4. These primers were designed by Dr Scott Edwards (Edwards, 1993) and were synthesised as it was thought that these primers amplify a homologous segment of mtDNA in a wide range of passerine and non-passerine bird species. It was hoped that these primers could also amplify weka. These primers were initially tested using the protocol that worked for a wide range of passerine and non passerine species (Edwards, 1993). Amplifications consisted of 30 cycles, with each cycle consisting of 40 seconds at 94 °C, 1 minute at 50 °C, and 1 minute at 72 °C. However, this primer combination was not successful with weka DNA. These primers were tested extensively, with annealing temperatures down to 48 °C, and $MgCl_2$ concentrations up to 3 mM. Multiple bands were produced but a band of the appropriate size was never produced and this primer combination were abandoned.

Every primer combination that had not yet been trialed was then attempted (refer table 3.4). The annealing temperature was varied for every primer combination depending on the G/C content of the primers.

Table 3.4 Combination of all PCR primers not previously tested and their corresponding annealing temperatures for attempting to amplify the control region in weka.

PRIMER COMBINATION	ANNEALING TEMPERATURE
ND6C + H417	55 °C
ND6C + 12SB	59 °C
ND6C + CRDR1	59 °C
ND6C + 12SF	52 °C
ND6E + HD4	54 °C
L437 + CRDR1	57 °C
L437 + HD4	55 °C
L437 + H417	53 °C

The only primer combination that worked under these standard conditions was that of ND6C and H417, although only a faint but clean and definite band, was

produced (refer figure 3.12). The rest of the PCR product was run out and a gel core taken. The excised bands were purified using a High Pure PCR Product Purification Kit (Boehringer Mannheim) following the manufacturer's instructions. The average DNA concentration was 15 ng/µl.

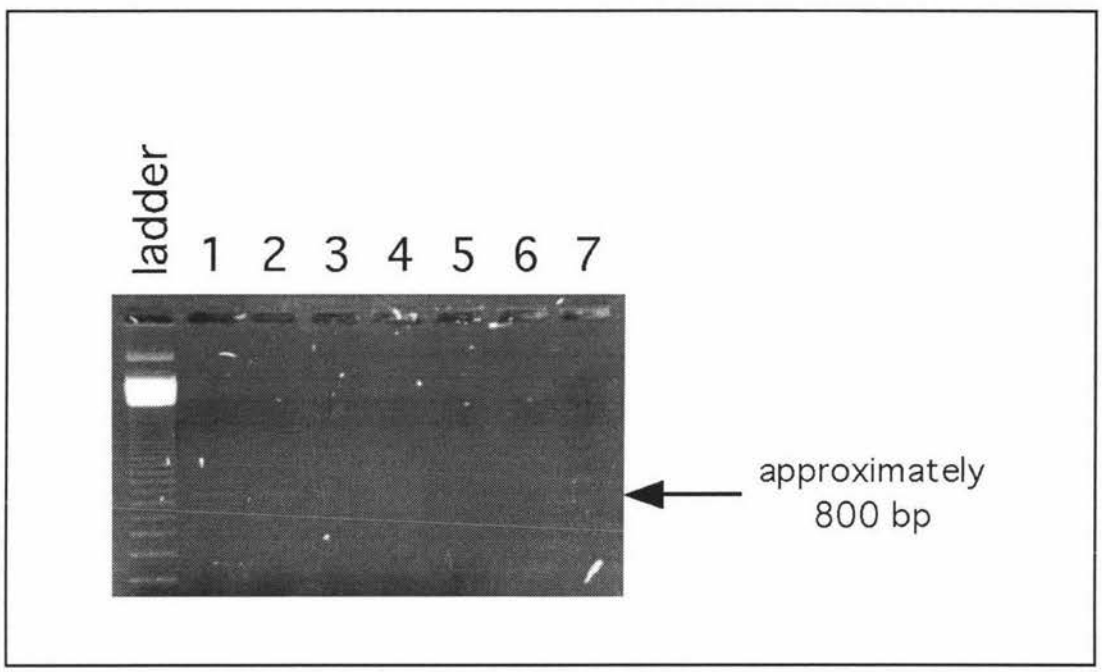

Figure 3.12 1% agarose gel with PCR products amplified from the primer combinations ND6C and H417. Lanes 1-4 contain weka DNA. Lane 5 is the negative control and a 123 bp ladder was used.

Four samples, one from each subspecies, were sent to the Applied Biosystems 377 automated sequencer and run according to the instructions of the manufacturer. None of these samples produced interpretable sequence. This could be due to several possible reasons. The possibility exists that not all the contaminating sequence in the sample was removed during the template purification procedure, that the primers did not anneal to the template properly, that the primers did not prime properly in the sequencing reaction or that there was perhaps not enough template.

Anticipating that it was because there was not enough template, 50 µl PCR reactions using the same conditions as before were performed in order to obtain

enough template for the automated sequencer which requires 20 ng/µl. A gel core of these PCR products was taken. The excised bands were purified using an High Pure PCR Product Purification Kit (Boehringer Mannheim) following the manufacturer's instructions. The amount of DNA measured in the PCR purified product was measured using the Gene Quant flurometer. Ensuring that there was 20 ng/µl in each sample, eight samples were run on the Applied Biosystems 377 automated sequencer, encompassing two samples from each subspecies. The sequence produced had low points with a lot of background noise.

This primer combination was discarded in favour of attempting primers designed in the tRNA on either side of the control region. These primers were tRNA-glu and tRNA-phe. These primers were designed from the kakapo (*Strigops habroptilus*) by Dr Bruce Robertson. A range of PCR conditions were used, including annealing temperatures ranging from 48-56 °C, and $MgCl_2$ (25 mM) ranging from 1-3 µl per 25 µl reaction. However, these primers were unable to amplify the control region in the weka.

The next method attempted was to perform a long PCR that would encompass the whole control region. Long PCR was attempted for two reasons. Firstly, so that weka specific primers could be redesigned closer to the control region, and secondly, so that it could be used as a template for other primer combinations. Two different primer combinations were used: ND6C + 12SA - H and CB1 + 12SA - H. Despite extensive trialing, neither of these primer combinations were able to amplify an approximately appropriate sized fragment in weka. In the latter primer pair, the CB1 primer was known to work on weka from earlier cytochrome b amplifications. Therefore, it was presumed that either it was the 12S sequence that was not annealing to the template properly, or that it the primer combination was poor. Therefore, a weka-specific 12S primer was designed from a previously obtained 12S weka sequence. It was anticipated that when combined with the cytochrome *b* primer the whole control region of the weka would be amplified.

The primer was called H-12S-Aves and it was designed by aligning weka and other species of rails, including the pukeko (*Porphyrio porphyrio*) and takehe (*Notornis mantelli*). Concurrently, a new ND6 primer was designed by Dr Bruce Robertson

called L-ND6-gb. It was designed in a conserved region of the ND6 gene as determined by aligning GenBank sequences for the chicken, duck and ostrich. This primer was chosen in favour of using a cytochrome *b* primer so that it would not be such a long PCR, which is more difficult to successfully amplify and would take longer and be more expensive to sequence.

Unfortunately this was also unsuccessful in sequencing the weka. In order to increase the possibility of amplification and also to ensure what was amplified is mitochondrial, mtDNA purification was performed.

3.7 PURIFICATION OF MtDNA

In order to improve the PCR amplifications, weka mtDNA was purified from nuclear DNA. It is believed that the entire control region amplifies more readily from mtDNA than from whole genomic DNA (Tarr, 1995), and that ND6E primer amplifies most successfully on purified mtDNA (S. Edwards pers comm.). This mtDNA purification procedure was performed using the protocol of Hillis et al. (1996) using a CsCI-PI gradient. This procedure was performed on a frozen liver that had been obtained from a dead weka that was found on Mokoia Island, Rotorua, in October, 1996. The weka had died from a predator control broadifacoum drop on the island.

MtDNA purification was performed because although mitochondria are common in cells, stoichiometrically there is still a much smaller proportion of the nucleotide bases of most cells that are mitochondrial as compared to nuclear DNA. Most mtDNA isolation procedures isolate the mtDNA by density-gradient ultracentrifugation (Chapman and Powers, 1984). This isolation procedure takes advantage of the closed-circular supercoiled structure of mtDNA, and uses intercalculating dyes, either ethidium bromide or propidium iodide, to differentiate between the different forms of DNA (White and Densmore, 1997). These dyes, when added to a DNA sample, slide between (intercalate) adjacent bases in the double helix causing a disruption in the periodicity of the helix. Linear DNA is able to intercalate more ethidium bromide or propidium iodide than the same length of supercoiled DNA due to the unrestrained rotation of the free ends. Since supercoiled DNA intercalates less dye, the density of the DNA-dye complex is

different from that of a linear complex, and it migrates to a different position in the density gradient. The most commonly used gradient is made with CsCl salt in a solution of Tris-EDTA buffer (TE) and, after 20-40 hours of ultracentrifugation at 200,000*g*, will differentiate among the various forms of DNA-propidium iodide complexes (White and Densmore, 1997). The resolution is sufficient to allow separation of complexes having buoyant densities that differ by less than 0.025g/ml. The mtDNA is isolated from the other components by bottom dripping the gradient and collecting only mtDNA bands (White and Densmore, 1997) (refer figure 3.13)

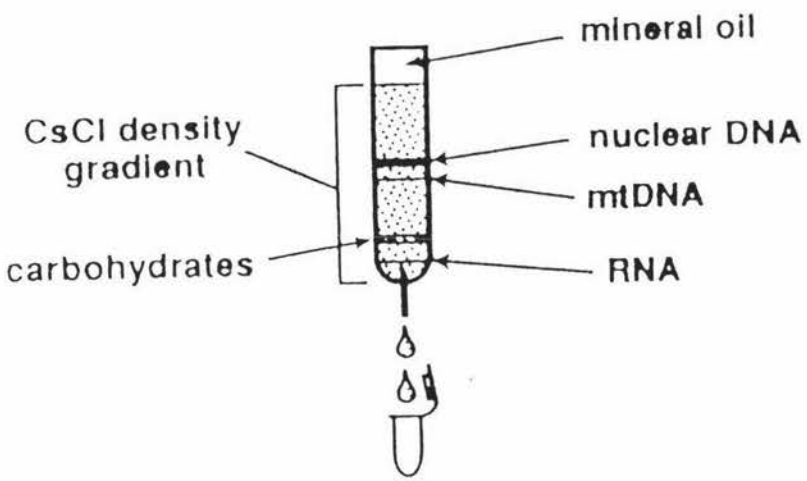

Figure 3.13 Schematic of CsCl gradient used to isolate mtDNA following 30h equilibrium ultracentrifugation run. Different nucleic acid and carbohydrate fraction correspond to correct relative mobilities in the gradient.(From:White and Densmore, 1997).

This purified mtDNA was trialed with a range of primers already trialed on genomic DNA, including the primer combinations:H417 + ND6C, l437 + CRDR1 and H-12S-Aves + L-ND6-gb. In some case, there was a cleaner product amplified, for example using the primer combination H-12S-Aves + L-ND6-gb. However, two attempts to sequence this failed. Figure 3.14 shows a cleaner product was obtained via purified mtDNA then with genomic DNA using the primer H-12S-Aves and L-

ND6-gb. This technique proved unsuccessful with weka as, although on occasion there was a cleaner product obtained visually on a agarose gel, consequent sequencing was not improved. The sequence was still very noisy and unreadable. Therefore, in terms of obtaining weka mtDNA sequence, no benefits were seen in the mitochondrial purified DNA over whole genomic DNA.

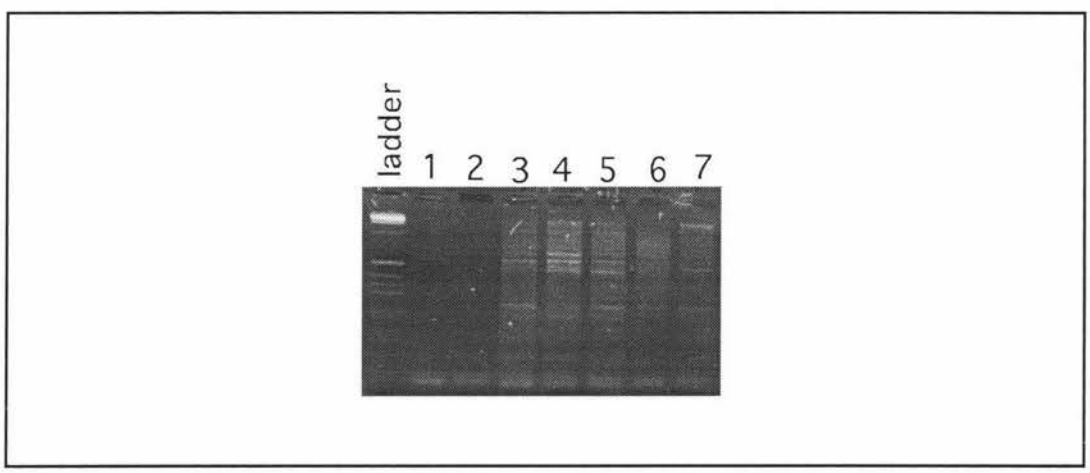

Figure 3.14 1% agarose gel containing PCR amplifications using the PCR primers H-12S-Aves and L-ND6-gb. Lane 1 was purified weka mtDNA. Lane 2 the negative control. Lanes 3-7 genomic DNA amplifications. There was a 100 bp ladder used.

The entire first domain of the control region of weka was amplified using two PCR primers: Ts 400H and L-ND6-gb. This produced multiple bands with a very faint band of the appropriate size of 700 bp. This band was band stabbed and reamplified using the same conditions as for the first PCR. Two samples, one from *G. a. greyi* and *G. a. hectori* were sequenced via an automated sequencer. Readable sequence was not obtained, therefore cloning of the control region was attempted.

3.8 CLONING THE CONTROL REGION

3.8.1 The cloning process

To begin the cloning process, the 700 bp amplified PCR product using primers TS 400H and L-ND6-gb were used to reamplify the sequences. Two individuals were used: Mokoia 13 and Mokoia 14 from *G. a. greyi.* The resulting PCR fragments

were purified using Pharmacia Biotech MicroSpin™ S-400 HR Columns and cloned according to the following procedure.

Fragments were ligated using the Promega pGEM®-T vector system 1. Ten µl ligations were performed using T4 DNA ligase following manufacturer's instructions with a 3:1 insert : vector molar ratio. These ligations were incubated overnight at 4 °C in 500 µl low DNA binding tubes. Control ligation and transformation experiments were also performed with the pGEM®-T Vector and Control Insert DNA. Background (negative) controls containing no insert DNA were also carried out.

A small amount of glycerol stock of JM109 cells were plated on to minimal medium plates and grown overnight at 37 °C. A single colony of cells were added to 5 ml of Luria broth (L-broth) and grown overnight at 37 °C. Five µl of the ligation mix was added to the JM109 cells and incubated on ice for 30 minutes. This was then heat shocked at 42 °C for 30 seconds and returned to ice for 30 minutes. 860 µl of L-broth, 20 µl of 10 mg/ml IPTG, and 20 µl 20 mg/ml X-gal were added to each transformation mixture and incubated at 37 °C for 40 minutes. The transformation mix was then plated onto 100 µg/ml ampicillin Luria agar (L-Agar) plates. The activity of ß-galactosidase can be detected on plates containing X-Gal. ß-galactosidase is not produced from plasmids which contain an insert and hence colonies containing these plasmids are white. Conversely, ß-galactosidase is produced when there is no insert and colonies containing these plasmids are blue. White colonies were chosen for DNA extraction.

3.8.2 DNA extraction

Eight white colonies were picked from each plate and were grown overnight at 37°C on an angled shaker in 3 ml of L-broth with kanamycin (6µl of 50 mg/ml). 1.5 ml of the culture was centrifuged for 30 seconds. The supernatant was discarded and the pelleted cells were resuspended in 100µl of cold TEG buffer (25mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose) and held at room temperature for 5 minutes. 200 µl SDS/NaOH solution (1% sodium dodecyl sulphate, 0.2 M sodium hydroxide) was added and the tube placed on ice for 10 minutes. 150 µl of 5 M KOAc solution (60% 5M potassium acetate, 11.5% glacial

acetic acid) was added and the tube placed on ice for five minutes prior to centrifugation at 13,000g at 4 °C. The supernatant, containing the plasmid, was transferred to a fresh tube and 1 ml of 100% ethanol was added and the tube placed in the -20 °C freezer for 10 minutes. This was then centrifuged at 13,000g for 10 minutes. After that the supernatant was vacuum dried and the pellet resuspended in 20-30 µl of Milli-Q water.

The plasmid DNA (10 µl) was digested at 37 °C with the restriction enzymes *Eco*RI and *Hin*dIII (5 units) (BRL) in the manufacturers recommended buffers, in the presence of RNAase (10 µl/ml) to a 20 µl volume. These enzymes cut at either end of the MCS and thus release the insert DNA. 10 µl of the digested DNA was electrophorised in a 1.2% agarose gel in TBE buffer (134 mM Tris-HCl pH 8.8, 74.9 mM boric acid 2.55 mM EDTA) at 40 volts. An ethidium bromide (0.5 μgml^{-1}) stained agarose gel was used to visualise the DNA under ultraviolet light. This enabled the identification of the recombinant cells.

Cells containing the recombinant plasmids were grown in 5 ml of L-broth, with kanamycin (50 µl/ml) overnight at 37 °C on an angled shaker. DNA templates were prepared using MagicMiniPrep™ columns (*Promega*). Approximately 3 ml of culture was centrifuged and the pellet resuspended in TE RNAase A (Resuspension Solution, *Promega*). The cells were then lysed with a NaOH/SDS solution (Lysis Solution, *Promega*) and the bacterial DNA precipitated with KOAc (Neutralisation Solution, *Promega*). A DNA purification resin was added to denature the proteins. The solution was then passed through a MagicMiniPrep™ column. This column was washed with Column Wash Solution (*Promega*), and spun dry in a microcentrifuge. The plasmid DNA was eluted off the column with 65 °C Milli-Q H_2O and the resuspended plasmid collected in an Eppendorf tube. 3µl of the recovered DNA was electrophorised in a 1.2% agarose gel in TBE buffer (134 mM Tris-HCl pH 8.8, 74.9 mM boric acid 2.55 mM EDTA) at 40 volts to estimate the quality and quantity of the template DNA. An ethidium bromide (0.5 μgml^{-1}) stained agarose gel was used in the agarose gel to visualise the DNA under ultraviolet light. The purified DNA was used for sequencing.

3.8.3 Sequencing of DNA templates

The plasmids were sequenced on an ABI 377 automated sequencer using M13 forward and reverse primers. These primers anneal to sites flanking the insert in the plasmid. Two clones were sequenced, and aligned by eye, using SeqEd and two weka specific PCR primers; L-Weka- and H-Weka-domII were then designed to amplify a ~900 base pair region of the first domain of the control region. This was done by aligning the weka sequences with the kakapo, duck, chicken, ostrich and some passerine sequences to observe conserved regions so that the primers may also be used for cross-species amplifications.

3.8.4 PCR amplifications

The two weka-specific primers described above were used to successfully amplify the weka control region. After optimisation the amplifications were carried out contained 2.5 µl of Perkin Elmer 10X PCR buffer II, 2.0 µl of $MgCl_2$ (25 mM), 2.0 µl of each primer (10 pmol/µl), 0.1 µl of dNTPs (20 mM of each deoxynucleotide), 0.1 µl Perkin Elmer Ampli-Taq Polymerase (5U/µl), 1 µl of DNA and Milli-Q H_2O to make up 25 µl.

This primer combination did not produce sufficient product for sequencing from one PCR product in all samples. Instead often PCR products had to be pooled in order to obtain enough template for automated sequencing (20 ng/µl). Also this primer combination worked better on some populations than others, working particularly well on the North Island populations. This could be due to the fact that the primers were designed from the sequences of two North Island weka.

The level of degradation of the samples could also affect the ability of the primer to amplify the samples. The Mokoia Island weka samples which produced the best amplifications were collected in November, 1997, and the blood samples were stored using non-clotting agents. The other samples were collected between 1987 and 1993 and were only stored in equal amounts of water. The age of some samples and poor storage conditions could perhaps result in the difficulty experienced in amplifying these individuals. When checking the quality of DNA on an agarose gel, the older samples did not appear as bright as the more recently collected samples. However, adding more DNA to these samples did not improve the brightness of their bands when visualised.

Two different size bands (~900 bp and ~1100 bp) were also observed among the samples (refer figure 3.15). All samples from Chatham Island had the larger sized fragment as did some individuals throughout New Zealand, encompassing each subspecies. In some populations both fragments occurred. This is presumed to be due to an indel event, but could also be the result of non-specific amplifications, heteroplasmy or contamination.

3.8.5 Template Purification

Amplified products were separated from non-specific PCR products and unincorporated PCR primers, by agarose-gel purification. In most samples, a few (2-4) 25 µl PCR products had to be pooled, ethanol precipitated and resuspended in 10 µl of Milli-Q H_2O and a gel core taken, in order to obtain enough template for sequencing. The excised bands were purified using a High Pure PCR Purification Kit (Boehringer Mannheim).

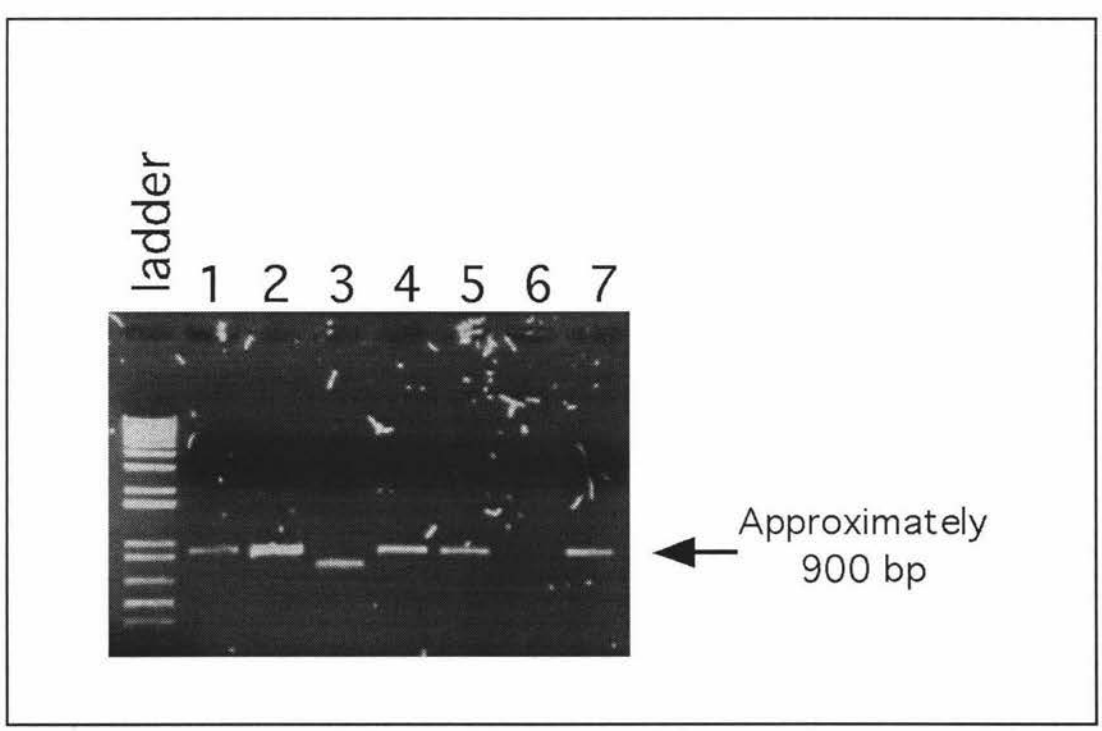

Figure 3.15 Weka specific control region PCR amplifications. This gel shows clearly the different sizes from weka from the Chatham Islands. Lanes 1 and 2 contain *G. a. Greyi.* Lane 3 contains *G. a. hectori.* Lanes 4 and 5 contain *G. a. australis.* Lane 6 is the negative control. Lane 7 is *G. a. scotti.* A 1kb ladder was used.

Following this, a total of 20 individuals encompassing seven populations were run on the Applied Biosystems 377 automated sequencer. The seven populations were Kapiti Island, Mokoia Island, Gisborne, Te Mahia, Nukuwaiata and Totaranui. The automated sequencing reactions were performed in the Molecular Ecology Laboratory, not at the automated sequencer. These reactions were carried out in 10 µl volumes using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Kit (PE Applied Biosystems). Cycle sequencing reactions were subjected to 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes for 25 cycles in a Hybaid thermal cycler. The excess DyeDeoyx terminators were removed from the reaction mix by ethanol/$NaOA_c$ precipitation, followed by a 70% ethanol wash. The precipitates were dried in a concentrator and resuspended in 5 µl of formamide/50 mM EDTA (pH 8.0). The samples were denatured at 90°C, transferred to ice, and immediately loaded onto the automated sequencer and run according to the instructions of the manufacturer.

The resultant sequences were aligned in SeqEd, and then copied into PAUP 4.0d64 (Swofford, 1998). When variation was found between individuals from the same location, the nucleotide site was double checked against the electropherogram. Approximately 216 bp of good, readable sequence was obtained for the 20 individuals (refer figure 3.13). A representative clamatogram is also show in appendix one.

3.9 RESULTS

Twenty weka from the North and South Island populations were sequenced for the 5' end of the control region using both automated and manual sequencing techniques. The complete data set is presented in figure 3.16. Phylogenetic relationship among the sequences were investigated using maximum parsimony, maximum likelihood and cluster analysis methods.

Maximum Parsimony is a method of construction of phylogenetic relationships among taxa. It is based on the principle of minimising the number of events needed to explain the data. In phylogenetic analysis, the optimal tree under the maximum parsimony criterion is the tree that requires the fewest number of character-state changes (which may be differentially weighted across characters and/or character

states. This method is often simply called parsimony. Of the existing numerical approaches to inferring phylogenies directly from character data, methods based on the principle of maximum parsimony have been the most widely used (Swofford et al., 1996). A heuristic parsimony analysis was performed on the aligned control region sequence. The consensus parsimonious phylogenetic hypotheses is presented in figure 3.17.

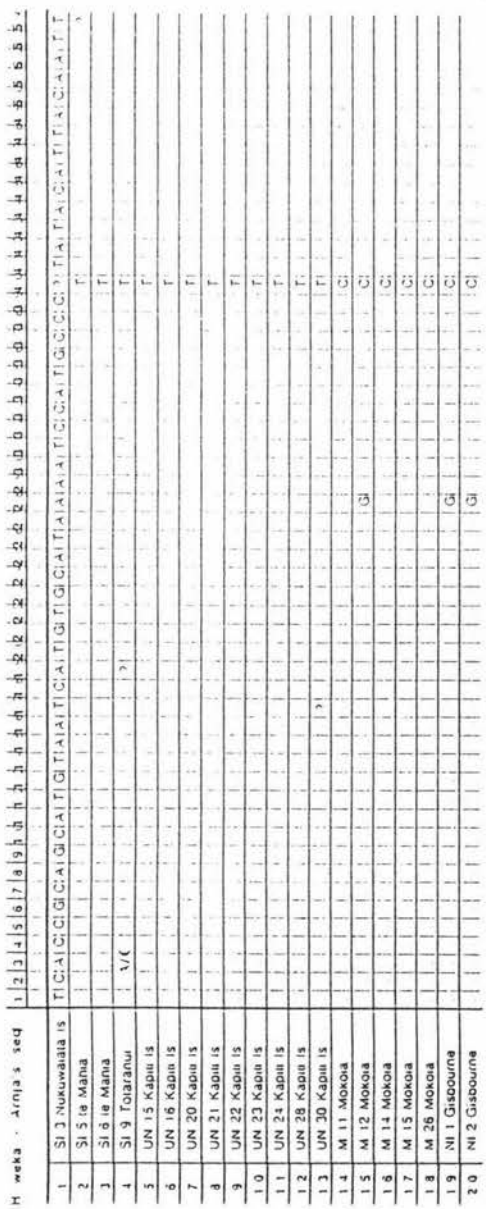

Figure 3.16 Aligned data set used in this analysis of variation among the the 20 indivdiuals sampled. (SI = South Island indivdiuals from Nukuwaiata, Te Mahia and Totaranui all located in the Marlborough Sounds, UN = Kapiti Island individuals, M = Mokoia individuals, NI = Gisbourne individuals).

	H weka - Arnja's seq	555555555555555777777777788888888889999999999000000000
1	SI 3 Nukuwaiata Is	?ATGTACTGAGACAACTAATTCATGCTATAAGACATACTATGTAATCGCTCCAC
2	SI 5 te Mania	?
3	SI 6 te Mania	A
4	SI 9 Totaranui	?.................... /G/?
5	UN 15 Kapiti Is	A
6	UN 16 Kapiti Is	?
7	UN 20 Kapiti Is	A
8	UN 21 Kapiti Is	A
9	UN 22 Kapiti Is	A
10	UN 23 Kapiti Is	A
11	UN 24 Kapiti Is	A
12	UN 28 Kapiti Is	A
13	UN 30 Kapiti Is	A
14	M 11 Mokoia	A
15	M 12 Mokoia	A.....................................C
16	M 14 Mokoia	A
17	M 15 Mokoia	A
18	M 26 Mokoia	A
19	NI 1 Gisbourne	A.....................................C
20	NI 2 Gisbourne	A.....................................C

Figure 3.16 Continued.

	weka - Arnja's seq	0 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 6 6 6
		3
1	SI 3 Nukuwaiata Is	C T A C T G T C T G C G T A C C C C C T A C T T T G ? A G G A T A C C C G G T T A T T G G A A C T A A C C A
2	SI 5 te Mania	C ?
3	SI 6 te Mania	C ?
4	SI 9 Totaranui	?
5	UN 15 Kapiti Is	? T G
6	UN 16 Kapiti Is	? T G
7	UN 20 Kapiti Is	C A T G
8	UN 21 Kapiti Is	C A T G
9	UN 22 Kapiti Is	C A T G
10	UN 23 Kapiti Is	C A T G
11	UN 24 Kapiti Is	C A T G
12	UN 28 Kapiti Is	C A T G
13	UN 30 Kapiti Is	? C A T G
14	M 11 Mokoia	C A T G
15	M 12 Mokoia	C A T
16	M 14 Mokoia	C A T G
17	M 15 Mokoia	C A T G
18	M 26 Mokoia	C A T G
19	NI 1 Gisbourne	C A T
20	NI 2 Gisbourne	C A T

Figure 3.16 Continued.

H weka - Arnja's seq#		6	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
1	SI 3 Nukuwaiata Is	C	A	T	G	G	C	A	C	A	A	C	A	C	T	T	C	C	C	T	C	G	A	C	T	A	A	G	G	G	T	A	C	A	A	A	C	C	C	C	T	T	?	A	T	T	T	C	A	C	A	C	A	A	C	T
2	SI 5 Te Mania																																											?									
3	SI 6 Te Mania																																											A				G	A				
4	SI 9 Totaranui																																											?				G	A				
5	UN 15 Kapiti Is				A		G														G									G				T									A				G	A				
6	UN 16 Kapiti Is				A		G														G			?						G				T									?				G	A	?		A	
7	UN 20 Kapiti Is				A		G														G									G				T									A									
8	UN 21 Kapiti Is				A		G														G									G				T									A									
9	UN 22 Kapiti Is				A		G														G									G				T									A									
10	UN 23 Kapiti Is				A		G														G									G				T									A				G	A				
11	UN 24 Kapiti Is				A		G														G									G				T									A									
12	UN 28 Kapiti Is				A		G														G									G				T									A									
13	UN 30 Kapiti Is				A		G														G									G				T									A									
14	M 11 Mokoia				A		G														G									G				T	T								A	G								
15	M 12 Mokoia			A	A		G														G									G				T									A									
16	M 14 Mokoia				A		G														G									G				T	T								A	G								
17	M 15 Mokoia				A		G														G									G				T	T								A	G								
18	M 26 Mokoia				A		G														G									G				T	T								A	G							G	G
19	NI 1 Gisbourne			A	A		G														G									G				T									A									
20	NI 2 Gisbourne			A	A		G														G									G				T									A									

Figure 3.16 Continued.

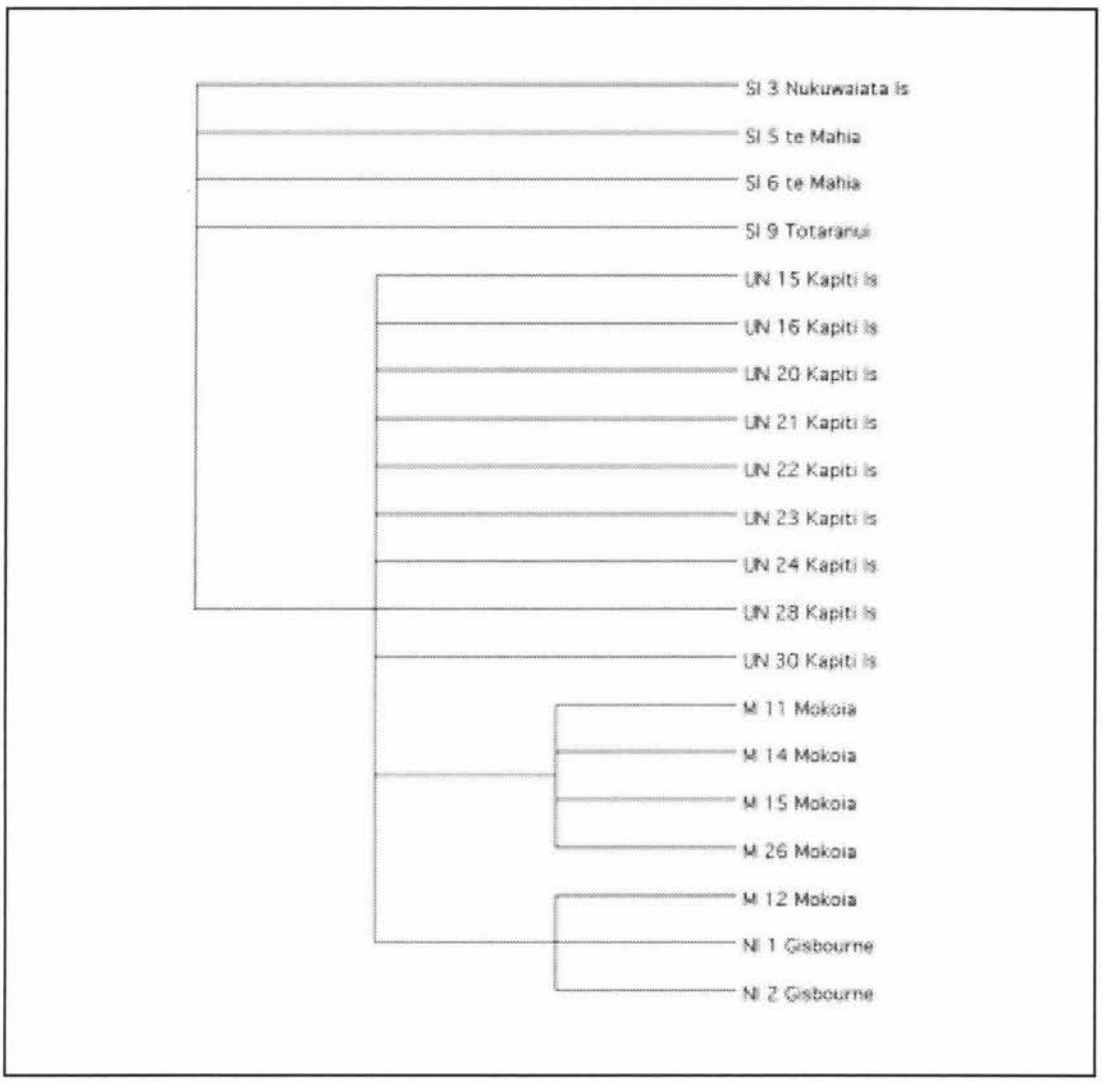

Figure 3.17. The consensus parsimonious tree for the 20 control region weka samples used in this analysis.

From this heuristic parsimony consensus tree, there are three observations that are able to be drawn. The first is that the North and South Island populations cluster apart. The second is that within the North Island cluster there is a cluster of four Mokoia Island weka and also a cluster of two Gisbourne weka and a Mokoia weka. The third is that the Kapiti Island individuals are identical.

A bootstrap analysis was also conducted on the 5' end of the control region of weka. The bootstrap analysis samples data aligned columns at random, with replacement, from the original data set until a new data set containing the original number of observations is obtained (Swofford et al., 1996). One thousand bootstrap replicates were performed. If the replicates of sub-sampled data result in trees that are statistically consistent with the original data set, then the entire data set has a consistent phylogenetic signal in it (Swofford et al., 1996). The 50% clear majority rule consensus bootstrap (Swofford, 1989: PAUP 4.0b2 Phylogenetic Analysis Using Parsimony) was identical to the most-parsimonious tree (refer fig 3.18). The bootstrap analysis showed that the consensus parsimonious tree is statistically justified.

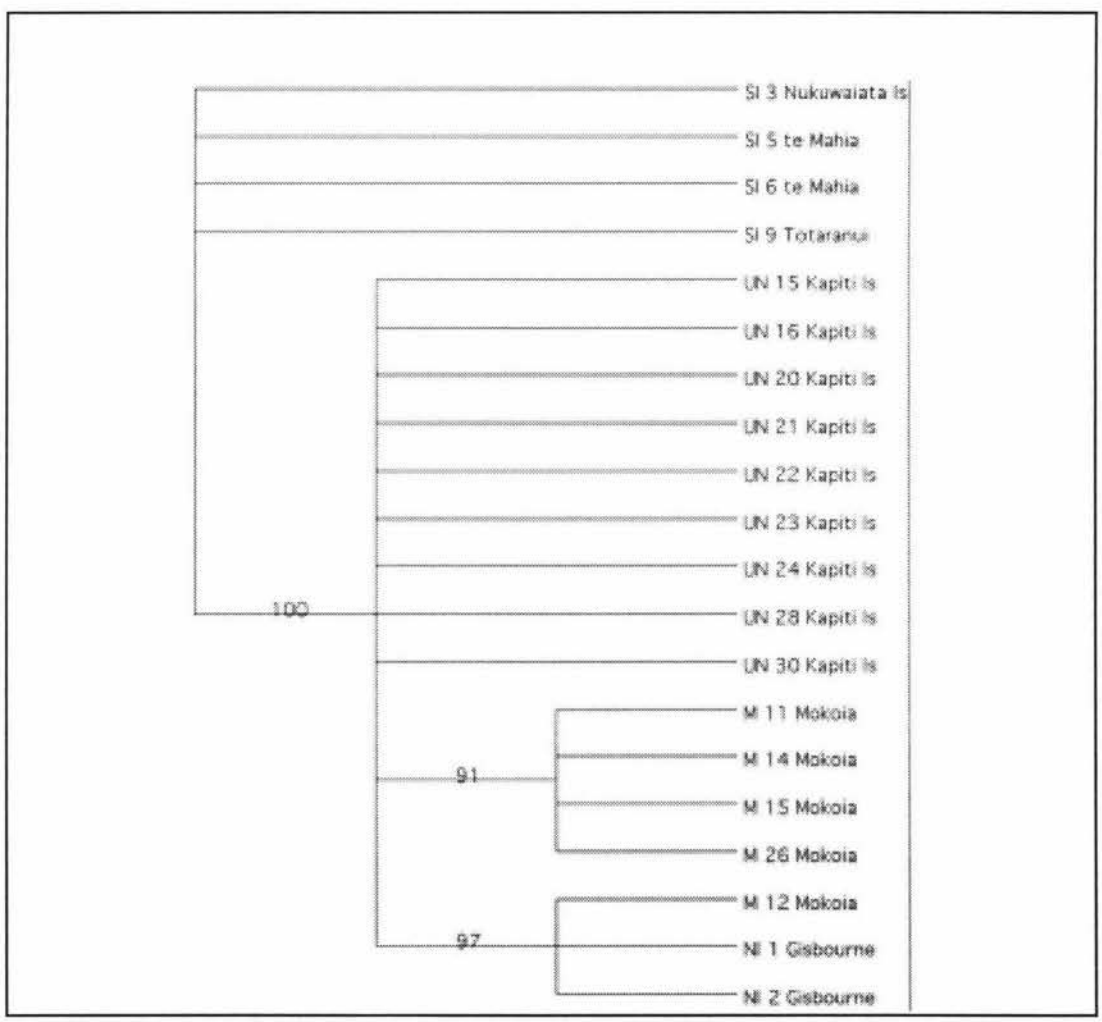

Figure 3.18 50% clear majority rule bootstrap of the 5' end of the control region in the 20 sampled weka, parsimony analysis. Bootstrap values are shown.

The two most commonly used distance methods are UPGMA (unweighted pair group method using arithmetic averages) (which asssumes a constant molecular clock) and neighbour-joining (which does not make this assumption). Both methods were used on the 5' end of the control region in weka.

Cluster analysis (UPGMA) is a method of hierarchical grouping of taxa or sequences on the basis of similarity or distance. The method of cluster analysis is conceptually simple. The raw data are provided as a table of distances between all pairs of the taxa. The tree is constructed by linking the least distant pairs of taxa, followed by successively more distant taxa, or groups of taxa. When two taxa are linked, they lose their individual indentities and are subsequently referred to as a single cluster. Initally, each taxon constitutes its own cluster. At each step in the process, as two clusters are merged into one, the number of clusters declines by one. The process is complete when the two clusters are merged into a single cluster containing all of the original taxa.

The results from the UPGMA are shown in figure 3.19. These results also show that the South Island individuals and North Island individual group in two clusters. Also within the North Island samples there are three clusterings evident. The first is among the Kapiti Island individuals, the second the two Gisbourne individuals joined by Mokoia 12, and the third cluster is an aggregate of the remaining four Mokoia individuals.

The neighbour-joining method was also performed on the 5' end of the weka control regions used in this study. Neighbour Joining is a heuristic method for obtaining a point estimate of a minimum evolution tree. Neighbour Joining is conceptually related to traditional cluster analysis, but removes the assumption that the data are ultrametric. Ultrametric distances are pairwise distance values that precisely fit a rooted tree with a constant molecular clock. In practical terms, neighbour joining does not assume that all lineages have diverged equal amounts. In contrast to cluster analysis, Neighbour Joining keeps track of nodes on a tree rather than taxa or clusters of taxa. The raw data are provided as a distance matrix and the initial tree is a star tree. A modified matrix is constructed in which the separation between each pair of nodes is adjusted on the basis of their average

divergence from all other nodes. The tree is constructed by linking the least distant pair of nodes as defined by this modified matrix. When two nodes are linked, their common ancestor node is added to the tree and the terminal nodes with their respective branches are removed from the tree. This pruning process converts the newly added common ancestor into a terminal node on a tree of reduced size. At each stage in the process, two terminal nodes are replaced by one new node. The process is complete when two nodes remain, separated by a single branch (Swofford et al., 1996).

Figure 3.20 shows the results of a neighbour-joining analysis. This method also resulted in a divisional split between the North and South Islands and the three intra-North Island splits grouping the Kapiti individuals, the two Gisborne and one Mokoia individual, and the remaining four Mokoia individuals. This is consistent with the other methods of analyses tried.

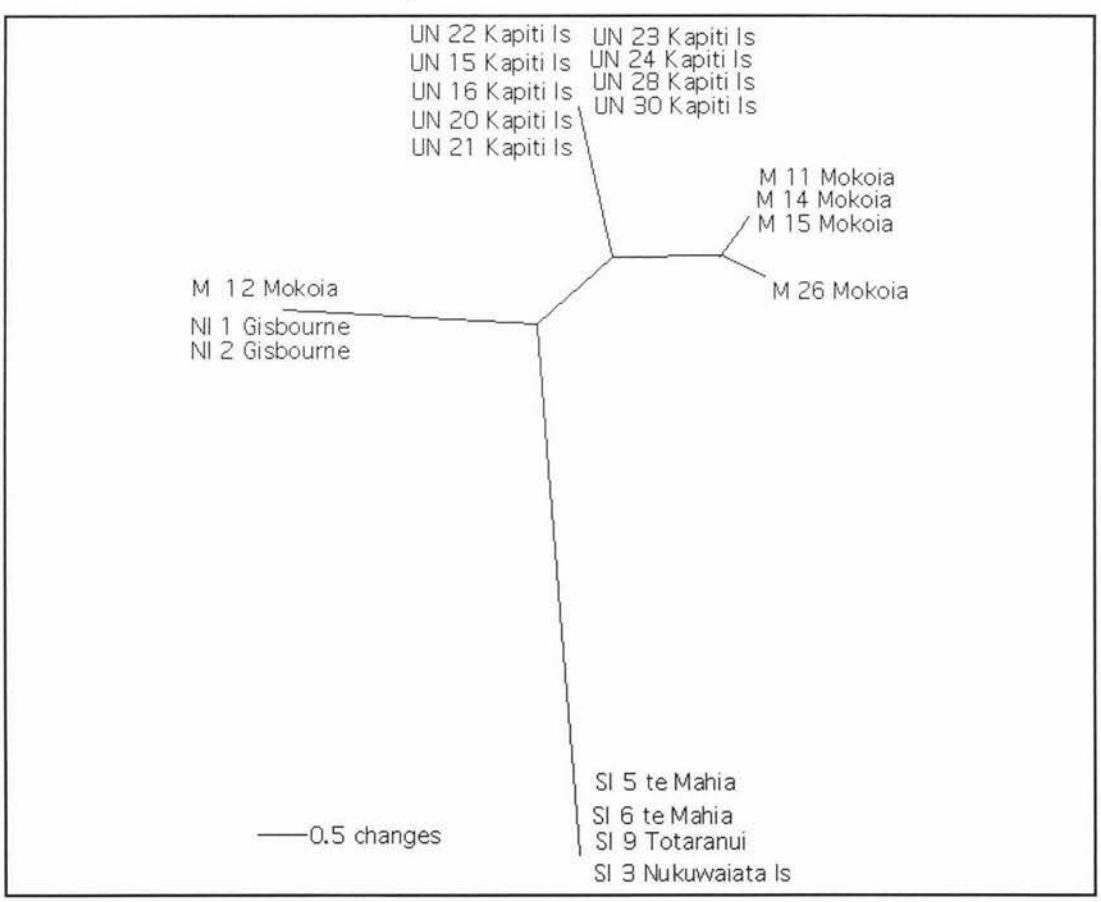

Figure 3.19 Unweighted phylogram of the UPGMA analysis of the 5' end of the control region in weka.

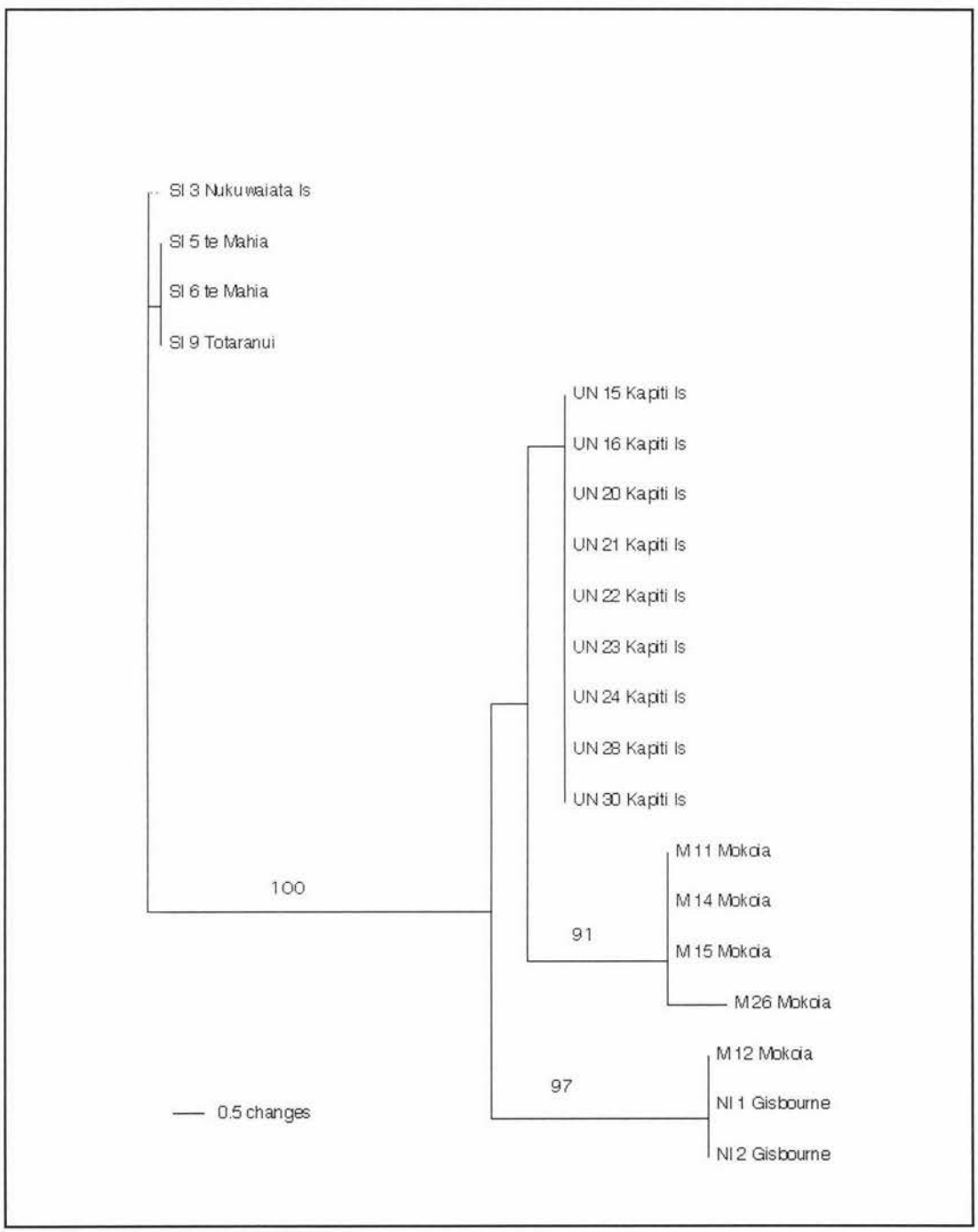

Figure 3.20 Neighbour Joining analysis of the 5' end of the control region of the 20 weka used in this study. The results are the consensus of 1000 bootstrap samples. The bootstrap values are shown.

Percentage sequence difference between the individuals and populations was also analyzed using PAUP*. Refer figure 3.21 for substitution differences between pairwise combinations. Table 3.5 shows the mean substitutional difference between pairwise combinations and the percentage sequence difference between the various populations.

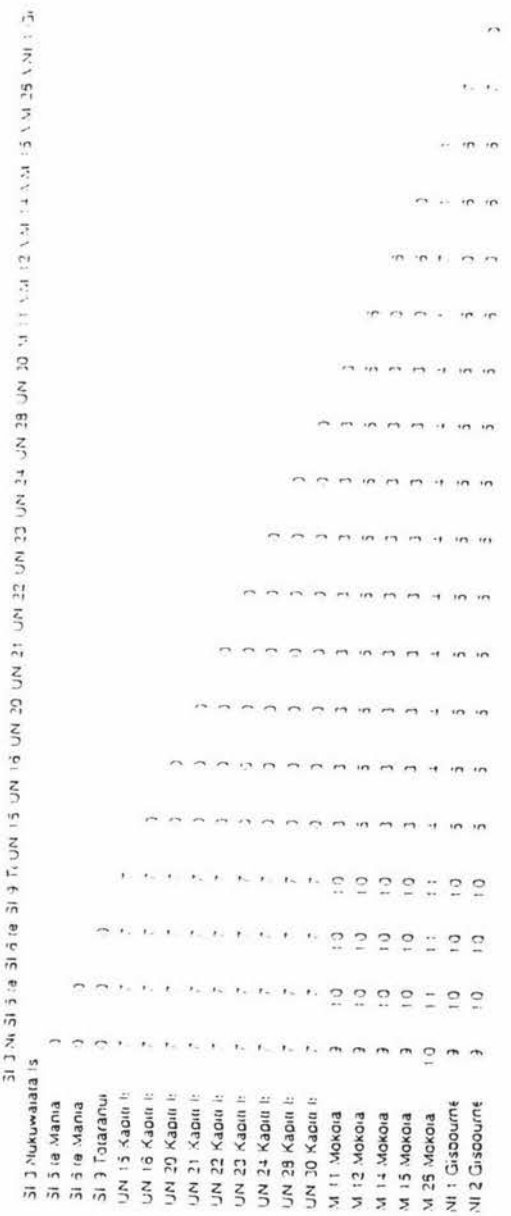

Figure 3.-21 Substitution differences between pairwise combinations of the 5' end of the weka control region (calcualted using PAUP*). (SI = South Island indivdiuals from Nukuwaiata, Te Mahia and Totaranui all located in the Marlborough Sounds, UN = Kapiti Island individuals, M = Mokoia individuals, NI = Gisbourne individuals).

Table 3.5 This shows the mean substitutional difference between pairwise combinations for populations. This table also shows the percentage sequence difference between the different populations.

Populations	Mean substitution difference between pairwise combinations	Standard deviation of mean substitutional difference between pairwise combinations	Percentage sequence difference
North Island/ South Island	8.27	1.49	3.83
South Island	0	0	0
Kapiti Island	0	0	0
Mokoia Island	2.8	3.01	1.29
Mokoia/Gisbourne	3.71	3.07	1.72
North Island	4.6	2.3	2.16
Mokoia Island/ Kapiti Island	2.09	2.04	9.68
South Island/ Kapiti Island	3.32	2.48	1.49
Gisbourne + M12	0	0	0
Mokoia (M11, M14, M15, M26)	0.33	0.58	1.53

Within and between population sequence diversity was also analysed. These results are shown in Table 3.5. The within population diversity of mtDNA haplotypes ranged from 0% sequence divergence, where all birds had the same sequence, such as Kapiti Island, up to 1.29% sequence difference in the Mokoia Island population. There was a clear division between the North Island/South Island individuals. These two clades had a 3.83% sequence difference. The highest percentage sequence difference between populations was seen when Mokoia Island and Kapiti Islands were compared being 9.68%. The within North Island sequence difference was the second highest at 1.29% sequence difference which included the Gisbourne, Mokoia Island and Kapiti Island individuals. The within-population sequence variations levels within the South Island and Gisborne populations were 0% sequence difference. This pattern was also seen when comparing the two

Gisbourne weka with Mokoia 12 which clustered together in all of the analyses performed. However, when all five Mokoia Island weka (including Mokoia 12) were analysed for percent sequence difference, there was a percentage sequence difference of 1.29%, this was increased to 1.53% with the removal of the individual Mokoia 12 which clusters with the two Gisborne individuals.

3.10 DISCUSSION

There are six interesting findings that have arisen from this research on the mitochondrial genes of weka. Firstly, is the pattern of nucleotide variation among weka control region sequences resulting in the existence of two large clusters of mitochondrial haplotypes. The second interesting finding was the remarkable degree of homogeneity observed in the Kapiti Island population. The third is the relationship of the Mokoia Island and Gisborne region weka. The fourth is the very high percentage sequence difference between Kapiti Island and Mokoia Island (9.68%). The fifth is the large proportion (over 1000 bp) of near identical sequence between the populations of weka when employing regions other than the control region that evolve at a slower rate. The last relates to the determination of provenance of populations of unknown origins.

Firstly, the North/South Island divisions of 3.83% sequence difference, were supported by the three methods of phylogenetic analysis used in this study and had bootstrap values of 100. These two clades correspond with the two weka subspecies *G. a. greyi* and *G. a. australis*. From this analysis, it appears that these two subspecies are in fact real. No comment can be made regarding the remaining two subspecific designations in weka (*G. a. scotti* and *G. a. hectori*) until sequence variation is for these two subspecies is obtained. There is a similar North Island/South Island pattern is seen in the kiwi (Baker et al., 1995).

The level of sequence divergence between the North Island/South Island weka populations was relatively high at 3.83% compared to other studies that have found subspecific sequence divergence levels to be quite low. In the seaside sparrow subspecies (*Quiscalus quiscula*), the mean sequence difference was only 1% (Avise and Nelson, 1989), the fox sparrow had 2% (Zink, 1996), and the song sparrow, the chirping sparrow and the red winged black birds all had less than 1% sequence

divergence between their haplotype sequence divergence (Zink, 1996). Despite the reasonably high level of sequence divergence between the North Island/South Island weka haplotypes, the two subspecies are known able to be able to interbreed and produce viable offspring (Ward et al., 1992). Although there is no suggestion of these two weka clades representing distinct species, their subspecific status is almost certainly valid.

The second interesting result is the remarkable degree of homogeneity exhibited in the Kapiti Island weka. The nine Kapiti Island weka sampled were all genetically identical. Cluster analysis grouped these weka as a subgroup of the North Island weka. This lack of variation is an interesting result which fits in with anecdotal evidence regarding the origin of the Kapiti Island population. According to Beauchamp (1987) the initial Kapiti weka population is reportedly derived from three individuals released in 1896. Beauchamp hypothesizes that a pair of Stewart Island weka (*G. a. scotti*) were donated to a local Waikanae Maori chief, Wi Parata, by Captain Fairchild or Captain Ballons of the government steamer *Hinemoa*, but that the female died and the male was released on the island with a female weka and chick captured from the surrounding mainland region, a year or so prior to the island being declared a reserve. Wi Parata later liberated several wekas on Kapiti from birds caught in the Marlborough Sounds (Beauchamp, 1986). Thus, it was thought that the Kapiti population may be derived from wekas of three subspecies.

From the results obtained in this study, it suggests that although several forms of weka may have been introduced to Kapiti Island, they have not all have become established in terms of mtDNA. Instead, it appears that Kapiti Island weka may be derived from one or a few female weka from the *G. a. greyi,* due to their large degree of homogeneity. Although these birds are very similar for intrapopulation variation, the Kapiti Island weka do appear distinct from other *G. a. greyi,* but not to the degree where they do not cluster together using cluster analyses. It is, however, possible that there are other mitochondrial lineages present on Kapiti Island but that these other mtDNA lineages were not sampled. A paternal contribution from the subspecies to the nuclear genome is quite possible, but can not be detected by mtDNA. Refer section 3.11 for a preliminary study on microsatellite variation in weka.

The third interesting finding in this study was that one Mokoia weka grouped with the two Gisbourne weka. This was useful as serving as a indicator of their translocation history as the Mokoia Island weka were transferred from Gisbourne. From the fact that the four other Mokoia Island weka were variable, it can be hypothesized that the Gisbourne weka population itself may be variable, despite the large fluctuctations in populations size that it has experienced in the past.

The fourth interesting feature in this analysis was the high mean percentage sequence difference between the Mokoia Island and Kapiti Island individuals (9.68). This percentage difference is approximately two and a half times the sequence divergence of the North Island/South Island weka. In order to explain this unexpectedly high sequence divergence a simple biogeographic hypothesis is proposed. Up until the late Pleistocene there was a Cook Strait landbridge which was severed by rising water levels. Until this time the North and South Island populations were able to mix. This mixing of North Island/South Island populations via the Cook Strait landbridge up until the late Pleistocene also occurred in brown kiwi (Baker et al., 1995).

The volcanic ranges of the Ruahines and the Tararuas provided a geographical barrier to the mixing or migration of individuals from the East Coast area and the lower West Coast of the North Island. This theory fits in with the translocation histories of the two populations, with the Mokoia weka originating from the Gisborne area and the female lineage on Kapiti Island being thought to have originated from the lower East Coast of the North Island.

The fifth interesting feature is that in over 1000 base pairs that were sequenced for a number of samples across the mitochondrial genome in weka, prior to the control region sequence working, there was predominantly no variance. Yet in the faster evolving control region, there was evidence of subspecific structuring within weka. These results are consistent with a recent presence of weka in New Zealand. The slower protein coding regions of the mitochondria are still all very similar, yet the faster evolving control region is highlighting that variation does exist in weka. Assays of the populations for microsatellite variation may be able to identify very recent evolution of subdivision within weka. This possible subdivision may be able

to increase the resolution of the weka phylogeny as microsatellite loci will frequently accumulate mutations within populations before they are spread widely among populations (Baker, 1993).

The last interesting finding is that the provenance of an unknown population was established. This was Kapiti Island weka populations. Untill now their origin was assumed mainly upon anecdotal evidence. It was presumed that Kapiti Island was a hybrid of two or three subspecies of weka (Ward et al., 1992). Kapiti Island was found be part of the North Island weka subspecies.

There are also other populations of unknown provenance in weka. If representative individuals from all populations of known origin were sequenced using the weka-specific control region primers designed in this study, it is hypothesised that their provenance would also be able to be discovered.

3.11 MICROSATELLITE ANALYSIS OF WEKA

A pilot study into the variability of microsatellites in weka was also performed as a possible avenue of finding nuclear variation in weka populations. Microsatellites can offer greater resolution than mtDNA in population analysis, but need a larger sample size than what is needed for sequence analysis. This is because it is likely that more alleles will be encountered (McDonald and Potts, 1997). Microsatellites can compare favourably to mtDNA also, as both the paternal and the maternal background can be traced with nuclear markers (Primmer et al., 1996).

Microsatellites have among the highest rates of DNA mutation known and typically exhibit high levels of polymorphism in natural populations (Lambert and Millar, 1995). Microsatellite repeat motifs are regions of short (approximately 1-6 base pairs), simple (usually dinucleotide) sequence repeats. Microsatellite loci are detected using PCR primers designed to anneal to single-copy DNA flanking the repetitive polymorphic sequence. The primers amplify the block of repetitive DNA and the different length variants are detected using radioactively end-labelled primers and polyacrylamide sequencing gels.

High variation in microsatellite markers have been described in species with little mitochondrial genetic diversity (Hughes and Queller, 1993). This may be very useful if mtDNA variation could not be found in weka. There are examples of low variation in mitochondrial DNA sequences where microsatellites have proved effective population markers. For example, polar bears (Paetkau and Strobeck, 1995). Microsatellites have also been suggested as a tool for monitoring loss of variation in isolated or remnant populations (Paetkau and Strobeck, 1994).

The use of microsatellites for the study of natural populations is increasing steadily (Ellegren, 1995). This is not suprising due to the attractive features of the marker such as hypervariability, abundance and a PCR-based typing procedure. However, the fact that novel markers often have to be isolated when analysis of a 'new' species starts, acts as a hindrance for the widespread utilisation of microsatellites among ecologists who lack the facilities, time and money for cloning and sequencing. The usual need for species-specific markers relates to the fact that PCR primers require a high degree of homology to the target sequence in order to function. Microsatellites are generally embedded within non-coding sequences and because of the comparable high mutation rate found in silent DNA, accumulated mutations in microsatellite flanking sequences will eventually inhibit amplification in one species with primers developed from a distantly related species (McDonald and Potts, 1997).

It is clear that the chance of a successful cross-species (heterologous) amplification of any DNA sequence is inversely related to the evolutionary distance between the two species (Primmer et al., 1996). Several studies have shown that microsatellite loci isolated from various species amplify corresponding, polymorphic loci in closely related species, but not in more distant species (Moore et al., 1991 and Schlötterer et al., 1991). In a study by Primmer et al. (1996) they found that some microsatellite primers developed for swallow and pied flycatcher genomes are able to amplify to perform cross-species amplification in 48 different bird species spanning different orders.

With this in mind, the ideal situation would have been to develop microsatellite markers specific for weka by constructing a partial genomic library (a set of cloned

DNA fragments which together represent a portion of the entire genome). Then selecting the microsatellite-containing DNA sequences as required by using repeat DNA probes and subsequent sequencing in order to develop suitable primers from the flanking sequences. A library was not constructed for weka due to the lack of need as a region of the mitochondrial control region in weka was eventually successfully sequenced showing variation in the species. However, cross species amplification was tested using so-called universal avian microsatellite primers.

The possibility of performing cross species amplification with avian microsatellite markers is not only important for population studies of individual species, but will also serve as a tool in various applications where the genetic characteristics of two or more species are being compared. For example, in phylogeny construction, the high mutation rate found at microsatellite loci, compared to mitochondrial DNA make them very useful in dissecting genetic differences between very closely related species or within species (Weber and Wong, 1993).

3.11.1 Amplification and Results

PCR optimisation was attempted using chicken primers, designed by Moran et al. (1986) and Tasmanian Native Hen (designed by Sandie Degnan) primers. Optimisation included titrating a range of variables, such as $MgCl_2$ concentration and primer concentrations, and varying reaction conditions such as annealing temperatures and the number of cycles.

Trial amplifications were carried out under the following temperature conditions: an initial denaturation of 94 °C for 2 minutes, followed by 30 cycles of 94 °C for 1 minute, 52 °C for 50 seconds, and 72 °C for 30 seconds, ending with a final extension cycle of 72 °C for 5 minutes. The reaction contained 2.5 µl of Perkin Elmer 10X PCR buffer II, 2.0 µl of $MgCl_2$ (25 mM), 2.0 µl of each primer (10 pmol/µl), 0.5 µl of dNTPs (20 mM of each deoxynucleotide), 0.15 µl Perkin Elmer Ampli-Taq Polymerase (5U/µl), and Milli-Q H_2O to make up 25 µl.

These primers produced varying results (refer table 3.6). Some microsatellite primers displayed a strong amplification band (refer figure 3.22). Two Tasmanian

Native Hen primers (TM 18 and TM 20) worked very well, producing clean strong bands. Four individuals (one from each subspecies) for each primer pair were tested for polymorphism and the primers were endlabelled using -^{33P} ATP and run on a 6% denaturing polyacrylamide gel, and exposed to Kodak BioMax film for 3 days to test for polymorphisms. These two loci were found not to be polymorphic but several other microsatellite primer combinations were optimised and are ready to be endlabelled and run on a sequencing gel. It would be worth screening these for polymorphisms in weka as a future direction for research in weka populations.

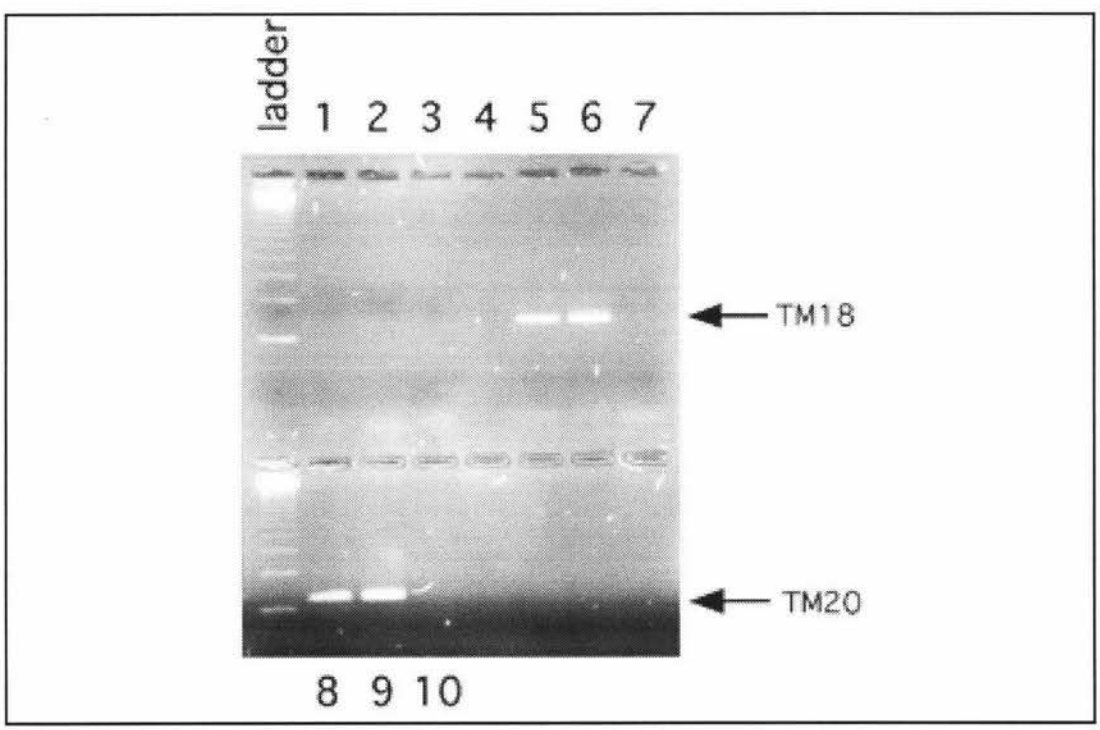

Figure 3.22 Agarose gel of three microsatellite PCR primers. Lanes 1 and 2 show the lack of amplifcations obtained from TM31, with Lane 3 the negative control. Lanes 5 and 6 show amplification using TM 18 with lane 7 a negative control. Lanes 8 and 9 show amplification of TM 20 with lane 10 a negative control. A 123 bp ladder was used.

Table 3.6 Preliminary results using Tasmanian Native hen and chicken microsatellite primers.

MICROSATELLITE PRIMERS	RESULTS OBTAINED
Tasmanian Native Hen	
TM 01	Multiple bands
TM 18	Strong single bands
TM 20	Strong single bands
TM 27	Weak single band
TM 31	No amplifications
TM 31a	No amplifications
Tm 36	Multiple bands
TM 105	Multiple bands
Chicken	
ACT	Multiple bands
AET	Multiple bands
MMWNF	Multiple bands

3.12 SUMMARY

In summary, a large proportion of the weka mitochondrial genome was invariant between populations. The variable control region, however, was able to identify genetic diversity both within and between the North Island (*G. a. greyi*) and South Island (*G. a. australis*) subspecies of weka. Further analyses needs to be performed to determine if the genetic diversity of *G. a. scotti* and *G. a. hectori* reflects their taxonomy also. Provenance of weka introduced to islands could only be accurately assigned to the subspecific level, not to the population level, and only for the two subspecies investigated. There is a high percentage sequence divergence between the two subspecies. The Kapiti Island population is remarkably homogeneous. Futher investigation of nuclear markers in weka, such as microsatellites, may be helpful in obtaining a higher resolution of the subspecific status of weka or at least provide concordance with the mitochondrial results.

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Chapter 4

Sexing of the Weka

4.1 INTRODUCTION TO AVIAN SEXING

This chapter describes the sexing of weka using a molecular technique. The results are compared with those of a morphometric sexing technique. The first of the six sections in the chapter explains the difficulties experienced when attempting to sex birds. The second discusses the advantages and disadvantages of behavioural, morphometric, surgical, chromosomal, hormonal and DNA based sexing techniques. The third section overviews the ZZ and ZW chromosome system of birds. The fourth reviews the currently used behavioural and morphometric techniques used to sex weka. The fifth presents the findings of a CHD based sexing investigation of weka. Lastly, it compares CHD-based sexing and morphometric-based techniques to sex weka.

Birds are generally difficult to sex. It is almost impossible to sex chicks and nestlings rarely show sex-linked morphology. It is estimated that in over 50% of the world's bird species, adult females appear identical to males (Griffiths et al., 1998).

Despite the difficulty involved in sexing birds, there are many reasons why it is necessary to do so. Determination of sex is of considerable importance for the understanding of numerous features of behaviour, evolutionary ecology, genetics and evolution (Clutton-Brock, 1986). For example, the evolution of a communal breeding system, basic life history characteristics, dispersal, conservation genetics, and offspring sex ratio.

It is also important to sex birds in captive breeding programs which have become a major component of many attempts to conserve avian species. Knowledge regarding the sex of birds is important to ensure that mistakes such as same sex pair bonding are not made. Information regarding the sex of individuals is also important when supplementing existing wild populations so that biased sex ratios are not formed. The avoidance of biased sex ratios may be important when establishing a new wild population, however, this level of importance depends on the specific mating/social group of the species.

There are several possible methods for sexing birds. Methods include behavioural observation, morphometric techniques, surgical laporoscopy, surgical laporoscopy, karyotyping, hormonal methods and DNA based methods. The advantages and disadvantages of each method are discussed below.

4.2 METHODS OF SEXING BIRDS

4.2.1 Behavioural Observation

In the past, biologists have often had to rely on sex-specific behaviours such as copulation or egg laying in order to sex birds. This method of sexing can prove difficult as in some species these behaviours are rarely performed (Ellegren and Sheldon, 1997). In addition, in some species, such as weka, both sexes share all responsibility of egg incubation and chick rearing, thereby making this method of sexing difficult. There are also examples in some species where same sex pair bonding and copulation occur. For example, at the Albatross Colony in Dunedin there is a pair of female birds which take turn in exhibiting female and male courtship behaviours (D. Emmerson, pers. comm.).

A further difficulty is that behavioural methods can only be applied to adult birds, and observations are sometimes not possible because the birds are not paired or not breeding. Behavioural sexing does have considerable advantages over other methods of sexing as it is noninvasive and the level of stress to the birds via this technique is low. Also, the data obtained using this method can often be used for other behavioural studies.

4.2.2 Morphometric Methods

Birds are often sexed using morphometric techniques (Fox et al., 1981). This method involves the analysis of external morphological measurements through the development of discriminant functions to find one, or a combination of measurements that can be used to accurately assign sex. Measurements of tarsus length, bill width and culmen length are frequently used as the measurements involved in morphometric studies (Fox et al., 1981), such as in the pukeko (*Porphyrio porphyrio*) (Craig et al., 1980).

Although morphometric sexing is not 100% accurate, it does have advantages over other methods of sexing avian species. Firstly, it is inexpensive when compared to laboratory-based methods. Secondly, it requires no tissue sampling of the individual. Thirdly, this sexing method alongside with behavioural sexing are the only sexing techniques that are able to produce immediate results in the field.

There are disadvantages associated with morphometric sexing also. This method can not be used for chicks and it has a reduced accuracy for juveniles from 3-12 months of age. It is also unable to be used for birds lacking measurements through injuries (for example, loss of limb). This method also encounters difficulties when attempting to sex sexually monomorphic birds, as it will only work where sexual variation is sufficient to produce two morphological classes. There are also problems associated with inter-measurer variation. Also this method of sexing does inflict a certain amount of stress on the birds as they have to be handled for a length of time in order to obtain an accurate measurement of all measurements required for the discriminant function developed to sex the particular species (Maho et al., 1992).

4.2.3 Surgical Methods

The most common surgical method of sexing birds is laparoscopy. This is an extremely invasive technique and involves the actual visualisation of the birds' sexual organs. This method is restricted to juvenile and adult birds only, being too invasive for chicks. It is an accurate method of determining the sex of the birds, but does represent a risk to the individual. Therefore, this technique is not commonly used on endangered species. There is a substantial cost involved with this method as it has to be performed by a veterinarian. It also inflicts a high amount of stress on the birds.

4.2.4 Chromosomal Methods

Karyotyping is another method of sexing that has been successfully used to identify the sex of many avian species (De Boer, 1984). A disadvantage with this method of sexing is that typically, for a significant proportion of samples, fibroblast cells, from which the chromosome preparations are made, can not be cultured and consequently no results can be obtained (Eason et al., in prep). However, when the results are obtained, they are said to be 100% accurate (Eason et al., in prep). This method is generally highly expensive and time consuming. It also requires blood or another tissue type resulting in a medium to high level of stress inflicted onto the bird.

4.2.5 Hormonal Methods

Sex plasma hormone concentrations (testosterone and estradiol) are another method able to be used to sex birds. However, radioimmunoassay of plasma testosterone and estradiol concentrations has several disadvantages. This sexing method is only able to sex adult birds that are actually breeding. Also tissue or blood samples are required which inflicts a moderate to high level of stress onto the birds. This stress in turn can affect the accuracy of the results as handling and capture stresses are known to affect plasma sex steroid concentrations in birds (Gratto-Trevor et al., 1991; Maho et al., 1992). Therefore, the handling of the birds should be keep to a minimum. Remote sampling may be possible in some cases using faecal material, although intrusive tissue sampling is normally required e.g. bleeding (Bercovitz and Sarvar, 1988). In order to obtain accurate results this procedure needs to be

performed on healthy, breeding, unstressed adult birds at the same time of the day as there are cyclic variations in daily sex steroidal levels (De Boer and Van der Gugten, 1987; Dunn et al., 1972).

4.2.6 Molecular Methods

DNA based methods are increasingly being used to sex a variety of bird species (Millar et al., 1992; Griffiths et al., 1998; Fridolfsson and Ellegren, 1999). Female birds are heterogametic (ZW) and males are homogametic (ZZ), therefore sexing can be made by the detection of the W chromosome or W chromosome sequences in a sample of unknown sex. The W chromosome is generally much smaller that the Z chromosome and shows other typical signs of a degenerated sex chromosome, i.e., a low gene content that is rich in heterochromatic, repetitive DNA of the satellite type (Fridolfsson et al., 1998).

There are many molecular methods that are able to be used for avian sexing. These include DNA fingerprinting, random genomic W chromosome probing, RAPD, CHD/restriction enzyme, CHD/SSCP and CHD/length differences. Table 4.1 briefly describes the main advantages and disadvantages to each of these molecular sexing methods.

The use of molecular sexing methods has several advantages over other methods of sexing birds. Firstly, there is a negligible failure rate regardless of the condition or age of the bird. Secondly, it is applicable to a wide variety of tissue and preservation types. For example, this method of sexing has advantages over karyotyping as the blood can either be stored in a freezer or in ethanol for a long period of time and used for sexing at a later date. Thirdly, there are extensive other potential uses from the samples obtained.

Table 4.1 Advantages and disadvantages of molecular methods used for avian sexing (From: Ellegren and Sheldon, 1997)

Method	Main Advantages	Main Disadvantages
DNA fingerprinting	Minimal development required if a probe is available	Works in very few species
Random genomic W chromosome probes	Minimal development required if a probe is available	Does not work across species, may be laborious to isolate a useful probe
RAPD	PCR based; internal positive control provided	Does not work across species, may be laborious to identify a useful primer, difficulty with repeatability
CHD/restriction enzyme	PCR based; works across all species (except ratites)	A suitable enzyme must be found by either trial or error and by sequencing
CHD/SSCP	PCR based; works across all species (except for ratites)	Requires facilities for running SSCP
CHD/length differences	PCR based; very simple; works across all species (except ratites)	No primer pair working across all species developed yet

There are also disadvantages associated with DNA based sexing methods. For example, the techniques used in the laboratory analysis can be expensive and time consuming. The tissue sampling is an intrusive process causing a moderate to high amount of stress to the birds (Maho, 1992). However, remote sampling techniques from discarded tissue is possible, for example the use of faeces (Robertson et al., 1999) and the use of urine (Nota and Takenata, 1999).

The most attractive techniques used for DNA-based gender identification rely on the polymerase chain reaction (PCR), of which an important advantage is the minute sample required for genetic analysis (Ellegren and Sheldon, 1997). Initial attempts in this direction were based on screening for W-linked repetitive (Quinn et al., 1990; Rabenold et al., 1991) or anonymous, e.g. RAPD, DNA sequences (Griffiths and Tiwari, 1993). However, it was found that these sequences were not well conserved between species and the associated sexing systems are only applicable to a few closely related species.

In 1995, the first W-linked avian gene was discovered (Griffith and Tiwari, 1995). This gene encodes for a chromo-helicase-DNA binding protein (CHD) and is shown to be present on the W chromosome of most birds (Ellegren, 1996; Griffith et al., 1996). Delmas et al. (1993) found that CHD proteins act as overall transcription regulators at the chromatin level. This function was suggested due to their unusual property of containing three conserved domains, each member of a discrete gene family. The CHD gene is a unique protein as it shows signatures of two separate protein families involved in global transcriptional activation at the chromatin level (Delmas et al., 1993).

The three domains of the CHD gene include: The chromo (C) domain which is thought to mediate chromatin structure and organisation during transcription. The helicase/ATPase (H) domain is found in a wide group of proteins involved in various interactions with DNA or RNA. The third domain is the DNA-binding (D) domain contains short sequence motifs similar to that found in other proteins (Griffiths and Tiwari, 1993).

There are at least four CHD genes known in humans and the avian W-linked gene is closely related to the human CHD gene CHD1 (Woodage et al., 1997). Ellegren (1996) discovered that this CHD gene exists in a second, but not W-linked copy, in most birds. To avoid confusion, the first gene has been called CHD1W and the copy has been called CHD1Z (Fridolfsson and Ellegren, 1999). Both of these genes show a very high degree of sequence conservation, among themselves and in relation to their mammalian homologues (Ellegren, 1996).

The existence of two avian CHD1 genes is both problematic and advantageous from the perspective of molecular sexing. There are three PCR based approaches to distinguishing these two copies (refer figure 4.1). Firstly, distinguishing can be done by using PCR primers that amplify a particular part of the gene, followed by the use of specific restriction enzymes that cut only one of the genes. This is done through the use of an endonuclease with a recognition sequence present in one but not in the other gene copy (Griffiths and Tiwari, 1993; Griffith et al., 1996). The most commonly used enzyme to cut the CHD gene PCR products is *Hae* III. The

discriminatory *Hae* III site usually cuts CHD1Z but not CHD1W. If *Hae* III fails to discriminate, other restriction sites are able to be used. Alternatively, the CHD1W and CHD1Z PCR products can be sequenced to look for an alternative restriction site (Griffiths et al., 1996).

The second approach is to distinguish the two copies by single-strand conformation polymorphism (SSCP) analysis (Ellegren, 1996; Ellegren et al., 1996). This uses an electrophoretic protocol for separating PCR fragments of identical length which vary slightly in sequences.

Fridolfsson and Ellegren (1999) have developed the third method of distinguishing CHD1W and CHD1Z through the use of a pair of highly conserved primers which consistently amplify a different sized intron of CHD1W and CHD1Z in birds throughout the whole avian phylogeny (with the exception of ratites). Ratities are thought not to have the CHD chromosome as they are the basal of birds in the avian phylogeny (pers. comm. Leon Huynen).

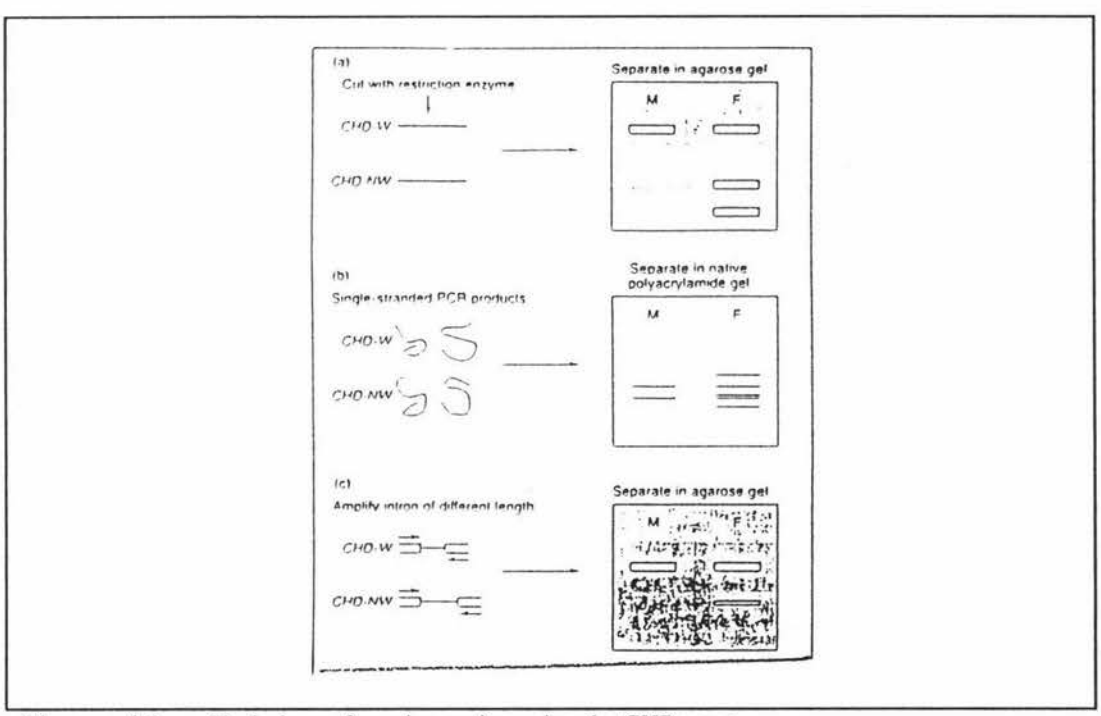

Figure 4.1 Techniques for avian sexing using the CHD gene.
M=male, F=female (From: Ellegren and Sheldon, 1997)

4.3 CURRENTLY USED TECHNIQUES FOR SEXING WEKA

Weka are visually monomorphic and until now have only been able to be sexed using behavioural and morphometric techniques (Beauchamp, 1987). Weka are only able to be sexed behaviorally if they are in a pair and only then during their courtship and breeding period. Positions during courtship, the provision of food and the rate and pitch of their 'spacing calls' are utilized to do this. A spacing call is a loud, rising repeated "coeet". The call is given by single territory holding birds or as a duet by pairs (Beauchamp, 1987). Duets can be used for pairs over 400 metres, with the male call being lower and slower than the females (King et al., 1999). During copulation, males always mount females, and provide food during courtship feeding (Beauchamp, 1987). Assigning sex using other behaviours such as nest building, egg incubation and chick rearing is difficult as both male and female weka share these responsibilities. There have also been reports of polygamy in weka which complicate this method of sexing even further (Guthrie-Smith, 1914; Beauchamp, 1986). Beauchamp (1987) found that assigning sex from paired weka courtship displays and size was correct in 98% of cases on Kapiti Island.

Weka are also able to be sexed using morphometric techniques through the development of a discriminant function. To determine the sex of a weka individual, a linear combination of variables was calculated in the form;

$$Z = Ai.Vi + Aj.Vj + ...An.Vn - C$$

where Ai is the co-efficient of the discriminant function for the ith variable ,Vi is the value of the ith variable for any individual, and C is the constant associated with the set of variables being used. If Z is positive then the bird was deemed to be male, and if negative, a female (Beauchamp, 1987).

Weka are unable to be reliably sexed by the use of any single morphological variable, instead a combination of variables is used (King et al., 1999). Three population specific discriminant functions for sexing weka have been developed. The three population specific discriminant functions were based on weka from Kapiti Island, Codfish Island and the Marlborough Sounds. It has been found that sexing using a function developed from one population may lead to the incorrect

sexing when used on another population. Population specific discriminant functions are necessary for three reasons. Firstly, island weka are often smaller than their mainland counterparts. Secondly, all Western weka measurements are considerably larger than other forms, with the exception of tarsus length, which was larger in the North Island weka (Beauchamp, 1987). Thirdly, weka weight cycles in populations with restricted breeding seasons, for example, weka are heaviest in late summer and decline in weight in late winter (King et al., 1999). Beauchamp (1987) recommends that if all three combinations of sets of variables give the same result for any one individual then it could be assumed with confidence that the weka was of that sex

The three population specific discriminant functions can be compiled from a combination of the four variables. These are: culmen length, bill depth, tarsus length and tarsus width. Figure 4.2 shows where on the birds these measurements are taken. Table 4.2 explains the precise method of measuring. Some of these measurements are not standard and they have to be taken from the positions indicated to be used in the already developed discriminant function of sex for weka. Table 4.3 contains the morphometric measurements using the Kapiti Island discriminant function and the assigned sexes of the thirty randomly selected weka used in this research.

Table 4.2 Exact methods for measuring variables for the morphometric analysis of sex in weka

Measurement	How to measure
Culmen length	Measure from the distal tip of the mandible to the proximal rhampotheca base of the mandible.
Bill depth	Measure the bill vertically in the line that would just strike the tip of the feathers that project onto the bill in front of the superciliary stripe.
Tarsus length	Measure from the back of the leg at the distal notch (top of the tarsometatarsus as opposed to the top of the joint) to the front of the leg where the major anterior tarsometatarsal scales split at the distal end (base) of the mid toe.
Tarsus width	Measure horizontally, in the bill-tail line, from the middle of the third large anterior front facing distal tarsometatarsal scale to the back of the tarsus.

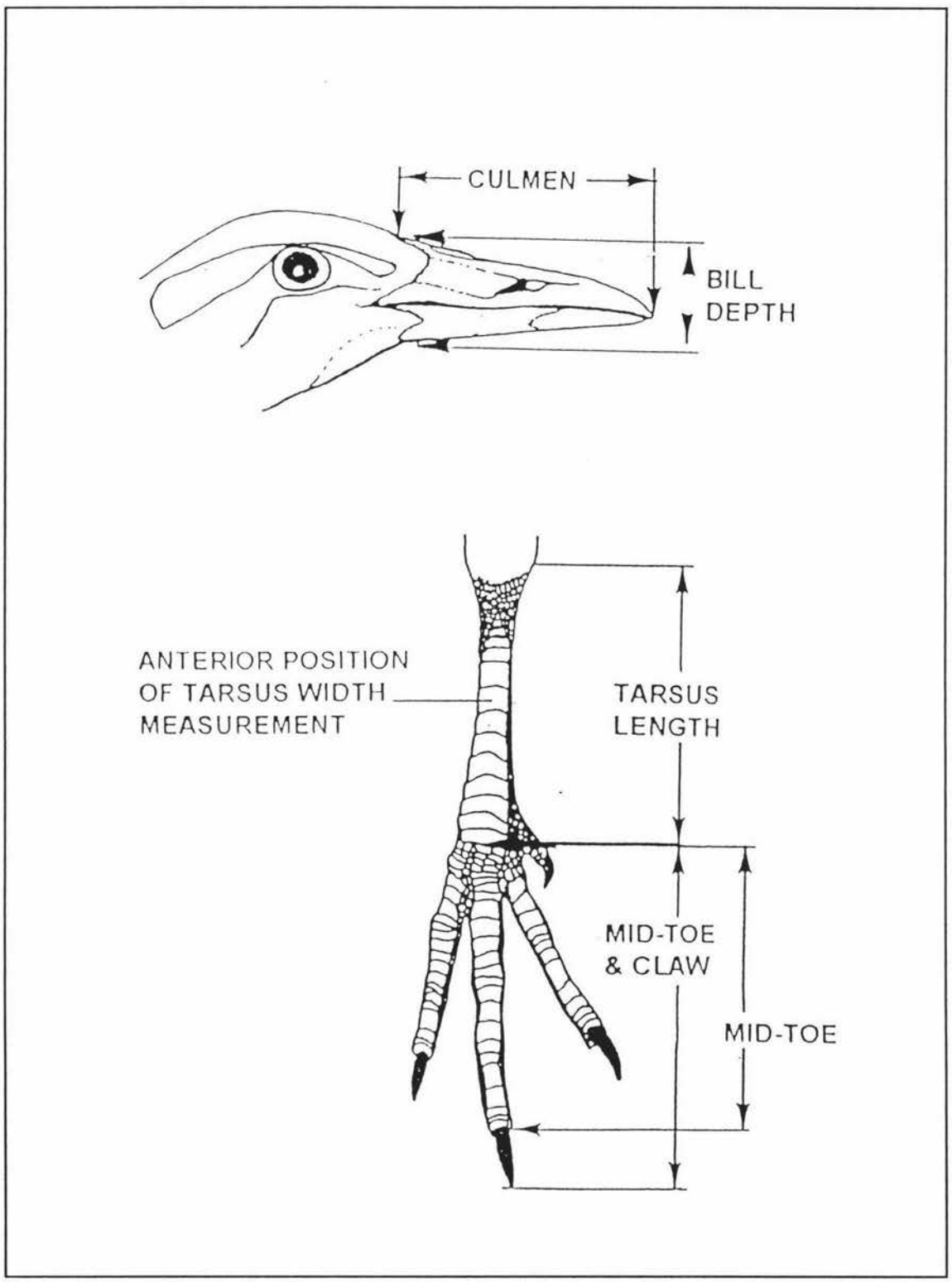

Figure 4.2 Position of bill and leg measurements used in weka morphometric sexing analysis (A = Culmen, B = Bill Depth, C = Tarsus Width and D = Tarsus Length) (From: King et al., 1999)

Table 4.3 Morphometric measurements of weka

Bird	Culmen Length (mm)	Bill Depth (mm)	Tarsus Width (mm)	Tarsus Width (mm)	Weight (grams)	Approx. Age (years)	Assigned Sex
M-44592	51.20	23.00	10.87	61.90	1185	4	Male
27-09664	43.00	15.40	10.39	60.00	885	3	Female
27-09663	44.20	17.50	9.73	53.00	785	3	Female
27-09661	44.70	18.40	11.21	51.00	985	2	Female
27-09662	46.1	12.0	9.40	55.00	985	2	Female
17-98300	42.9	15.6	10.9	53.3	985	2	Female
17-98296	49.6	16.7	11.58	60.4	1132	4	Male
17-98298	44.0	17.4	11.32	52.6	982	3	Female
M-44590	46.9	11.9	11.09	56.2	982	2	Male
17-98297	46.39	16.74	11.10	71.99	1185	3	Male
M-44591	40.80	14.54	9.07	62.30	885	2	Female
27-09665	52.05	18.47	11.82	72.92	1085	4	Male
17-98290	40.22	15.02	9.78	62.55	780	3	Female
17-98294	47.03	11.62	10.18	74.50	1080	2	Male
M-44588	48.23	18.71	11.09	78.42	1230	3	Male
17-98282	44.56	16.52	9.64	74.74	982	3	Female
17-98287	47.80	19.42	10.45	77.60	1130	3	Male
17-98293	45.03	16.23	10.31	66.63	830	3	Female
M-44585	42.71	18.58	10.86	75.68	1280	2	Male
L-33232	43.63	16.58	9.64	66.08	980	2	Female
M-44551	48.42	19.44	11.12	82.61	1280	3	Male
M-44583	45.58	19.19	11.53	74.29	1180	3	Male
17-98288	47.15	19.13	10.51	76.90	1230	3	Male
17-98286	45.69	18.70	11.84	71.55	1180	3	Male
L-33251	40.36	16.77	8.4	67.17	730	3	Female
M-44589	46.35	12.10	11.25	62.50	1090	4	Male
M-44586	47.40	19.26	10.89	80.86	985	3	Male
M-44584	46.55	16.98	10.95	75.75	1185	3	Male
17-98291	45.13	17.52	11.12	72.66	980	4	Male
M-44587	47.00	18.81	10.97	76.68	980	3	Male

4.4 MOLECULAR SEXING OF WEKA

In order to obtain a 100% accurate method of sexing weka of all ages and health CHD/restriction enzymes sexing methods were trialed.

4.4.1 Collection of Samples

Blood samples were collected from thirty *Gallirallus australis greyi* (refer table 4.3) that were held in captivity at Rainbow Springs, Rotorua, prior to a broadaficoum poison drop on Mokoia Island (refer figure 3.1 for location). Following morphometric measurements of the birds, the area containing the brachial vein of either wing was cleansed using cotton wool and 70% ethanol. This allowed the vein to be clearly seen and blood to form droplets at the puncture. Birds were bled by vein puncture of the brachial vein, using disposable 25-gauge needles. The blood was collected in 32 x 0.8 mm heparinised capillary hematocrit tubes as long as blood flowed or until 3 tubes were filled (maximum of 60 µl). The filled tubes were placed into 1.5 ml screw-topped *Nunc* tubes containing 100% ethanol. Tubes were then shaken rapidly by hand to homogenise the blood and alcohol solution. Each tube was labelled with the bird's band combination, band number, and the date and time of the blood collection. Cotton wool was placed over the puncture wound until bleeding had stopped. Prior to release, the wound was checked to make sure that the bleeding had stopped. Samples were stored at 4°C in the field at the time of collection, then eight hours later stored permanently at -80 °C in the Molecular Ecology laboratory, Massey University. The DNA extraction protocol was the same as that followed in Chapter Three.

4.4.2 CHD Amplifications

PCR amplifications were done in a total reaction volume of 25 µl. This included PCR buffer II (PerkinElmer: 1 x 10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$ (25 mM), 0.125 µl of dNTPs (20 mM of each deoxynucleotide), P2 (5' - TCT GCA TCG CTA AAT CCT TT - 3') and P3 (5' - AGA TAT TCC GGA TCT GAT AGT GA - 3') primers (Griffith et al., 1996), 50-100 ng of whole genomic DNA and 0.5 units of *Taq* DNA polymerase (*Amplitaq*: Perkin Elmer). The thermal profile was 95 °C for 2 minutes followed by 35 cycles of 94 °C for 45 seconds, 50 °C for 1 minute and 72 °C for 1 minute, with a final cycle of 72 °C for

10 minutes. Eight microlitres of each PCR reaction was size fractionated on 2% *NusSieve*/1% agarose gels in Tris acetic acid pH 8.0 buffer containing ethidium bromide (0.5 $\mu g ml^{-1}$) and run at 10 V cm^{-1} for 30 minutes, and visualised under ultraviolet light (312 nm). Negative template controls were run with all experiments. Eight microlitres of the remaining reaction was digested with *Hae* III (5 units: Gibco Life Technologies) in 1 x Gibco Life Technologies enzyme buffer 2, BSA (100 ng ml^{-1}) and spermidine (4 mM final concentration) to a total volume of 10 µl. Samples were incubated at 37°C for 2 hours. The digested samples were then size fractionated and visualised again under UV light (312 nm) (Refer figure 4.3).

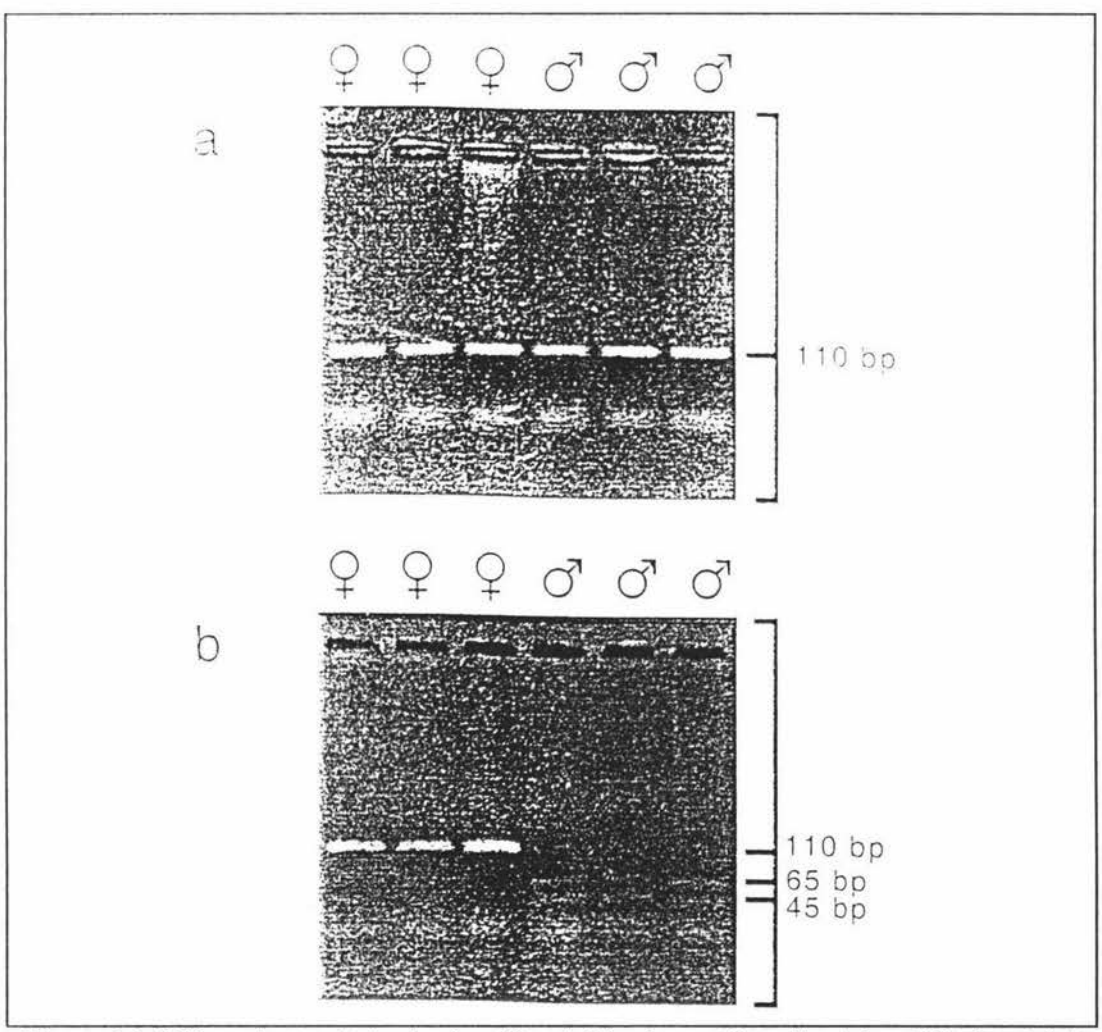

Figure 4.3 PCR products obtained using P2 and P3 primers, (a) undigested and (b) digested. It can be seen that in (a) bands of both males and females are 110 bp in size. Once digested with the restriction enzyme *Hae* III, the male is cut into two bands of 65 and 45 bp respectively. Thus males can be easily distinguished from females.

As figure 4.3 shows, the male band cuts into two bands, while the female band remains uncut. This pattern is similar to that found in a range of other New Zealand species (B. Stephenson, unpublished data).

4.5 Results

A pilot study of the CHD primers was initially performed on six weka of known sex identified through behavioural methods. These weka were: M-44592, 27-09665 and M-44551 being male and 27-09663, M-44591 and l-33251 being female. All six weka were found to be the same sex as their behavioural designation. From this pilot study it was regarded that this method of sexing is accurate for weka. The remaining 24 samples of the morphometrically sexed weka were sexed using the CHD/restriction enzyme method. When compared to the morphometric sexing 27 of the 30 weka had a concordant assignment of sex (refer table 4.4). Three indivdiuals were involved in discrepencies between the two methods of sexing. These individuals were:17-98300, 17-98296 and 17-98298. These three individuals were analysed twice more to remove the possibility of laboratory error (refer figure 4.4). In all cases the DNA result was consistent.

The high degree of concordance between morphological and molecular methods is partially due to the age of weka used in this study. All weka were adults and were aged between two and four years. If chicks or juveniles were used, it is likely that there would have been a much larger discrepancy between the two sexing methods, as morphometric analysis is less accurate when applied to juveniles or chicks.

The three discrepancies involved two males being mistaken for a female (band number 17-98300 and 17-98298), and one female (band number 17-98298) being mistaken for a male. Both males were comparatively smaller than the other males weka involved in this study. Both males had weights that were approximately 150 grams below the average of the sampled males. The weight of male weka ranged from 980 grams to 1280, with an average weight of 1129 grams.

Table 4.4. Results from the comparison of morphometric and molecular sexing techniques. It can be seen that 27 out of the 30 adult weka were correctly sexed using morphometric methods. A tick indicates the same result for the two different techniques and a cross indicates discrepancies between the two methods of sexing.

Band No.	Age of bird in years	Morphometric sex	Molecular sex	Result
M-44592	4	M	M	√
27-09664	3	F	F	√
27-09663	3	F	F	√
27-09661	2	F	F	√
27-09662	2	F	F	√
17-98300	2	F	M	X
17-98296	4	M	F	X
17-98298	3	F	M	X
M-44590	2	M	M	√
17-98297	3	M	M	√
M-44591	2	F	F	√
27-09665	4	M	M	√
17-98290	3	F	F	√
17-98294	2	M	M	√
M-44588	3	M	M	√
17-98282	3	F	F	√
17-98287	3	M	M	√
17-98293	3	F	F	√
M-44585	2	M	M	√
L-33232	2	F	F	√
M-44551	3	M	M	√
M-44583	3	M	M	√
17-98288	3	M	M	√
17-98286	3	M	M	√
L-33251	3	F	F	√
M-44589	4	M	M	√
M-44586	3	M	M	√
M-44584	3	M	M	√
17-98291	4	M	M	√
M-44587	3	M	M	√

The third discrepancy involved a male that was classed as a female using the morphometric technique and male using the molecular technique. This molecular sexed female weka had a comparatively large body weight then the other females used in this analysis. This female weighed 1132 grams, almost 200 grams above the average weight for the female weka sampled, with their weights ranging from 730 grams to 1180 grams, with an average weight of 921 grams.

These three particular discrepencies indicated that it appears that the size and therefore the weight of weka has a large bearing as to what classification the weka are given when sexed morphometrically. Figure 4.5 shows the weight distribution of the weka sampled .

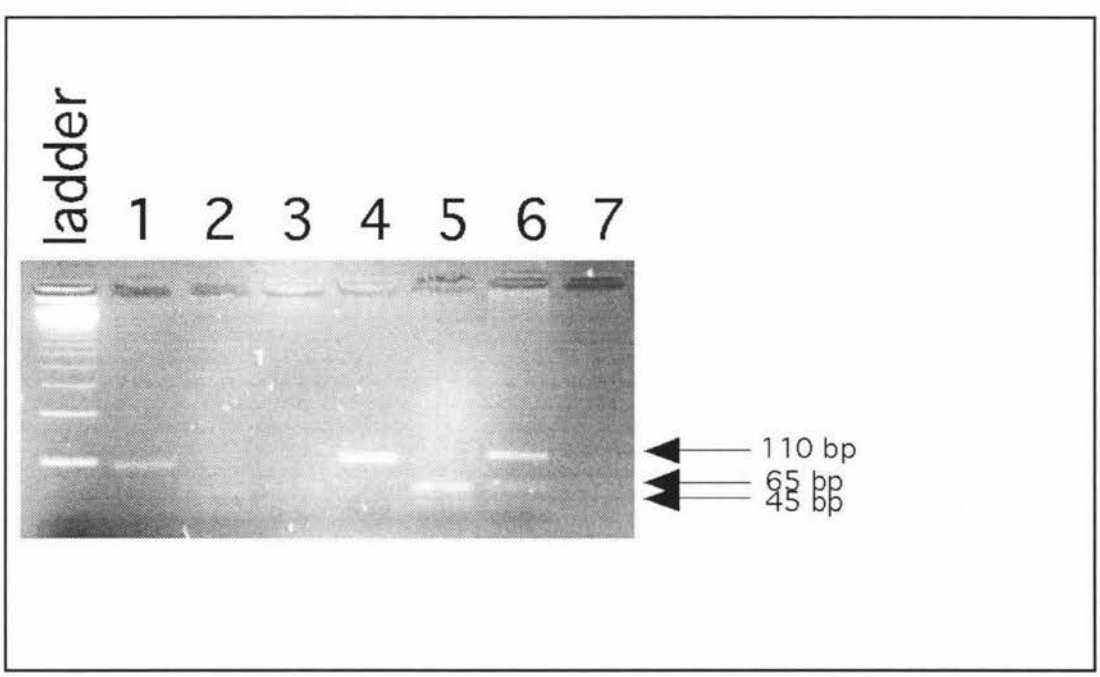

Figure 4.4 1% agarose gel containing P2 and P3 PCR primer products that have been cut with *Hae* III. This gel contains the three weka that had discordant molecular and morphometric results. Lanes 1 and 4 contain females. Lane 4 contains individual 17-98298 that was classed using morphometrics as a male but classed as a female using molecular methods. Lane 5 and 6 contains 17-98300 and 17-98298. These individuals were classed as females using morphometric techniques and males using molecular techniques. A 123 bp ladder was used.

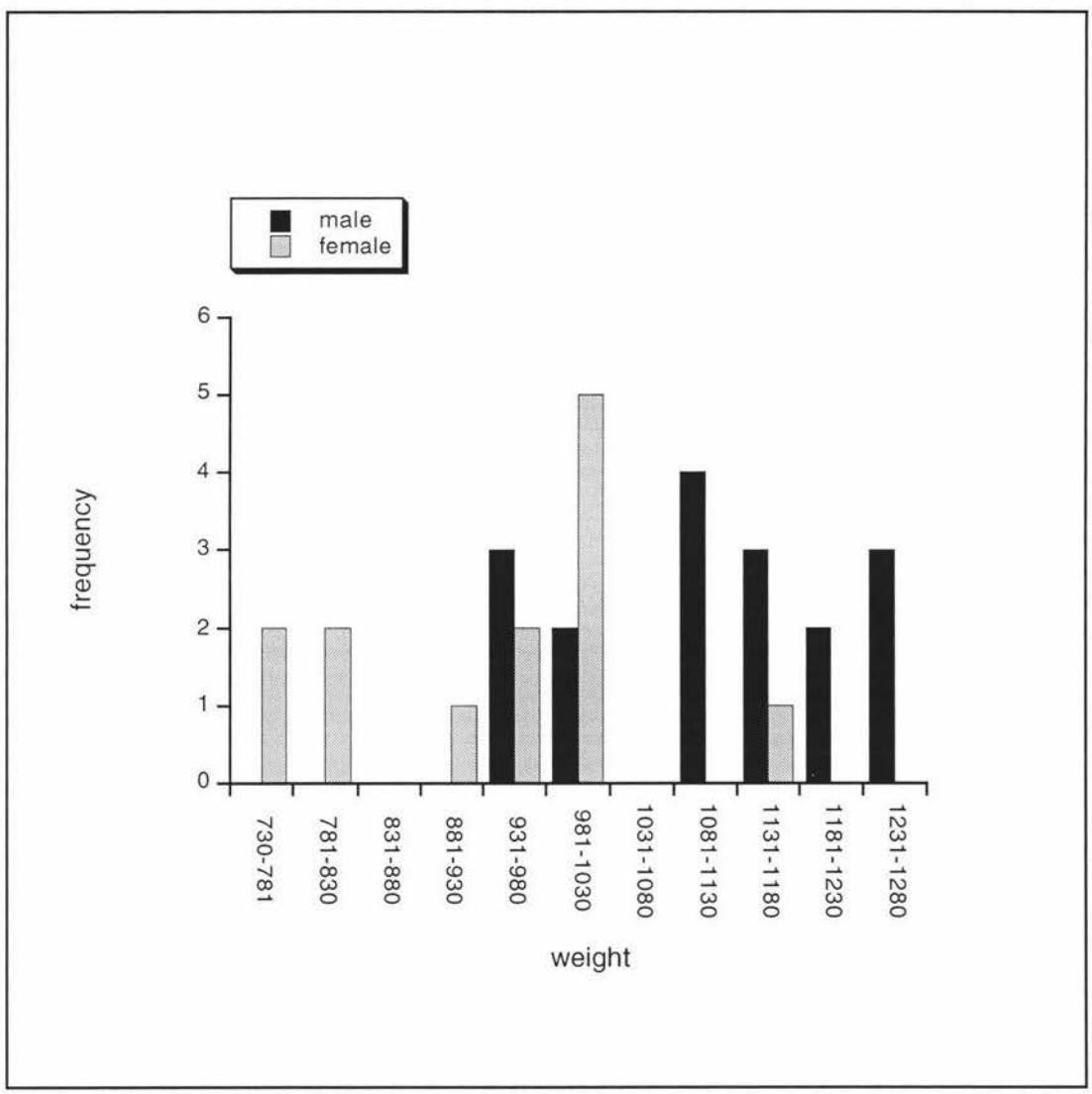

Figure 4.5 Graph to show the distribution of weights (in grams) of the 30 weka morphologically sexed from Mokoia Island in November,1996.

4.6 CONCLUSION

In conclusion, these two methods of sexing weka result in approximately a 10% discrepancy when sexing adults. Although this method of sexing is often invasive requiring tissue sampling, it does have the advantage of being able to sex weka of all ages unlike morphometric sexing. Sexing using CHD has recently been shown to work using noninvasive tissue sampling through the use of faeces and urine (as seen in Robertson et al., 1999 and Nota and Takenata, 1999).

It is important to be able to accurately sex weka for many reasons. Firstly, when being breed in captivity it is imperative to do accurate pair bonding, as weka bond for life from a very early age. It is also important to be able to sex weka accurately when understanding numerous features of their behaviour, evolutionary ecology and evolution, for example, life history characteristics, breeding systems and offspring sex ratio.

Due to the fact the molecular sexing can be done regardless of the age of the birds or their health status and that noninvasive techniques for obtaining DNA have been developed, I recommend the use of CHD sexing as a rapid and accurate method of sexing of this, and other, monomorphic avian species. I believe that CHD sexing is an important tool that has great significance for avian researchers

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Chapter 5

Conservation Management: The Implication of Subspecies

5.1 INTRODUCTION

The category of subspecies has always generated a great deal of controversy among biologists (Wilson and Brown, 1953; McKitrick and Zink, 1988; Avise and Ball, 1990). Some consider it to be a sacred unit of taxonomy, reflecting a discrete category of geographic variation, perhaps incipient variation, within a species (Weins, 1982). Others regard the category to be an "artefact that has reality only in museum trays and to those who delight in fiddling with nomenclature" (Cracraft, 1983).

This chapter achieves nine tasks. Firstly, it provides a brief historical background of the category of subspecies. Secondly, it examines the definition of subspecies by Mayr (1953) and highlights the major flaws with it. Thirdly, it implicates the arbitrary nature of the category, with no defined lower limits as the major problem associated with the past and current usage of subspecies. Fourthly, it reviews examples of genetic techniques attempting to classify subspecies. Fifthly, it examines some major considerations involved in the genetical classification of subspecies. Sixth, it examines the evolutionary significance of the subspecies

category. Seven, it looks at hybridisation in relation to subspecies. Eight, it gives suggestions as to a new, useful definition of subspecies that places the category in an evolutionary important context. Finally, these eight factors will be discussed with specific reference subspecific relationship of weka.

5.2 HISTORICAL BACKGROUND

The recognition of variation in natural populations has had a long history in taxonomy (Mayr, 1996). Linnaeus recognised variation in natural populations as the 'variety' and defined it as any deviation from the type of that species (Mayr, 1982). Other subspecific divisions with definition of varying precision and utility have occasionally been used, such as cline, isophene, deme, phenon, and variant (Wilson and Brown, 1953; Barrowclough, 1982). However, subspecies is the most common subspecific definition, incorporating trinomials. For example, *Gallirallus australis greyi.*

Since the early 1950's, there has been widespread doubt about the efficacy of trinomials for the description of subspecific variation. In 1953, Wilson and Brown stated that subspecies do not convey the actual patterns of geographic variation. They believe that a widespread pattern of actual variation consists of a lack of concordance of clines in different characters (independent geographic variation), reoccurrence of characters in several geographic areas (polytypic subspecies), and the problems of virtually every population differing in some character or other (microgeographic races).

Once a Latin binomial or trinomial is in the literature, the group of organisms to which it refers almost automatically assumes an aura of reality that may or may not be commensurate with its true evolutionary distinctiveness (Lanyon, 1982). It is important that population managers keep an open mind regarding taxonomic realignments, particularly for populations within the vast majority of species that have remained poorly studied.

Avise (1992) believed that it is abundantly clear that most species should not be viewed as monotypic entities, but rather as a series of geographically differing

populations with a hierarchical and sometimes deep genetic and historic structure. Futuyma (1986) claimed that the level of variation increases with the size of the geographic area encompassed. Large geographic areas often lead to the occurrence of clines. Clines are the gradual change in a character along a geographic transect which may extend over the whole geographic range of a species (Futuyma, 1986). Wilson and Brown (1953) believed that attempting to categorise this clinal variation that occurs over large geographic areas is dangerous as it could led to the inaccurate classification of subspecies. An example of a well known cline is the white-tailed deer (*Odecoileus virginianus*) which exhibits gradual size increase with increasing latitude over most of North West America. This relationship between body size and latitude is so common in mammals and birds that it has been dubbed "Bergmans rule" (Futuyma, 1986).

Many authors have refrained from describing this clinal variation in terms of subspecies because of the inherent danger of "biological distortion" (McKitrick and Zink, 1988). Due to the phenomenon of clines, Parker (1982) believed that the subspecies concept is not suitable for continuously distributed continental species and is inherently misleading and should only be used for discrete populations. These clinal gradients cause problems when attempting to define subspecies. For example, the Grey-Crowned Babbler (*Pomatostomus temporalis*) was traditionally classified into several subspecies, until recent analysis showed that much of the morphological variation was clinal (Edwards, 1993). Edwards (1993) suggested that the populations should be grouped into two major taxa, the eastern "grey-crowned "(*P. t. temporalsi*) and western "red-breasted" (*P. t. rubeculus*). This division was supported by phylogenetic analysis of sequences from a portion of the cytochrome *b* gene (Edwards and Wilson, 1990).

5.3 DEFINITION OF SUBSPECIES BY MAYR et al. (1953)

The most accepted definition of subspecies is that of Mayr et al. (1953). Mayr defined subspecies as "an aggregate of phenotypically similar populations inhabiting a geographic range of that species and differing taxonomically from other populations of that species". There are two major downfalls with this definition.

They culminate to form the major problem associated with the misuse of this terminology which is arbitrariness.

The first major downfall is that it does not define the level of differentiation required for naming a subspecies. This exemplifies the fact that the sets of rules for what constitutes a subspecies are not defined. There is no mention in this definition of what level of differentiation is sufficient. Thus the category is extremely subjective and depends on whether you are a "lumper" or a "splitter" and therefore has no theoretical significance (Storer, 1982).

The lack of a lower limit is problematic because some combinations of characters will distinguish every population from others (Futuyma, 1986). Individual subspecies could in turn be divided into an array of microgeographical races. An extreme of this could turn every population, or even individual, into a species (Storer, 1982).

The only way to resolve this situation is to establish a lower limit above which populations will be formally recognised as subspecies. However, no lower limit will ever be completely satisfactory for even if only one character is used, there will always be borderline cases of an extremely vexing nature.

Some biologists have tried to solve this problem by using statistical methods with the level of statistical reliability most neatly conforming to their own preconceived notion of what should constitute a valid subspecies in a particular group of study (Wesson, 1991). Austin (1952) believes that unless at least three-quarters or more of the individuals of a population can be correctly assigned by their morphological characters alone, then designating a subspecies is of no practical use. However, this does not take into consideration the occurrence of cryptic species.

At present there are thousands of formally named subspecies which range in distinctiveness from groups of populations barely discernable on the basis of weak divergence in a single character to geographic differences in morphology, behaviour and genetics. With such a breath of variation in levels of differences under one

umbrella, it is no wonder biologists question this category (Johnson, 1982). The same could be said of the species taxon also.

The species status of closely related taxa can not be determined unequivocally solely by reference to some arbitrary standard of genetic divergence, but information on genetic distance can contribute to systematic inference. There are examples where genetic distances between subspecies is the same as that for species level divergence. For example, protein distances between the dowitches subspecies *Limnodromus scolopaceus* vs *L. griseus* is D=0.060, and the titmice subspecies *Parus bicolor bicolor* vs *P. b. atricrstatus* is D=0.063. Although small, are of a magnitude frequently observed between some avian taxa whose status as species has not been subject to debate (Avise and Zink, 1988).

The various categories used to class subspecies is also a major problem. This is the second major downfall of Mayr's 1953 definition. Weins (1982) believes that Mayr's usage of phenotype in this definition indicates that the division of species into subspecies has been made largely by visual analysis, and thus subjective criteria, and not by adaptive (behavioural) or objective (genetic) criteria. However, behaviour can also be subjective and genetic criteria objective. For example, the actual bands on a visualisation gel are objective, but the level of difference is still subjective.

There are a number of categories which can be used to classify subspecies. For example, morphological, biochemical, behavioural, geographical, and genetical. Using a number of categories would reduce the arbitrary naming of subspecies due to one difference, traditionally morphometric. This would also ensure that differences on a broad range do in fact exist and produce a more hierarchical, categorical system with more significance.

Using a number of techniques to name subspecies that have concordant results would obviously reduce the number of subspecies present. However, it would still be possible to identify populations for which a trinomial would be useful, but this would require extensive study of geographic variation of many characters to delimit the taxa. For most species no trinomial would be justified. But this search for

trinomials should not be an end in itself, instead their description ought to be the occasional result of studies aimed at understanding the patterns and processes associated with geographic variation (Barrowclough, 1982).

Seizing upon a single arbitrary difference without looking at a broad range of characters is one of the most common taxonomic errors (Caughley and Gunn, 1996). An example of the incorrect designation of this category is the King Cheetah (*Acinonyx jubatus* spp.). The king cheetahs tabby coat was first identified as a different separate species, later it was considered a subspecies. It is now known to be the result of the expression of a single recessive gene, which has different frequencies in different cheetah populations (Caughley and Gunn, 1996).

Increasing the stringency of subspecies is not just shifting words around, by using different names to explain the same phenomenon. Instead it would ensure the correct usage of words. Various words could be used to exemplify the relative importance of the populations, such as ecotype. This would also help to ensure that polytypic species are not divided into different subspecies as is often the case.

It would be useful if the subspecies concept has the same end result as other taxonomic categories. The goal of classification is to provide a hierarchical framework to be used in the retrieval of information. The usefulness of the subspecies concept lies in the power to predict the extent to which new generalisations may hold, based on inexhaustive sampling of taxonomic hierarchy (McKitrick and Zink, 1988).

In the past, some taxonomists have divided species up into subspecies when they felt that further study might discover some trait or characteristic that varies between the two populations in some biologically distinct way (O'Neill, 1982). This does not seem to be valid justification in calling a population a subspecies. However, retrospectively, this may be advantageous for the conservation of many populations as O'Brien and Mayr (1991) believe that the listing of taxa as endangered has encouraged an investigation into these taxa, notably in molecular genetics and field ecology.

5.4 THE USE OF GENETICS TECHNIQUES TO CLASSIFY SUBSPECIES

The relatively new techniques of molecular genetics have been used to compare genetic subspecies categorisations with that of other methods of categorising subspecies, such as morphometric. There are a number of genetic techniques that are able to distinguish subspecies variation. For example, isozymes, RFLP's, mitochondrial DNA sequencing and microsatellites. All these techniques give different resolution, with the question that you are trying to answer dictating the level of resolution needed and therefore the most appropriate technique.

One of the most commonly used techniques for looking at subspecies differences is mitochondrial sequencing. MtDNA is often used as it offers a sensitive molecular probe at intraspecific evolutionary processes (Wilson et al., 1985; Avise et al, 1987; Shields and Wilson, 1987; Avise, 1989). This is due to the rapid evolution and uncomplicated, maternal inheritance of mtDNA (Suzuki, 1993). In many instances, mtDNA analysis has been able to reveal dramatic population differentiation. For example, the Canary Island endemic lizard genus, *Gallotia* (Gondzalez et al., 1996), and the kiwi (*Apteryx* spp.) (Baker et al., 1995).

There are instances however, where molecular techniques have revealed little or no geographic structuring where it is presumed to exist. Ball and Avise (1992) used mitochondrial DNA to assess whether genetic differences are concordant with traditionally recognised subspecies boundaries. They found that of the 16 morphometrically recognised subspecies, only two proved to be readily distinguishable from their conspecifics in terms of their mtDNA. A similar result was found by Sheilds and Wilson (1987). They recorded very low mtDNA variability within the goose (*Anser caerulescens* spp.) and hypothesise that this was due to a lower tempo of mutational divergence than in mammals or, alternatively because the goose subspecies are still very young.

Another example of mtDNA analysis finding little or no geographic structuring occurring is in the large scale mtDNA study of the Red Winged Blackbird (*Ageliaius phoeniceus*) (Ball et al., 1988). Although ample mtDNA variation was

found, the diversity was not strongly partitioned among geographic or subspecific populations (Ball et al., 1988).

5.5 IMPORTANT CONSIDERATIONS INVOLVED IN GENETICAL CLASSIFICATION OF SUBSPECIES

There are several major considerations associated with DNA sequencing that must be taken into account in the analysis. Since the adoption of DNA technology, the bulk of molecular analysis of animals have addressed variation only in the mitochondrial genome. Because of the lack of recombination, the entire mitochondrial genome acts as a single genetic locus, providing only a single 'gene tree' which might not accurately reflect the 'organismal tree' (Pamilo and Nei, 1988). This is of vital importance because the correct inference of the organismal tree is the goal of most phylogenetic reconstructions. Although several authors acknowledge that lineage bias may be associated with single gene genealogies (e.g. Avise et al., 1983; Avise et al., 1984; Wilson et al., 1985, Dittmann and Zink, 1991), empirical demonstration of this bias is scarce, and studies of genetic variation relying solely upon mitochondrial DNA (mtDNA) apparently have become widely accepted (Degnan, 1993).

Another problem with sequencing only one gene of the mitochondria is that different genes have higher resolution than others. This is because genes evolve at different rates. Some genes, such as cytochrome B and ATPase 6 and 8, are much more conserved that others because of constraints placed on them due to their different functions. For example, cytochrome *b* is more conserved then region I and III of the control region as it codes for a protein. Also, if only one gene is used then only a small proportion of the genome is examined. For example, Ball and Avise (1992) looked at only one gene (360 base pairs) which equates to 2.2% of the mtDNA genome. Different results may possibly be found if a larger portion of the genome is sequenced.

Several researchers are against the usage of single gene research, due to the number of perils associated with using results on single genes (Degnan, 1993). There are many examples of studies in which two genes give different answers as to the phylogenetic structuring of populations. For example, Gondalez et al. (1996) found

different phylogenetic relationships in their studies on lizards using the ribosomal 12S gene and the protein coding cytochrome *b* gene. Another example is Degnan (1993), where different markers used for silver eyes, *Zosterops* spp., were found to provide clear empirical evidence that single gene analyses can provide incomplete, and even misleading, phylogenies, and emphasises the need for population genetic analyses to employ a composite of genetic markers under different modes of inheritance and different modes and rates of evolution (Degnan, 1993).

It is often premature to reclassify species based on one gene analysis. In the first place, mitochondrial trees pertain only to the maternal lineage. When dealing with lineages that have diverged within the past few million years, the possibility exists that nuclear gene comparisons could reveal a more complex picture (Shields and Wilson, 1987; Van Wagner and Baker, 1990).

Nuclear DNA provides a source of numerous, essentially independent, genealogies, under varying degrees of selective constraint, with different mutational mechanisms and rates of evolution, and with a different (biparental) mode of inheritance to that of mtDNA (Degnan, 1993). The results obtained by mtDNA sequencing stem from only one maternally inherited locus. If males disperse more than females, as is often the case with mammals, the genetic structure observed may be less strongly correlated with the geographic distribution observed by screening nuclear genes which are transmitted by both sexes. Furthermore, mtDNA introgression can occur between conspecific populations or between closely related subspecies (Lehman et al., 1991; Carr et al., 1986; Ferris et al, 1983). Such introgression can hide the real phylogenetic relationships. For these two reasons, it would be desirable to confirm the results obtained by mtDNA control region sequencing by also studying nuclear loci. This would definitely be desirable in weka.

Despite the utility of molecular analysis, it is easy to lose sight of the fact that genetic techniques subsample genes, as do morphometric, biochemical and other attributes used to assign taxonomic criteria (Caughley and Gunn, 1996). For example, for morphological reasons, the polar bear (*Ursus maritimus*) and the Canadian brown bear (*Ursus americanus*) are not conspecific, despite one mtDNA

genotype of the brown bear being more similar to the polar bears than to the other four brown bear genotypes (Shields and Kocher, 1991).

Although molecular techniques are useful tools in answering the question of what constitutes a subspecies, they can not be used alone to decide the specific or subspecific status of populations. Instead molecular genetic results must be used in combination with other methods of analysis. Concordance among genetical and independent attributes should be the deciding criterion upon which to base formal taxonomic recognition of subspecies (Avise and Ball, 1990).

Molecular techniques are useful in identifying sets of populations that are likely to have been separated for significant periods of evolutionary time. Johnson (1982) believes that for a category to have evolutionary significance, it is imperative that the molecular techniques support it. This is especially true when the mtDNA differences are concordant with those registered by nuclear genes and their products, including morphological and behavioural traits (Avise and Ball, 1990). There is much controversy however as to whether subspecies should have evolutionarily significance or not. Several researchers have argued that infraspecific categories are evolutionarily important and deserve taxonomic recognition (Johnson, 1982; Parker, 1982). Opposing this, many researchers question the validity of subspecies designations having evolutionary significance (Mayr, 1953; Wilson and Brown, 1953; O'Neill, 1982; McKitrick and Zink, 1988; Zink, 1989).

5.6 THE EVOLUTIONARY SIGNIFICANCE OF SUBSPECIES

Reasons for the scepticism of subspecies having evolutionary significance are varied and include failure of genetic data to corroborate distinction among morpho-subspecies (Barrowclough, 1982; Ball et al., 1988; Avise and Nelson, 1989) and indications that rearing environments can directly influence the development of some morphological traits of traditional importance (Suzuki, 1993).

At present, the concept of subspecies does not have an evolutionary significance and is simply a handle of convenience (Mayr, 1982). Many researchers agree with

Mayr and believe that if the subspecies concept is void of evolutionary processes then the usefulness of the concept is greatly enhanced. O'Neill (1982) believes that this would eliminate the need to differentiate between taxonomic and evolutionary implications that are so often misused. Although, if a subspecies definition is able to be reached in agreement, it would not be necessary to differentiate between taxonomy and evolutionary naming.

The length of time a population has been separated from others also has implications for the justification of naming the population a subspecies. Avise and Ball (1990) say that short term population separation should not be sufficient to justify formal taxonomic recognition of subspecies. They believe that subspecies names should be reserved for the major subdivisions of genepool diversity within species. However, evolutionary separation should not matter if differences are registered by concordance of a number of categories.

A decrease in the number of subspecies named would have significant repercussions for conservation. A number of conservation agencies, such as the Carnivore Preservation Trust, do not recognise the subspecific distinction due to controversy involved with subspecies categorisations. Their reasons for not using the category are that they do not believe that the category has any biological relevance and that the present reality would inhibit saving species below the species distinction. There are often huge numbers of subspecies defined, such as 30 subspecies of the cougar (*Felis rufus*), but, until more research is done on this species, the correct number will remain unknown.

5.7 HYBRIDISATION AND SUBSPECIES

A reduction in the number of subspecies listed would affect conservation strategy because of the issue of hybridisation relating to conservation law. Hybridisation between species and subspecies is regarded in America as likely to jeopardise that species' continued existence. Therefore, according to the Endangered Species Act (USA) of 1973 which has a hybrid policy stating that the protection of hybrids would not serve to recover listed endangered species, hybrids of species and subspecies are not protected in America. The hybrid policy should attempt to discourage hybridisation between species, but should not be applied to subspecies

because subspecies retain the potential to interbreed which is why they are not species.

If the number of classified subspecies was reduced in America, some subspecies would inevitably still exist. However, the conservation focus would have to shift from species and subspecies to populations and ecotypes.

In New Zealand, the law regarding subspecies is quite different. The Department of Conservation does not have a set policy on species, subspecies and hybridisation and instead refers to the guidance from the scientific community for management (Pers. comm. Alan Baker, DoC). Challenges to the particular status of our native species, subspecies, and hybrids is unlikely to occur due to the Wildlife Act structure. Under this Act all the native land vertebrates are protected. Therefore, even if there was a reduction in the number of subspecies, in reality it would not matter as all endemic fauna in New Zealand are protected.

Until the subspecies controversy has been resolved, the conservation of present day categorised subspecies should continue. This is particularly important in the weka and other endangered species where the subspecific relationship has not been completely analysed. All endangered populations should be protected, whether or not they represent species, subspecies or ecotypes. Separate populations often have different structures with regard to their ecosystems, and this ecological structure is important for the same reasons that genetic diversity is important within populations.

It is possible that the misuse and misinterpretation of the subspecies category warrants the abolishment of the term subspecies. Instead a thorough review is needed with stricter criteria for its classification. Although Phillips (1982) claimed that the utopian ideal of attaining agreement on the details of subspecies is unlikely, it is possible that a new subspecies definition can be achieved, as only one author (Cracraft, 1982) in the forum of fifteen authors discussing the utility of subspecies in the scientific journal Auk in 1982 recommends the abandoning of the concept. All the other authors advocate modification of the category. Of these authors, Lanyon (1982) points out a simple yet important piece of advice which he claims is

the one useful attribute of subspecies. He states that the category can be omitted if not essential and regarded optional if deemed burdensome.

5.8 PROPOSED NEW DEFINITION OF SUBSPECIES

I propose a new definition of subspecies in order to provide the concept with utility and ensure that it has evolutionary significance.

> *Subspecies are an aggregate of similar populations inhabiting a geographical range that share a number of concordant features, such as morphological, genetical, behavioural, physiological etc., to a level where 95% of the individuals can be recognised.*

This proposed definition of a subspecies would provide the category with a useful, evolutionarily significant meaning. It has advantages over Mayr et al.'s (1953) definition as it states a number of factors which should be used to categorise subspecies, rather then just phenotype. It also provides limits by which the number of individuals can be recognised. Although implementation of this new definition would reduce the number of subspecies and therefore impact on conservation it would promote the importance of ecotypes as integral participants of species and subspecies. Moreover, regardless of the distinction any endangered population is given, it is worth saving. These changes would ensure that subspecies gain the significant evolutionary recognition that they deserve, as well as being a useful classification point of reference.

5.9 THE SUBSPECIFIC RELATIONSHIP OF WEKA

Weka have four morphometrically recognised subspecies at present, although the Department of Conservation is trying to get recognition of a fifth subspecies by dividing *Gallirallus australis australis* into two subspecies. In order for these weka subspecies to withstand my definition other features such as behavioural or genetical need to be concordant with these divisions to a level where 95% of the individuals can be recognised. Using genetical techniques, none of the four weka mitochondrial genes sequenced were, at this stage, able to conclusively agree with

the morphometrically defined subspecies. However this may be a failing of the choice of the marker used.

Cytochrome *b* and 12S were unable to distinguish any differences at all between the population/subspecies. ATPase 6, however, was able to produce a similar result to that of a previous isozyme study (Miskelly et al., unpub.), in that a North Island/South Island difference was able to be established. The 5' end of the control region was able to identify the highest resolution of variation in weka. There was distinct clustering between the *G. a. greyi,* and *G. a. australis* able to be observed. There was 3.83% sequence divergence between these two clades.

The apparent absence of phylogenetic structure in weka haplotypes of the first three genes sequenced (cytochrome *b*, 12S and ATPase 6) implies that there has been no long-term geographic isolation of subspecies. However, the control region of the weka mitochondrial genome was able to give a higher resolution because has a faster evolution rate. The sample size used in this study could also have an effect on the outcome. It is unlikely that all the weka haplotypes were identified from sampling the populations sampled. There are two other subspecies that were not investigated (*G. a. hectori* and *G. a. scotti*). Unsampled haplotypes might provide more structure to the mtDNA gene tree, perhaps even revealing more clusters of closely related genes within the subspecies, or perhaps even revealing more *Gallirallus* subspecies.

Another caveat is that mtDNA is a single linkage group that reveals only matrilineal relationships. It is possible that other loci (e.g. from the nuclear genome) would reveal a more stringent geographic pattern. Although the genetic basis for morphological differences among weka subspecies is unknown, variation in genes influencing these traits may have a geographic component. Thus, it is concluded not that the subspecies are "invalid", but only that they are not distinct on the basis of mtDNA variation in the genes that have been sequenced in this study, except for the control region. A more complete sequence analysis of the region of the weka samples would perhaps be able to reveal some haplotypes structuring, as just over 10% of the extracted DNA samples were used. A faster and cheaper option would be to perform RFLP analysis on the remaining samples.

Trewick (1997) stated that weka are the only rail where there is any evidence of radiation within a flightless taxon, and in this case variation in plumage coloration is allopatric and not accompanied by significant genetic variation. Trewick (1997) thought that this indicates that the weka subspecies are of very recent origin, perhaps within the time frame of the current postglacial period which began approximately 14,000 years ago. This is despite an ancient derivation of the weka lineage itself (Trewick, 1997). However, Trewick used only the 12S gene to make this assumption. Although weka do appear to be of very recent origin, the control region is certainly able to uncover some geographic variation.

Provenance was able to be established for one of the unknown origin populations. As only a pilot study was performed on variation in the control region of weka, it was not able to identify all, or the majority, of the haplotypes in the weka. Therefore, the identification of the exact provenance of the "unknown" populations was not able to be resolved. These unknown origin populations are weka that have been introduced to islands without adequate documentation of the weka source populations. At this point in time it is possible to tell if the bird(s) in question are from the North Island or the South Island, but not a more specific date on location is available. Further analysis of the control region should be able to estimate the provenance of many of the "unknown" populations to a more convincing resolution.

Information relating to subspecies and provenance will aid the development of management guidelines for weka on the mainland and offshore islands, including the likely consequences of local extinctions. This knowledge will benefit any translocation of weka. This information may be able to be obtained through the processing of the remaining weka samples that have been obtained. It is simply a matter of processing these, as all the systems have been developed and are in place.

Kapiti Island weka, an example of a weka population of unknown origin showed a remarkable degree of homogeneity, and were very similar to the North Island weka. There have been several documentations of weka being introduced to Kaptiti Island from all around New Zealand, although it appears that only North Island weka became established in terms of mtDNA. The existence of only one mitochondrial

haplotype suggests that the existing Kapiti population might have been derived from a small number of North Island females only, perhaps even a single female, since mitochondria are typically inherited only through females. It is possible that there are other mitochondrial lineages present on Kapiti Island, but that they are simply rare or have not been sampled in this study.

It is recommended that in order to preserve the genetic diversity of weka, all populations of weka throughout New Zealand be treated as separate conservation units. At least until more research in all facets, behavioural, genetical, morphological and physiological, has been done on weka so as to adhere to the newly proposed definition of subspecies.

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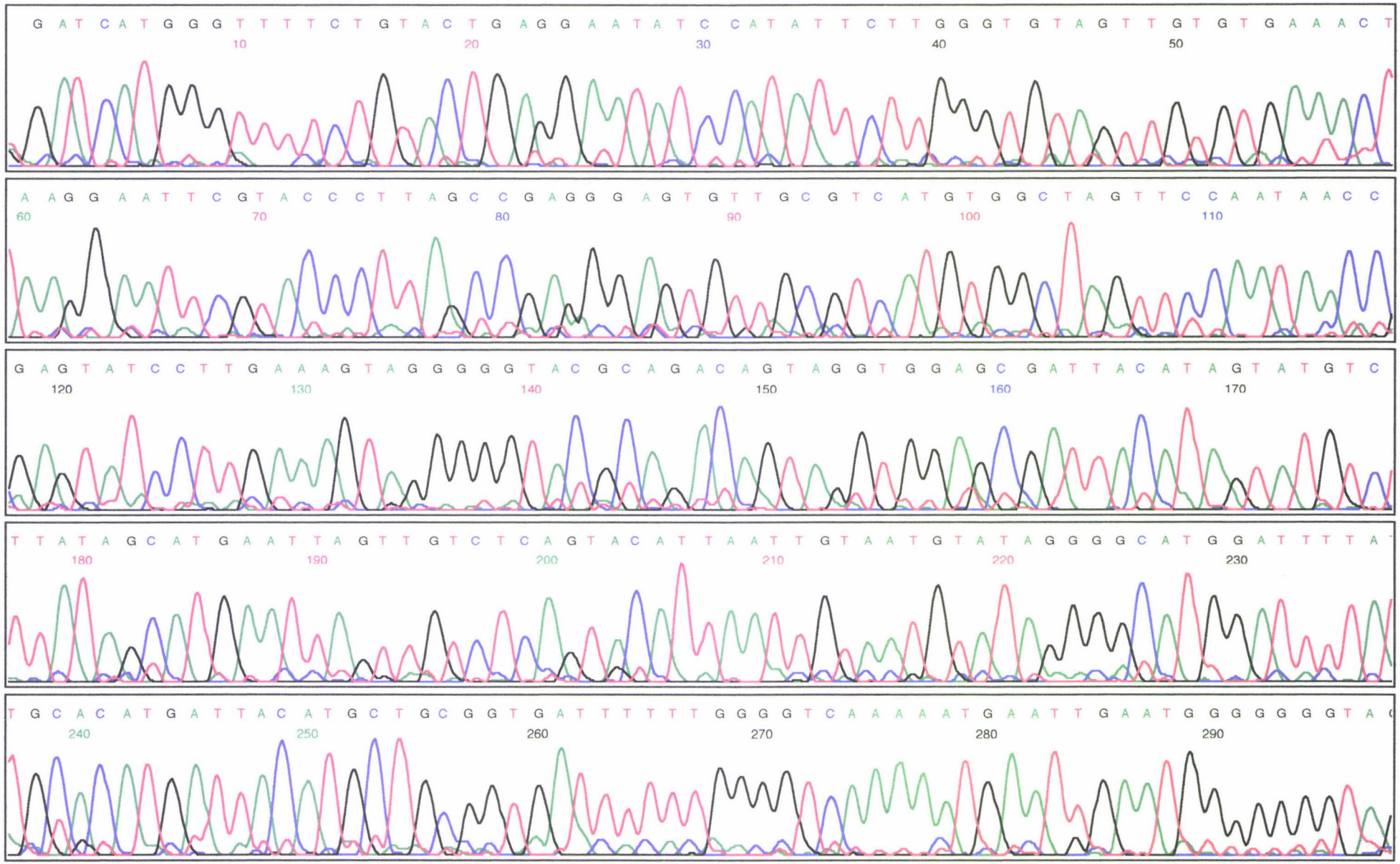
Appendix

Electrophoretogram of Sequence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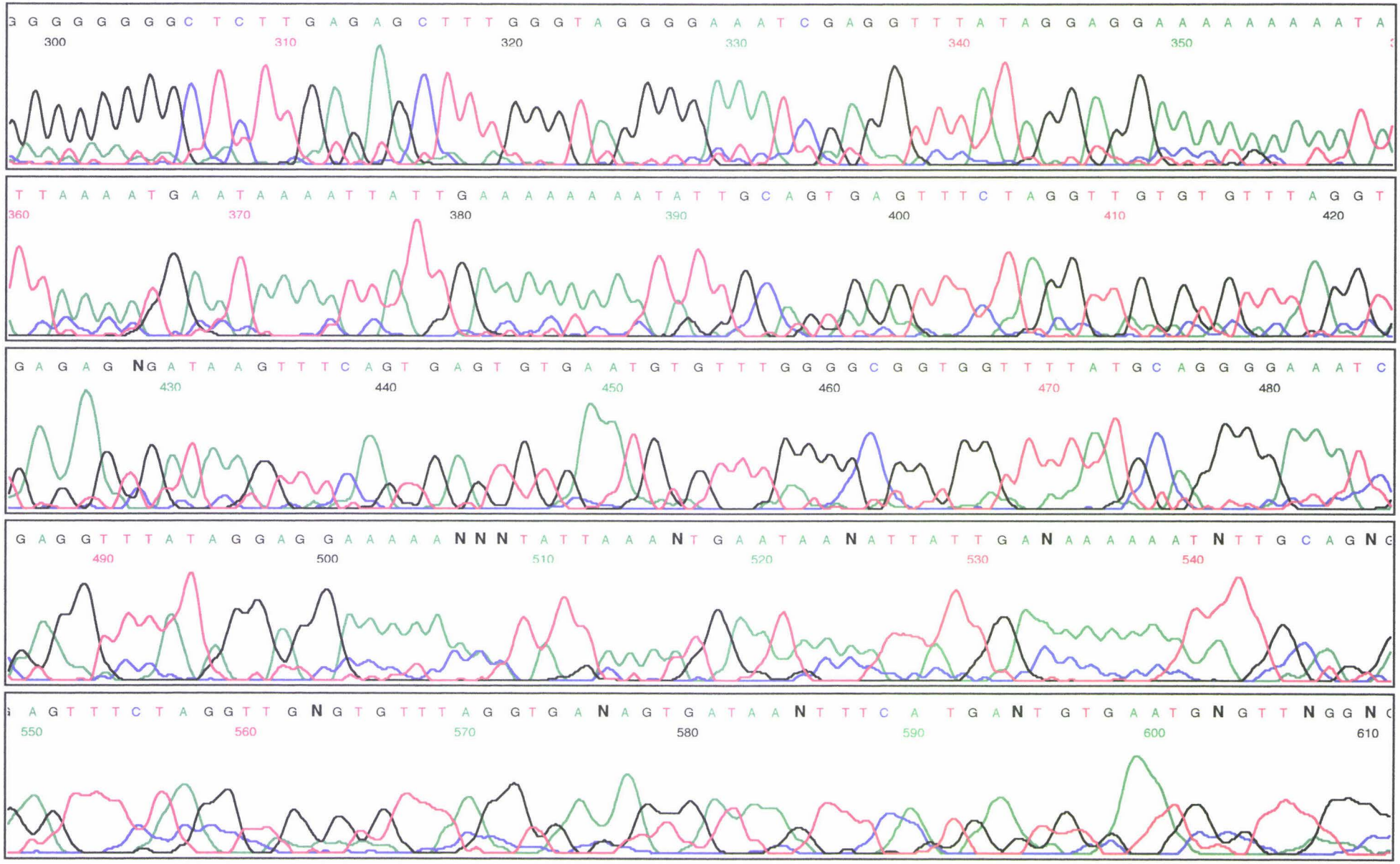
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The End.