The Ecology and Molecular Ecology of the New Zealand Lesser Short-tailed Bat *Mystacina tuberculata*

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GENERAL ABSTRACT

The distribution, ecology, and molecular ecology of short-tailed bats *Mystacina tuberculata* were investigated in the central North Island, New Zealand. Special emphasis was given to providing information for conservation management of the species. Seven large populations containing between 2000 and 10 000 bats were found to persist in the area. Populations require numerous large cavities in the main trunk of mature trees for colonial roosts and are therefore restricted to extensive stands of tall old-growth forest. There was no evidence of predation by introduced mammals at roosts where the bats are most vulnerable. In summer, large group size, aggressive behaviour, and frequent movement between colonial roosts reduce the probability of predation. During winter, roosting bats remain in torpor for long periods and are vulnerable to predators, but the entrances to winter roosts are usually too small for predators to enter. Although field trials demonstrated that the bats may be at risk of secondary poisoning during pest control operations, close monitoring of a bat population during a control operation revealed no mortality. The species' intraspecific phylogeny was investigated using a 2878 bp sequence alignment from multiple mitochondrial genes. Six sympatric phylogroups were identified with estimated divergences of 0.93–0.68 My ago. The phylogroups do not correspond to the existing subspecific taxonomy. The phylogeographic structure and demographic history of the phylogroups were investigated using control region sequences modified by removing homoplasic sites. Phylogeographic structure was generally consistent with an isolation-by-distance dispersal model. The observed pattern is not typical of microbats, which generally exhibit low levels of genetic structure over continental ranges. Coalescent based analyses (mismatch distributions, skyline plots, lineage dispersal analysis, and nested clade analysis) indicated the three phylogroups found in central and southern North Island expanded before the last glacial maximum, presumably during interstadials when *Nothofagus* forest was most extensive. Genetic structure within a central North Island hybrid zone was consistent with range expansion from separate refugia following reforestation after catastrophic volcanic eruptions. Discrepancies between estimates of historic and current population size (> 12 million cf. 50 000) indicate the species has undergone a massive population decline.

ACKNOWLEDGMENTS

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STUDY BACKGROUND

The lesser short-tailed bat *Mystacina tuberculata* is a moderate sized (14 g) microbat that inhabits relicts of indigenous old-growth native forest throughout New Zealand. It is the sole extant species of the Australasian family Mystacinidae, a distinctive, and possibly basal group, within the diverse New World super-family Noctilionoidea (Pierson *et al.*, 1986; Kirsch *et al.*, 1998; Kennedy *et al.*, 1999; Van den Bussche and Hoofer, 2000). The species is ranked as a "Category A" endangered species (i.e. the category with highest priority for conservation action) because of both taxonomic isolation and perceived rarity (Molloy and Davis, 1994).

Historically, short-tailed bats were a difficult species to study. As well as being nocturnal and cryptic, they inhabit extensive and generally inaccessible areas of unmodified forest. Populations and individuals are highly mobile and their colonial roosts are transitory (Lloyd, 2001). Consequently, until recently, published information on their distribution and ecology was largely based on casual sightings and opportunistic studies (e.g., Dwyer, 1960, 1962; Daniel, 1976, 1990; Daniel and Williams, 1984). In the last ten years a variety of technological developments have provided a selection of tools that make field studies of short-tailed bats feasible (automated bat-detectors, miniature radio-transmitters, aerial radio-tracking, infra-red illuminated video surveillance systems, dataloggers and PCR based molecular methods). Fortuitously, the advent of these new technologies coincided with the publication of the Department of Conservation's bat recovery plan (Molloy, 1995) and the development of a recovery programme. The work described in this thesis is part of a wide ranging investigation I undertook to provide information and develop techniques identified in the bat recovery plan as necessary for conservation management of short-tailed bats. The diverse objectives of the investigation included: developing survey and monitoring techniques required to determine the species distribution and monitor population trends; investigating the general ecology of *M. tuberculata* to identify threats and possible management options; and determining the species taxonomy to define appropriate conservation management units.

The main field study area was Rangataua Forest on the southern slopes of Mt. Ruapehu, central North Island (Fig 2.1), where a population of approximately 6000 short-tailed bats inhabit 10 000 ha of mature old growth *Nothofagus* forest. Work at Rangatauau including the development of study methods, and investigation of the bats'

activity patterns, roosting and reproductive behaviour, habitat use, demography, and diet. At the start of the study urgent political requirements necessitated priority being given to an investigation of the threats posed by the broadcast of toxins during pest control operations. The detailed investigation at Rangataua was followed by widespread surveys throughout central North Island to establish the species status and distribution and to collect samples for genetic analyses (Lloyd and Whiteford, 1998).

INTER-POPULATION DISPERSAL

In the course of the study it became apparent that information on current and historic inter-population dispersal levels was crucial for assessing the impact of recent forest fragmentation on the remaining populations, and for interpreting the results of management practices and population census data. Colonial microchiropteran species are generally good dispersers (Altringham, 1996; Hill and Smith, 1984). In many species high levels of gene flow give rise to continent sized populations (Ditchfield, 2000). Short-tailed bats are physically capable of widespread dispersal, as they are strong fliers (Lloyd, 2001). During field studies, radiotagged bats routinely commuted up to 10 km from their day roost to foraging sites. However, they are rarely found far from forested areas and dispersal over long distances outside of indigenous forest has not been documented.

Prior to European settlement in the nineteenth century, short-tailed bats were widespread throughout the almost continuous tract of old growth forest that covered central North Island and probably belonged to a single metapopulation, with local populations linked by high levels of dispersal. Extensive deforestation during the period 1890 to 1980 (McGlone, 1988) restricted populations to remnant patches of forest. In the contemporary fragmented landscape, deforested areas almost certainly hinder dispersal between populations. Observations from the field study were contradictory. Sudden massive changes in minimum population estimates, and exceptionally low recapture rates for marked individual observed at Rangataua, were both consistent with ongoing high levels of dispersal between populations. On the other hand, radiotagged bats were never found roosting outside of old growth forest and remained within about 10 km of colonial roost trees clustered in the core of the population's home range.

Population fluctuations caused by ongoing large-scale dispersal could confound the results of monitoring programmes designed to determine census trends and

conservation management outcomes on local populations. Alternatively, if recent deforestation has fragmented previously continuous populations there could be critical demographic and genetic consequences for the species conservation (Lacy, 1987; Lande and Barrowclough, 1987; Lande, 1988).

Methods of measuring dispersal

Field trials showed that direct methods of measuring dispersal, using either radiotelemetry or capture-recapture methods, were impracticable for short-tailed bats. Radio-transmitters small enough to be fitted to short-tailed bats have a life expectancy of three weeks and an effective range of about 2 km, which is considerably less than a bat's normal nightly commuting distance of 10 km or more. The effort and expense of maintaining an adequate-sized sample of radiotagged bats and searching potential dispersal areas were prohibitive. Permanent marking methods used on other bat species proved unsatisfactory for short-tailed bats. Both forearm bands and passive insertable transponders caused an unacceptably high incidence of trauma. Build up of dirt under forearm bands caused by the bat's burrowing behaviour resulted in infection and swelling of the forearm. The smallest available passive insertable transponders were too large for humane insertion. In addition, the low recapture rate (< 1%) observed for field trials indicated massive effort would be required to identify even relatively high levels of dispersal between the large central North Island populations (> 5 000 individuals) using capture-recapture methods. A more general limitation of direct methods is that because of the limited time scale for the measurements, infrequent events are unlikely to be detected (Slatkin, 1985). This is a serious problem as occasional dispersal of individuals, historical changes in patterns of dispersal, or episodic high levels of dispersal in response to unusual conditions, may all have profound demographic and genetic consequences.

Indirect methods using molecular markers seemed a more practicable method for detecting rates of dispersal between the relatively large and widely spaced populations of short-tailed bats in central North Island. In the past, such methods have involved analyses based on estimation of the *F* statistic and its analogues (Avise, 1994; Weir, 1996; Templeton, 1998) which have severe limitations. They have poor resolution, being unable to either estimate the order of magnitude of dispersal, or separate genetic history from present day patterns of gene flow (Neigel, 1997; Bossart and Prowell,

1998; Templeton, 1998). However, recently developed analytic techniques, such as the analysis of molecular variance (Excoffier *et al.*, 1992) and cladistic analysis of phylogeographic data (Neigel *et al.*, 1992; Templeton, 1998) appear to overcome these problems.

Thus, although initially it was only planned to use molecular methods to determine the species taxonomy, it became apparent that molecular methods might be the most effective method to obtain information on current and historic inter-population dispersal levels.

THESIS FORMAT

Chapter 2 is a published paper (Lloyd, 2001) which summarises key findings from the wider investigation into the ecology of short-tailed bats in central North Island and provides a review of literature published on *Mystacina* up to 2000. Chapters 3 and 4 are the main body of the thesis and present the results of phylogenetic and phylogeographic studies using molecular methods. The two appendices are published papers on the likely impacts on short-tailed bats of toxins used in possum control operations (Lloyd and McQueen, 2000, 2002).

Because chapters 2–4 and the appendices were prepared as separate papers for publication in journals, each contains an abstract, introduction, methods, results, discussion, and references. This unavoidably leads to repetition, but does allow each chapter or appendix to be self-contained and achieve better focus than within the traditional thesis format. Minor differences in terminology among papers reflect different requirements of the papers and are dealt with in the text. Inconsistencies among formats used, especially for citations and reference lists, arise from differences among styles of the journals the papers were prepared for.

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Chapter 2:

Advances in New Zealand mammalogy 1990–2000: Short-tailed bats

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Brian D. Lloyd

SUPERFAMILY NOCTILIONOIDEA

There have been many attempts to determine *Mystacina*'s phylogenetic relationships using morphological features. These attempts have produced conflicting results with the genus placed in four families by different authors (Kirsch *et al.* 1998a). Recent classifications based on morphological features place *Mystacina* in the superfamily Vespertilionidae (e.g., Van Valen 1979; Koopman 1994; Simmons 1998), but four molecular analyses place *Mystacina* in the superfamily Noctilionoidea (previously Phyllostomoidea), a placing that is now generally accepted (e.g., Altringham 1996). Immunological comparisons place *Mystacina* close to *Noctilio*, a genus in the Noctilionoidea (Pierson *et al.* 1982, 1986). DNA-DNA hybridisation indicates *Mystacina* is the most basal group in the Noctilionoidea (Kirsch *et al.* 1998a; Kirsch & Lloyd, 1998). Phylogenetic analyses of mitochondrial DNA sequences from cytochrome *b* (Kennedy *et al.* 1999), 12s and 16s rRNA, and tRNA Val (Van den Buscche *et al.* 1999) also place *Mystacina* in the Noctilionoidea.

Difficulties in determining *Mystacina*'s phylogenetic relationships probably stem from the mosaic of morphological adaptations, including derived and convergent features, which have arisen in the genus as a result of its diverse lifestyle (Kirsch *et al.* 1998a). Alternatively Kennedy *et al.* (1999) suggest that conflicts in classifying *Mystacina* may be a result of the rapid chiropteran radiation which did not allow for phylogenetically meaningful morphological differentiation between families.

The Noctilionoidea is a large superfamily containing 158 extant species, divided into four families: Mystacinidae (1 species), Noctilionidae or fishing bats (2 species), Mormoopidae or moustached bats (8 species) and Phyllostomidae or New World leaf-nosed bats (147 species). With the exception of *Mystacina*, the superfamily is now restricted to central and southern America. It is thought to have evolved in the neotropics and exhibits more diversity than any other superfamily of bats. The group includes insectivorous, carnivorous, sanguinivorous, nectarivorous, frugivorous and omnivorous species. Carnivorous species prey on a variety of small vertebrates including small mammals (including other bats), birds, reptiles, amphibia and fish. All members of the superfamily echolocate, though many also use prey-generated sound to locate prey.

FAMILY MYSTACINIDAE

The family Mystacinidae is a distinctive southern lineage represented by the endemic New Zealand genus *Mystacina* which contains a single surviving species, the lesser short-tailed bat *Mystacina tuberculata*. A second species, the greater short-tailed bat, *M. robusta*, is believed to have become extinct recently. Three fossil species attributed to Mystacinidae have been identified from early to middle Miocene deposits in Australia (Hand *et al.* 1998). The fossil species, at present placed in the genus *Icarops*, are representated by isolated teeth and dentary fragments, which exhibit a combination of apomorphies shared only with *M. tuberculata* and *M. robusta* (in particular, the loss of two lower incisors, a large single rooted P_2, and a moderately reduced M_3).

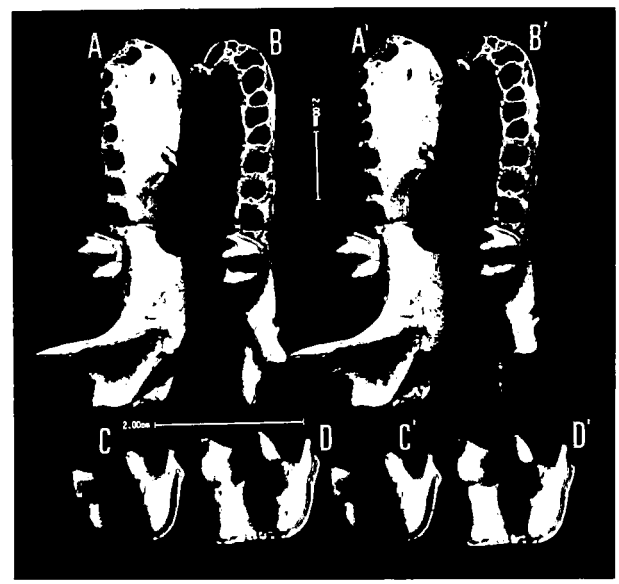


Plate 2.1 Dentary fragments of fossil mystacinids, *Icarops* spp., from Miocene deposits in northern Australia (from Hand *et al.* 1998)

GENUS *MYSTACINA*

Type species *Mystacina tuberculata* Gray, 1843.

Description

Mystacina are medium-sized microbats. Their fur is grey-brown, short (*c.* 7 mm), dense, and velvety, sometimes appearing frosted. The bare skin of the ears, wings, nose, legs and tail is pigmented grey-brown. The forehead slopes steeply and the muzzle is relatively long (*c.* 11 mm) with a conspicuous array of whiskers radiating around the mouth and nostrils. The nostrils are prominent and tuberculate, or wart-like. The ears

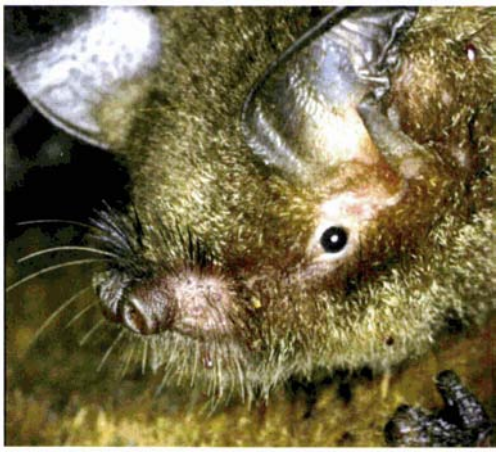


Plate 2.2 (above) Close up of short-tailed bat's face showing the tuberculate nostrils and the conspicuous array of whiskers

Plate 2.3 (right) Short-tailed bat roosting with the delicate flight membranes folded under thickened proximal membranes for protection

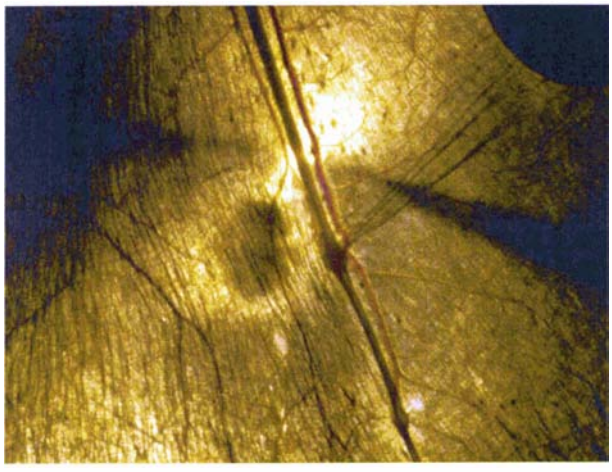
are large (*c.* 18 mm long x *c.* 9 mm at base) and simple, with a long (*c.* 10 mm) tragus. When bats are handled they will often fold their ears to protect them. The propatagium (the leading edge of the wing) and proximal regions of both the plagiopatagium (hand-wing), along the arm and body, and uropatagium (tail-wing), close to the legs, are thickened and striated. When not in flight the wing is folded under these thicker regions so that the delicate flight membranes are completely protected. The proximal section of the tail lies within the uropatagium, but the distal end projects *c.* 7 mm from its dorsal surface. The legs are unusually robust for a microbat, and the feet are stout and broad (*c.* 6 mm). A trivial, but unique, characteristic of the genus is the presence of very fine talons, only just visible to the unaided eye, at the base of the inside curve of the claws of the thumbs and toes.

The male's penis is slightly tapered. Its length varies from *c.* 2 mm in juveniles and subadults, to *c.* 6 mm in large breeding males. Testes are internal. In contrast to many other species of microbats, testes and epididymides (sperm storage vessels) are not visible externally at any time of the year. There is a pronounced clitoral pad above

the female's vagina, which inexperienced obervers may mistake for a penis, but it is smaller and more domed. Females have a single pair of subaxillary teats located *c.* 4 mm from the armpit. In nulliparous females the teats are a bare patch of skin, often darker than the surrounding skin. They are either flat or slightly concave discs, *c.* 1.5 mm diameter. The teats of nulliparous females are well hidden by the surrounding fur and can be extremely difficult to find. The teats of non-breeding parous females are slightly domed or convex disks, *c.* 2 mm diameter. Usually they are the same colour as the surrounding skin, but have dark rings around them and a small white spot in the centre. Although the teats are hidden by the surrounding fur, they are easily found by blowing or brushing the fur to one side. During pregnancy and lactation the teats become enlarged and are obvious. Their form varies: usually they are protuberant, pink with an obvious white centre, though sometimes they appear wart-like with a granulated brown top. Late pregnancy can be diagnosed easily as the female's body mass increases by up to 5 g (i.e., 35%) and the lower abdomen becomes distended. The single foetus lies transversely and can be felt easily. The vulva may be blood stained for a day or two after parturition. During lactation, the teat may look raw with teeth marks and milk can sometimes be extruded by gentle pressure.

Juveniles can be reliably distinguished from adults until about 12 weeks after birth by the presence of cartilaginous epiphyseal plates in the finger joints; these are visible when the wings are trans-illuminated. The finger joints of adults are knobby whereas those of juveniles are evenly tapered. Forearm length and body mass may be less in juveniles than in adults, but this is not always the case. Subadults are young bats that have achieved full adult skeletal size, but not breeding condition. Their finger joints are fully adult. Subadult females may be recognised by their nulliparous teats. There is no reliable method for distinguishing subadult males, although generally subadults have long, sharp, unworn teeth.

Plate 2.4 Trans-illuminated wing showing a fully developed adult finger joint



The two species may be identified using the key following (Hill and Daniel 1985):

> Length of forearm 40.0–45.0 mm, condylobasal length 17.3–19.1 mm, canine to third molar (c-m^3) 7.3–8.0 mm, ears relatively long extending to or beyond the tip of muzzle when laid forward.....***M. tuberculata***
>
> Length of forearm 45.3–47.5 mm, condylobasal length 21.0–22.5 mm, canine to third molar (c-m^3) 8.9–9.4 mm, ears relatively shorter, not reaching to the tip of muzzle when laid forward.....***M. robusta***

Forearm length is the only one of these criteria that can be measured reliably with living bats. The range of forearm lengths for *M. tuberculata* caught in central North Island (39.9–46.9 mm; $n = 999$, mean = 43.64 ± 1.24 mm) (B.D. Lloyd pers. obs.) encompasses most of the variation in both species cited by Hill and Daniel (1985). Thus, the key is of limited use in the field.

Colonisation

Although the oldest known fossil remains of *Mystacina* are probably < 20 000 years old (Worthy & Holdaway, 1994a, 1995, 1996), the genus is considered an endemic archaic element of New Zealand's fauna, having no close relative in other countries (Fleming, 1979). Estimates for separation of *Mystacina's* ancestors from the other noctilionoids vary from 35 to 68 million years ago (Pierson *et al.*, 1986; Kirsch *et al.*, 1998a; Kennedy *et al. 1999*). As all estimated divergence times are considerably later than New Zealand's separation from Gondwanaland (*c.* 80 million years ago), *Mystacina* was probably not present in New Zealand at the time of the formation of the Tasman Sea. Most probably the mystacinid-noctilionoid lineage became widespread in Gondwanaland after New Zealand's separation and divergence between the mystacinids and the other noctilionoids followed after Australia and Antarctica moved apart (Kirsch *et al.* 1998a). Ancestral mystacinids subsequently dispersed from Australia to New Zealand. Evidence to support this theory is the identification of three fossil mystacinid species in Miocene deposits in Australia (Hand *et al.*, 1998) and the occurrence on *Mystacina* of an *Argas* tick closely related to species present on some Australian bats (Daniel 1979). Dispersal over the 1600 km from Australia to New Zealand with assistance from the prevailing westerly wind seems more plausible than direct dispersal from either south America or Antarctica. There are historic records of Australian bats arriving in New Zealand (Daniel 1975); and many of New Zealand's bird species

Plate 2.5 Short-tailed bat's co-mensal bat-fly *Mystacinobia zeelandica*. Although superficially similar, the species is not related to other bat-flies.

(Cooper & Millener 1993) as well as New Zealand's third bat species *Chalinolobus tuberculatus* (Daniel 1990b), originated in Australia. Hand *et al.* (1998) suggest that, because much of New Zealand was submerged during the Oligocene (25–37 million years ago), mystacinids arrived from Australia some time after the Oligocene. Estimates for the timing of mystacinids arrival in New Zealand are largely conjectural. Though *Mystacina*'s unique adaptations for the New Zealand environment, together with the independent and convergent evolution of its co-mensal bat fly *Mystacinobia zeelandica* from taxa unrelated to other bat-flies (Gleeson *et al.* 2000) are persuasive arguments for a lengthy *in situ* evolution within the New Zealand region.

LESSER SHORT-TAILED BAT

Mystacina tuberculata Gray, 1843

Confusion over the nomenclature for *Mystacina tuberculata* stems from Gray (1843). Believing he was dealing with only one species of bat from New Zealand, Gray confused an illustration by G. Forster of New Zealand's long-tailed bat *Chalinolobus tuberculatus* (then called *Vespertilio tuberculatus* J.R. Forster, 1844) with two specimens of short-tailed bat, which he described as *M. tuberculata*. It was not until 1857 that it was realised there were two distinct species involved (Tomes 1857). Recently Kirsch *et al.* (1998b) and Mayer *et al.* (1999) proposed that *M. tuberculata*

should be replaced by the next available name, *M. velutina*, originally proposed to avoid confusion due to two species of New Zealand's bats having the same specific epithet (Hutton, 1871). Spencer & Lee (1999) argue for retention of the name *M. tuberculata.*

Field sign

Daytime

Occupied or recently occupied colonial roost trees may sometimes be recognisable, but the chance of finding an occupied colonial roost without radiotracking is low as there are usually only two or three in a large area of forest (> 100 km^2). Some occupied colonial roosts can be easily recognised by the accumulation of droppings (sometimes > 1 m high) at their base, the noise of bats within the roost, and a distinctive musky smell. This is not true for all roosts. Often there is little or no build-up of droppings at the roost base because the droppings remain within the roost tree, and there may be little audible noise because the bats may roost several metres above the roost entrance. There is a wide variety of solitary roosts and, with the exception of mating or singing roosts, they are not distinctive.

Roosting bats are usually quiet in the mornings, but during afternoons and early evenings they become noisy as a result of both the bats moving about within the roost and their social calls. Their 'chirupping' social calls have both audible and ultrasound components which allows short-tailed bats' presence to be confirmed using a bat detector.

Where fresh droppings are present, the individual droppings are distinguishable as dark brown pellets, 4–5 mm x 3 mm. Typically they contain finely comminuted insect fragments, only recognisable microscopically, but at some times of the year they may contain fruit pulp and small seeds. In late summer, when the bats moult, there may be large amounts of fur mixed in with the droppings.

Bat flies *Mystacinobia zelandica*, or their exoskeletons, may persist at roost entrances or in the gauno at the base of the roost trees for several weeks after roosts have been abandoned by the bats. They provide convincing evidence that short-tailed bats have used a roost.

Night-time

Short-tailed bats do not usually fly until after it becomes dark in the forest, and return to their roost before first light. Their flight is fast and direct, usually among trees, under the canopy of large forest and often less than 2 m above the ground. Thus, they are not easily seen or identified in flight.

The most effective way to detect short-tailed bats is by using bat detectors at night. Because long-tailed bat and short-tailed bat echolocation calls are very different, it is usually possible to distinguish the species reliably using narrow band (heterodyne) bat detectors. Short-tailed bat echolocation calls can be detected over a wide range of frequencies extending from 20 to 100 kHz, the optimum frequency being 28 kHz. At this frequency short-tailed bat echolocation calls are a series of short staccato clicks (20–50 s^{-1}), whereas long-tailed bat echolocation calls are a leisurely series of soft 'thwacks' (*c.* 8 s^{-1}). Changes in the nature of long-tailed bat calls during prey capture (feeding buzzes) can confound species identification, but feeding buzzes rarely occur in the absence of other, more characteristic, calls. Feeding buzzes are not often heard from short-tailed bats.

Ecroyd (1994) and Winnington (1999a) have suggested that short-tailed bats can be located by monitoring flowering plants fed on by them (e.g., *Dactylanthus taylorii* and *Metrosideros* spp.). This has not proven to be as effective as wide-scale surveys of large stands of old growth forest with automatic bat detecting systems.

Adult male bats may be heard singing from November through until April. The audible component of the song is a high-pitched repetitive call resembling the song of rifleman *Acanthisitta chloris*. A strong ultra-sound component to the song can be heard using a bat detector. Both audible and ultra-sound components can be detected over a range of 50 m. The males generally sing in small clusters and can be either stationary in small cavities in trees (singing roosts) or moving around in the forest canopy. Singing is usually quite localised and does not provide a reliable method for finding short-tailed bats in large areas of forest.

Measurements

Measurements for adult *M. tuberculata* (B.D. Lloyd unpub. data) are: pre-foraging body mass 10–19.7 g (n = 300; mean 13.6 g; sd 1.3); forearm length 37.0–46.9 mm (n = 1068; mean 43.3 mm; sd 1.4); wing span 280–300 mm; body length with tail furled 60–70 mm (when the tail membrane is extended there is an extra c. 20 mm); ear length 17–19 mm; tibia or lower leg 14.5–17 mm. The body mass of individuals can increase by 20–30% after foraging trips. During autumn, when individuals put on condition to survive hibernation, pre-foraging body mass of individuals can increase by 20–30%. Female's body mass can increase by up to 35% during late pregnancy.

Variation

Hill and Daniel (1985) defined three subspecies of *M. tuberculata* on the basis of morphological characteristics. This has been supported by phylogenetic analysis of mitochondrial control region sequences (Winnington 1999b). Although the mean forearm lengths of the three subspecies (Table 2.1) are significantly different ($P < 0.001$), the range of forearm lengths for *M. t. rhyacobia* nearly encompasses the variation in all three subspecies. Thus, discrimination between the subspecies using forearm length is not possible.

Table 2.1 Forearm lengths of subspecies of *M. tuberculata* (B.D. Lloyd unpub. data)

	n	Mean (mm)	*s*	Max. (mm)	Min. (mm)
M. t. aupourica	49	40.90	0.89	42.00	36.95
M. t. rhyacobia	999	43.64	1.24	46.90	39.88
M. t. tuberculata	304	42.23	1.01	45.10	39.44

Distribution

Palaeological and historical evidence is sparse, but consistent with the view that the species was once very widespread but has been eliminated from many areas by forest clearance. Recent fossils (i.e., < 20 000 years old) of *M. tuberculata* have been found in

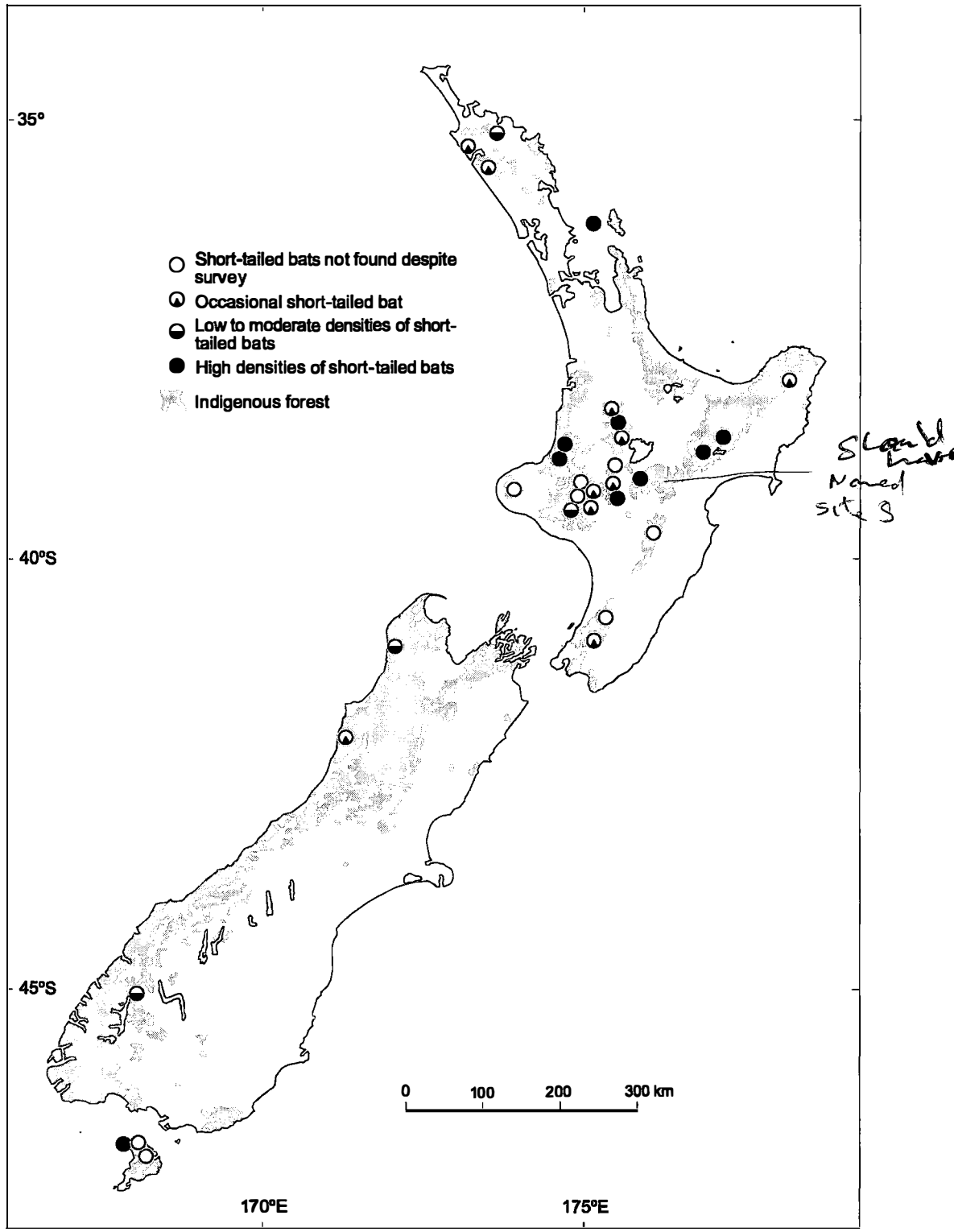


Figure 2.1 Distribution of *Mystacina tuberculata* as known in 2000

sites at Waitomo, Hawkes Bay and Wairarapa in the North Island, and north-west Nelson, Canterbury and Fiordland in the South Island (Daniel 1990a; Worthy & Holdaway 1993, 1994a, 1995, 1996; Worthy *et al.* 1996). There are nineteenth-century

accounts of large colonial roosts, found during logging in Kaipara, Thames and Wairarapa in the North Island and north-west Nelson in the South Island (e.g., Buller, 1893; Cheeseman, 1894). Short-tailed bats are no longer present at any of the reported locations, and in many cases the sites are no longer forested.

Many location records between 1930 and 1984 (Dwyer 1962; Daniel & Williams 1984; Daniel 1990a) are from areas where the species persists to this day, but a number are from mainland areas where there are no current records even though unmodified indigenous forest survives. The latter areas include: Coromandel, Rotorua district, Pirongia in the Waikato, East Cape, western Tararuas, Nelson Lakes, Richmond Range, Okarito in South Westland, Longwood Range and Catlins in Southland. The species may persist in some of these areas, as none of them have been surveyed.

Because short-tailed bats are forest-dwelling, cryptic, volant and nocturnal, the probability of casual sightings is remote. Consequently, short-tailed bats were considered rare on the New Zealand mainland. The recent development of automated bat-detecting systems that identify flying bats to species has revised our knowledge of the species' distributions. The results of ongoing surveys and other work (e.g., Hunt & Anderson 1996; Lyall 1996; Arkins 1998; Lloyd & Whiteford, 1998; Coats & Torr 1999; Lloyd 1999; O'Donnell *et al.* 1999; Williams 1999) show that populations of short-tailed bats survive in several areas in the North Island, and at least two areas in the South Island. None have been located on Stewart Island.

In Northland, a moderate-sized population remains in Omahuta-Puketi Forest, and individuals have been recorded in Waipoua and Warawara Forests. In the rest of the North Island, short-tailed bats have been found throughout the tract of indigenous forest remnants which extends from north Taranaki, across the central volcanic plateau to East Cape and south to the Tararuas. Significant populations have been found at Waitaanga, Pureora, Waitotara, Rangataua, Kaimanawa, Whirinaki, and south-east Urewera. They occur occasionally in forests in the Waimarino district, on the western slopes of Ruapehu, Raukumaras, and eastern Tararuas. Despite survey effort, none have been recorded from forests around Mt Taranaki, Wanganui River, Matemateonga, Tongariro, and Ruahines. There have been few, or no surveys, in most Northland forests, and in forests in Coromandel, Kaimai, Mamaku, Rotorua district, northern Urewera, Kaweka, and western Tararuas.

In the South Island, populations of short-tailed bats have been found in the Oparara Basin (north-west Nelson) and the Eglinton Valley (Fiordland). Calls have been

recorded at Punakaiki (West Coast). No other short-tailed bat calls have been recorded during small-scale surveys of forests in the north of the West Coast, South Westland, Fiordland and north-west Stewart Island. Extensive areas of forest in South Island and Stewart Island have not yet been surveyed.

There are large populations of lesser short-tailed bats on two offshore islands: Little Barrier Island (off the coast of Northland) and Codfish Island (off the coast of Stewart Island).

Habitat

Short-tailed bats live in forest areas from close to sea level (Codfish Island, Northland and Little Barrier Island) up to the upper altitudinal limits of forest (central North Island and Fiordland). Large populations have been found only in extensive (> 1000 ha) areas of undamaged old-growth forest, which include many large trees suitable for colonial roosts (> 1 m girth and > 25 m high), numerous epiphytes and deep leaf-litter. The species composition of the forest is not important. Low numbers of bats have been recorded in a variety of habitats (logged forest, scrubland, pine plantations and farmland) in areas close to areas of undamaged old-growth forest.

Food

Diet

The lesser short-tailed bat is remarkable among the Chiroptera for the diversity of its diet. Although the species is primarily insectivorous, it also eats nectar, pollen and fruit. It is the only temperate microbat known to consume plant matter (Daniel 1976; McNab 1982). Daniel's (1990a) statement that short-tailed bats are carnivorous, preying on nestlings and small birds, and scavenging carrion was based on inconclusive observations by Stead (1936) and unsubstantiated reports from mutton birders (Dwyer 1960). In both cases the species observed was probably the greater short-tailed bat *M. robusta*. Captive lesser short-tailed bats could not be induced to consume meat despite having it offered to them on many occasions (Blanchard 1992; B.D. Lloyd pers. obs.).

Lesser short-tailed bats consume a wide variety of volant and non-volant arthropod taxa (Table 2.2). Most of their diet is from five orders of insects: Coleoptera (beetles), Lepidoptera (moths), Diptera (flies), Blattodea (cockroaches) and Orthoptera

Table 2.2 Arthropod taxa identified in droppings from *M. tuberculata*. Data are from four faecal analysis studies in three areas: a. Arkins *et al.* (1999), Little Barrier Island; b. McQueen and Lloyd (unpublished), Rangataua Forest central North Island; c. Arkins (1997), Codfish Island; d. Lloyd and McCartney (unpublished), Codfish Island.

	Study			
Arthropod taxa	**a.**	**b.**	**c.**	**d.**
ACARINA (mites)	*			*
ARANEA (spiders)	**	*	**	*
OPILIONES (harvestmen)	*	*	*	*
AMPHIPODA		*		
MYRIAPODA (millipedes & centipedes)	*	*		
INSECTA				
BLATTODEA (cockroaches)			**	**
ORTHOPTERA				
Anostostomatidae (tree & ground weta)	**	**	**	***
Rhaphidophoridae (cave weta)	**	***		
HEMIPTERA	*			
THYSANOPTERA	*			
NEUROPTERA	*			
COLEOPTERA				
Curculionidae (weevils)	***	*	*	
Carabidae (carab beetles)	**	*		
Scarabaeidae (scarab beetles)	***	*	***	***
Chrysomelidae (leaf beetles)	**			
DIPTERA				
Tipulidae (crane flies)	**	*		***
Chironomidae (midges)	**	*		
Psychodidae (moth flies)	*			
Muscidae (house flies & blow flies)	**	***		
LEPIDOPTERA (moths)	**	***	*	**
HYMENOPTERA (wasps, bees ants)	*			*

Key: * occasional; ** common; *** abundant.

(weta). Other arthropod groups also fed on regularly are: Aranea (spiders), Amphipoda, and Myriapoda (centipedes and millipedes). Arkins *et al.* (1999) reported seasonal changes in the arthropod component of the diet of short-tailed bats on Little Barrier Island. Higher proportions of flying insects (coleopterans, lepidopterans and dipterans) were consumed during summer when they were more plentiful. During winter, araneans and orthopterans were more common in the diet. This, together with the high degree of

Plate 2.6 Short-tailed bats feeding on weta from the forest leaf litter (left) and flowers of New Zealand flax *Phormium tenax* (right)

variation, indicates that the species is probably an opportunistic forager (Arkins *et al.* 1999).

In captivity, lesser short-tailed bats eat virtually any arthropod presented to them (Dwyer 1962; Blanchard 1992; McCartney 1994; McQueen & Lloyd 1997, 1998; B.D. Lloyd pers. obs.). They appear to prefer soft-bodied insects to heavily sclerotised insects (McCartney 1994) and often clip off the legs and wings of large items before consuming the body (B.D. Lloyd pers. obs.). They have been maintained in captivity for long periods on a staple diet of honey water and cultured mealworm (*Tenebrio molitor*), moths (*Galleria mellonella*) and locusts (*Locusta* sp.), supplemented with small amounts of wild-caught arthropods. They regularly consumed 5–7 g of insects in a night (i.e., 36–50% of pre-feeding body mass) as well as the honey water (B. Blanchard, *pers. comm.*; B.D. Lloyd & S.M. McQueen, unpubl. data). This consumption rate is close to the estimated daily food intake for free-living bats of 5.6 g (i.e., 40% of body mass) calculated using an allometric equation (Lloyd & McQueen, 2000).

Mystacina is adapted for nectarivory: the tongue can protrude for at least 5 mm beyond the muzzle, and has a brush of fine papillae on its tip well suited to feeding on nectar and the reduced lower incisors facilitate use of the extensile tongue (Daniel 1976, 1979). The only observations of short-tailed bats taking nectar in the wild are by Ecroyd (1995), who obtained nocturnal video recordings of lesser short-tailed bats taking nectar from wood rose *Dactylanthus taylorii* (an endangered, low-growing root-parasite). In captivity they take nectar from a wide variety of flowers including native and exotic species (Blanchard 1992; McCartney 1994; B.D. Lloyd pers. obs.). While taking nectar from flowers they crawl across the inflorescences excitedly and when feeding on larger

flowers they may tear the flowers apart (Blanchard 1992; B.D. Lloyd pers. obs.). As a result of this behaviour the bats become covered in pollen, which they subsequently groom out of their fur and ingest.

Pollen from a wide variety of plant taxa has been identified in the droppings, stomachs and on the fur of lesser short-tailed bats from Northland (Daniel 1976, 1979), Little Barrier Island (Arkins *et al.* 1999), and central North Island (P. Peterson, A. Robertson & B.D. Lloyd, unpubl. data). Some pollen types are found only as traces, and may be contaminants picked up either from feeding on insects that have fed on the pollen, or by accidental contact with flowers. Other pollen types occur commonly and in large amounts, and are almost certainly an intentional part of the bat's diet, ingested after feeding on nectar. In Northland and on Little Barrier Island, pollen from *Metrosideros* spp., rewarewa *Knightia excelsa*, and the perching lily *C. hastatum* appear to be major components of the bat's diet during their flowering periods. In central North Island, the only pollen that appears regularly is the perching lily *C. microspermum*. Other pollen types, which are found only occasionally, but probably, consumed intentionally, are kiekie *Freycinetia bauriana*, and wood rose. Godley (1979) notes that the flower types visited by short-tailed bats have three characteristics in common: the pollen is exposed; nectar, if present, is easily available to the extensile tongue; and the flowers are aggregated in large or prominent inflorescences.

Lesser short-tailed bats are known to feed on the fruits of *F. bauriania*, *C. hastatum* and *C. microspermum*. Daniel (1976) found seeds from these species in droppings and on the fur and wings of bats caught in Northland. For some weeks during a period of prolific fruiting of *C. microspermum*, short-tailed bats in Rangataua Forest, central North Island, were feeding almost exclusively on these fruits (B.D. Lloyd pers. obs.). The droppings of almost all bats handled during this period comprised entirely the pulp and seed of *C. microspermum*, and there were numerous seeds adhering to their fur and wing membranes. The fruits of the three species that lesser short-tailed bats are known to feed on (*F. bauriania*, *C. hastatum* and *C. microspermum*) have small seeds that are either ingested, or adhere to the bat's fur and wing. It seems probable the bats also feed on other fruits with larger seeds that are not ingested and do not adhere to them, so evidence of feeding may have been overlooked.

Fern and fungal spores have been found on the fur of lesser short-tailed bats in Northland (Daniel 1976, 1979). In most cases there were only small numbers of spores, probably resulting from incidental contamination, but high numbers of spores of *Cyathea* spp., *Dicksonia squarrosa,* and a fungus, on some individuals, indicates that they may be have been fed on.

Foraging behaviour

Observations in captivity show that lesser short-tailed bats can display a wide range of behaviour when foraging for arthropods (Blanchard 1992; McCartney 1994; Jones 1999; B.D. Lloyd pers. obs.). Flying prey are caught by aerial pursuit which can involve either hawking or flycatching behaviour. Non-flying prey is caught either by gleaning from the ground and vegetation, or by hunting for them in crevices and under leaf litter. The bats are remarkably fast-moving and agile on the ground and when climbing. They have strong stocky legs, and the wing and tail membranes are furled out of the way allowing the bats to move around using their wrists like feet. Prey is usually caught in the mouth, but large items are often transferred to be held in the wing-claws and feet for processing before being consumed.

Lesser short-tailed bats locate non-flying prey on surfaces by either echolocation or passive listening, either while flying or while foraging on the surfaces. If the bats are flying they land near to the prey and then pounce. They use a combination of passive listening and olfaction to locate prey in crevices and under leaf litter. When foraging under leaf litter and humus, they often disappear underneath the leaf-litter and re-emerge only sporadically.

During the pursuit and capture of flying prey, microbats modify their echolocation calls to increase the amount of information available to them. Pulse length and interpulse interval are both reduced to produce characteristic feeding buzzes. Feeding buzzes have been recorded from captive lesser short-tailed bats during pursuit of flying prey (Jones 1999) and are occasionally heard in the wild. The rarity of feeding buzzes from lesser short-tailed bats in the wild indicates that aerial foraging is relatively uncommon (B.D. Lloyd pers. obs.).

Captive lesser short-tailed bats spend very little time flying, preferring to forage on the ground or in trees. Radiotagged free-living lesser short-tailed bats spend long

periods moving around in the same location presumably also foraging on the ground and in trees rather than flying (B.D. Lloyd pers. obs.). Wilson (1973), Daniel (1979), and McCartney (1994) provide unsubstantiated estimates of the relative proportions of different foraging categories for lesser short-tailed bats.

Social organisation and behaviour

Flight

In mature beech and hardwood forests lesser short-tailed bat usually fly in the relatively uncluttered layer below the main canopy and above the understorey, but they can also fly at high speed through relatively cluttered environments. They are also able to fly quite slowly, manoeuvering easily in restricted spaces and can take off from flat surfaces easily, leaping into the air before flying almost vertically upwards (B.D. Lloyd pers. obs.). Radiotagged bats at Rangataua and in the Eglinton Valley frequently commute > 10 km from their day roosts when foraging (O'Donnell *et al.* 1999; B.D.

Plate 2.7 Short-tailed bat in flight (photograph Stephen Barker)

Lloyd pers. obs.). One radiotagged bat was estimated to be flying at 60 km h^{-1} while commuting (O'Donnell pers. comm.).

The differing wing morphology of various bat species are thought to have evolved to suit each species' flight strategies, which in turn are adapted to the species' foraging modes and habitat types (Norberg & Rayner 1987; Norberg 1994). Norberg & Rayner (1987), Norberg (1994), and Webb *et al.* (1998) compared short-tailed bat's wing morphology with other microbat species. Estimates for all the wing parameters examined by Webb *et al.*(1998) were within the 95% confidence interval of expected values for microbats of similar body mass ($n = 35$, wing loading 9.68 ± 0.76, aspect ratio 5.57 ± 0.24, tip length ratio 1.4 ± 0.09, tip area ratio 0.90 ± 0.06 and tip shape index 1.93 ± 0.59). The results indicate that short-tailed bat's wing morphology is not specialised for any particular flight strategy. Lack of specialisation probably represents a compromise between the different adaptive pressures resulting from the wide variety of flight strategies required for the diverse foraging modes and habitat types used by short-tailed bats. The range of foraging modes found in lesser short-tailed bats is the widest known in any bat species, and includes frugivory, nectarivory, as well as hawking, flycatching, gleaning and terrestrial foraging for insects. Old growth forest, the species' typical habitat types, includes both the relatively uncluttered layer between the upper canopy and undergrowth as well as the extreme clutter found within the canopy and undergrowth.

Echolocation calls

The lesser short-tailed bat's echolocation calls are broad-band frequency-modulated calls with steeply descending frequencies, a high pulse repetition rate and a low duty-cycle (Pye 1983; Parsons 1997 & 1998; Lloyd 1998; O'Donnell *et al.* 1999) typical of bats that forage in forests. The calls usually contain a fundamental and two other harmonics, occasionally a fourth harmonic is present. The fundamental begins at *c.* 30 kHz and descends to 20 kHz. The second harmonic descends from 60 to 40 kHz, the third from 80 to 60 kHz and the fourth from 105 to 85 kHz. Maximum power is usually at about 28 kHz in the fundamental but may be in the first harmonic. The call duration is relatively constant, 3.5–4.5 ms, whereas the interpulse intervals in sequences of calls may vary widely from 10 to 100 ms. Parsons (1997) suggests there may be geographic variation in the structure of the echolocation calls but I have recorded high levels of variation in a single population and believe Parsons' (1997) conclusion is not supported

by existing data. Bats adjust the structure of their calls to suit both the local environment and their activity. Indeed, Parsons (1998) demonstrated significant differences in echolocation call-structure between individual lesser short-tailed bats and in different recording situations.

Roosting

Lesser short-tailed bats spend the daytime and long periods at night in roosts. The roosts may be either colonial or solitary. Colonial group sizes vary: the largest colonial roost recorded thus far contained > 6000 bats. All contemporary records of colonial roosts have been in cavities in the main trunks of old-growth forest trees. Large colonial roost trees are traditional, being used intermittently for several decades or possibly even hundreds of years. There are usually numerous large colonial roost trees in the home range of a bat population. For instance, in Rangataua Forest the bat population uses more than 30 large colonial roost trees scattered throughout 10 000 ha of forest (Lloyd & McQueen 1998). During summer individual colonial roost trees are usually occupied for only relatively short periods, from a few days to a few weeks, before the bats move on to other roost trees. Occupation periods tend to be longer during winter and may extend to months. During early summer the entire population of bats in an area can often be found occupying only two or three colonial roosts, but at other times of the year roosting groups tend to be smaller and many bats roost solitarily. In winter most bats roost alone and colonial roosts rarely contain more than 100–200 bats (Lloyd & McQueen, 1998).

Individual bats move freely between the different occupied roosts and may also shift from roosting solitary to colonial roosting. When colonial groups move between roosts, almost all members of the group move on the same night, even though the roost trees may be several kilometres apart. A roost tree occupied by several thousand bats one day may be completely deserted on the following day. The pattern of movements between roost sites appears seasonal but is not entirely predictable. The stimulus initiating movement between roosts is generally unknown, but disturbance at the roost by predators or humans can cause an occupied roost to be abandoned (B.D. Lloyd pers. obs.).

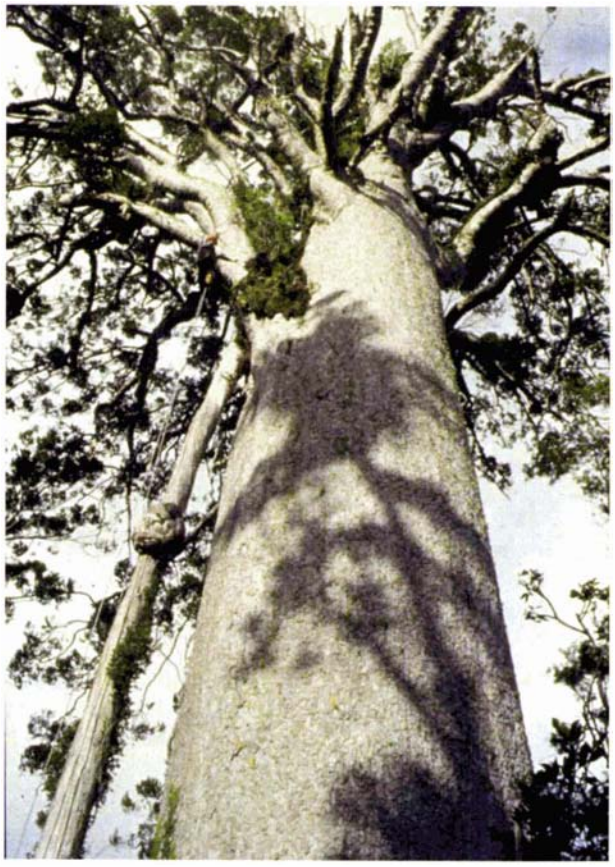


Plate 2.8 Typical colonial roosts found in different regions: (above) kauri *Agathis australis* in mainland Northland, (top right) red beech *Nothofagus fuscus* in beech forest throughout mainland New Zealand, and (bottom right) Hall's totara *Podocarpus hallii* on Codfish Island

Usually colonial roost trees are alive, though very occasionally short-tailed bats continue to use a traditional roost tree after it dies. Typically, the roost entrance is on the main trunk less than 7 m from the ground, and the roost cavity extends upwards. Roost entrances vary from slits or holes only just large enough for bats to enter (25 mm diameter) to massive openings more than 3 m high and up to 1 m wide. The roost cavity is usually a chimney-like, sometimes extending several metres up inside the main trunk.

Bat teeth marks are often apparent on the inner surface of the walls, indicating that lesser short-tailed bats burrow into the rotten wood inside hollow trees to excavate cavities for roosting. Further evidence for this behaviour are the seasonal occurrence of droppings composed entirely of chewed wood at the base of a roosts, and the presence of wood fragments in the mouths of captured bats.

The preferred tree species for colonial roosting varies regionally and in different forest types. In mainland Northland colonial roosts have only been found in kauri *Agathis australis*, whereas in the lowland forests on nearby Little Barrier Island colonial roosts occur in pohutakawa *Metrosideros excelsa*, and puriri *Vitex lucens*. In the hardwood-podocarp of the North Island the preferred species is matai *Podocarpus spicatus*, though historically short-tailed bats were frequently reported as roosting in the now relatively uncommon kahikatea *Podocarpus dacrydioides*. In beech forest the bats favour red beech *Nothofagus fuscus* and hard beech *N. truncata*, silver beech *N. menziessi* is used occasionally, but mountain beech *N. solandri* is not used for colonial roosts. On Codfish Island most colonial roosts are in Hall's totara *Podocarpus hallii*. Other tree species occasionally used for colonial roosts are southern rata *Metrosideros umbellata*, kamahi *Weinmannia racemosa*, and rimu *Dacrydium cupressinum*.

There are no contemporary reports of colonies of lesser short-tailed bats roosting in caves, although large concentrations of mystacinid remains have been found in caves, indicating that they formerly roosted in them (Worthy & Holdaway 1994a). Large numbers of both species of *Mystacina* roosted in the granite sea caves on small islands off south-west Stewart Island before the populations went extinct in the mid 1960s (Stead, 1936 Daniel, 1990a).

When roosting solitarily, lesser short-tailed bats commonly roost in small cavities in the main trunks of a variety of tree species. They have also been found in many other locations (B.D. Lloyd pers. obs.) including: large, otherwise empty, colonial roosts; under bark flakes; in small cavities in rotted wood; inside the trunk of dead tree-ferns; at the base of perching lillies; and in holes in the ground.

Although there may be many factors determining whether an individual bat roosts alone or in a colony (Altringham 1996; Vonhof & Barclay 1996), the primary determinant for short-tailed bats is probably the energetic costs of the different strategies. During summer, when all bats are active nightly, clustering in colonial roosts reduces the energetic costs of thermoregulation. In contrast, during winter, when bats

Plate 2.9 Inside colonial roosts short-tailed bats form tight clusters of hundreds or thousands of bats to reduce the energetic costs of thermoregulation

are hibernating, disturbance by other bats in colonial roosts will result in increased energetic costs. Advantages of colonial roosting include additional opportunities for mating and maternal care and a reduction in the individual predation risk resulting from being a group member. Disadvantages include an increased incidence of disease and ectoparasites and increased local competition for food.

In colonial roosts the bats form tight clusters, with bats stacked on top of one another. During summer the bats become very active in the afternoon and roost temperatures rise, reaching as high as 39°C (Daniel 1979). At this time, the bats make a great deal of noise scuttling around inside of the roost, echolocating and squeaking. Bats begin leaving their roosts approximately 30 minutes after civil twilight ends in the evening (defined as when the geometric centre of the sun is 6° below the horizon). During summer, almost all bats leave the roost within a 40-minute period but in winter the departures are scattered throughout the night and some bats do not leave the roost at all. Departure rates from large summer colonial roosts can reach 200 bats per minute. Bats often visit daytime roosts intermittently through the night, sometimes in large

numbers. In the morning, all bats are in roosts approximately 30 minutes before civil twilight begins.

Torpor and hibernation

Lesser short-tailed bats use torpor to conserve energy (Webb 1999; B.D. Lloyd pers.obs.). When in torpor a bat's metabolic rate is reduced to a small fraction of the normal resting metabolic rate and its body temperature falls below the normal homeothermic level and approaches ambient temperature (Altringham 1996). Arousal from torpor can take 10 to 20 minutes. Most temperate-zone microbats use torpor but *Mystacina* is the only genus in the Noctilionoidea known to use it (Ransome 1990). Torpor may vary from daily torpor, lasting a few hours during the daytime resting period, to seasonal hibernation, lasting many days after a fall in the ambient temperature or a reduction in food supply (Altringham 1996). At Rangataua Forest in the central North Island seasonal hibernation extended from late April through until mid-October (B.D. Lloyd pers. obs.), a period of about five months, though onset of seasonal hibernation may be delayed by a plentiful supply of autumnal fruit. During hibernation, periods of torpor lasting up to 10 days are interspersed with periods of activity lasting from 1 h to several nights. Longer activity periods are likely during moist, unsettled weather, when night-time temperatures are relatively high and invertebrate activity levels approach summer levels. These intermittent periods of intense activity throughout the winter led Daniel & Williams (1984) and Daniel (1990a) to conclude that lesser short-tailed bats do not hibernate; however periodic arousal from hibernation is typical of all hibernating animals (Lyman *et al.* 1982).

Reproduction and development

During courtship, sexually active male lesser short-tailed bats occupy mating or singing roosts in trees on commuting routes, or close to major colonial roosts and call from them at night to attract passing females for copulation. The song is a high-pitched repetitive call with a strong ultra-sound component. Mating roosts generally have a small entrance *c.* 20 mm diameter in the main trunk of a tree, usually < 4 m above ground. The bark around the entrance is polished with a brown oily secretion from the males' throat glands. The secretion has a distinctive musky smell, which presumably serves to attract females.

Males appear to vary their mating strategies according to circumstances. In areas with predictable high densities of females (e.g., Codfish Island and Little Barrier Island) mating roosts are clustered and males compete for possession of them. Daniel & Pierson (1987) and Daniel (1990a) describe this behaviour as a lek mating system. In large mainland populations, where home ranges are larger and the location of female congregations are less predictable, mating roosts in the vicinity of active colonial roosts are occupied only transiently. Large numbers of males congregate nightly in the vicinity of maternity roosts throughout the lactation period. These males move around in the forest canopy singing, presumably attempting to attract females for copulation as they enter and leave the maternity roost.

Singing begins during spring and early summer (September to December) but courtship activity peaks during late summer and autumn (January to May). Most females mate in late summer. Daniel & Pierson (1988) observed mating on Codfish Island during March, and vaginal plugs have been observed in females caught in Rangataua Forest during April (B.D Lloyd pers. obs.). Vaginal plugs are formed from secretions produced by the male's urethral gland during mating. They harden in the vagina soon after mating to block further matings. Gestation does not begin until several months after mating, in the following spring, probably at the time bats begin feeding nightly (i.e., mid October). The mechanism allowing the delay between mating and the onset of gestation has not been investigated in short-tailed bats. In other species of microbats it may be achieved by sperm storage in the female, delayed implantation, or embryonic diapause.

Lesser short-tailed bats are monoestrous, giving birth to a single pup some time between mid December to mid January throughout New Zealand. Births within a population are closely synchronised with most births occurring during a one-week period. Pups born outside of the main birthing period are probably at a disadvantage. All the pregnant females in a local population either give birth to their pup in a maternity roost or carry the pup to the maternity roost at an early age. When being carried the pups hang by their milk teeth from one of the mother's teats. Pups up to two weeks old can be carried long distances between roosts in this way, but they are not carried on foraging flights. There are usually several roost trees that may be used as maternity roosts within a population's home range, but only one maternity roost tree is used at any one time. All pups in a population are gathered together in the active maternity tree.

This aggregation of individuals elevates ambient roost temperatures and humidity. Maternity roosts are occupied for periods from two to four weeks (B.D. Lloyd pers. obs.).

During the first two weeks after birth, the lactating females roost in the maternity roost with the pups during the day and return to feed the pups several times at night. Later the lactating females roost in other colonial roosts during the day and merely visit the maternity roost at night to feed their pups. Some males and non-breeding females use the maternity roost as a day roost during this period.

At birth the pup's body mass is *c.* 5 g, (*c.* 30–35% of the mother's body mass), and the forearm length is *c.* 19 mm. Their eyes are closed and they have no fur, other than bristles around the muzzle. Skin pigmentation is present at birth: the back is grey-brown and the front paler. By two weeks after birth the back, sides, and head are covered by short silver-grey velvety fur and the eyes are beginning to open. The pups are born with milk teeth at the front of the mouth (canines and incisors). These milk teeth are very fine and have a backward curve. They are replaced by permanent teeth about three weeks after birth. At about this time the pups begin exercising their wings; they begin flying in the vicinity of the roost at about four weeks, by which time they are completely furred. About six weeks after birth they leave their maternity roost. By eight weeks they are skeletally almost fully-grown, but their body mass usually remains less than that of an adult for some months.

Population dynamics

The population dynamics of lesser short-tailed bats have not been studied closely due to problems in attaching permanent individual markers. Forearm-bands, which can be used to study some bat species without problems, injure short-tailed bats wing and forearm. Most information comes from work undertaken by the author in Rangataua Forest, central North Island. In this population there are equal numbers of males and females, but the results of any given capture sessions are often heavily skewed to one sex or the other. Approximately 80–90% of reproductively mature females breed each year. During the breeding season *c.* 20% of females are reproductively immature, indicating that females do not breed in their first year.

Simultaneous video counts of departures from all active colonial roosts in an area provide population estimates. Pre-breeding estimates of the population at Rangataua Forest over a five-year period (1995–99) fluctuated between 5740 and 6977 (i.e., 22% from the minimum value). There was no trend in the population estimates. Minimum population estimates have also been obtained for populations at Waitaanga (2700 Williams 1999), Eglinton Valley (257 O'Donnell *et al.* 1999), and Codfish Island (1557 Sedgeley & Webb 1999). The estimates for the latter two populations are probably substantially lower than the actual population size because there were other occupied colonial roosts in the areas.

In the central North Island there are at least six local populations of lesser short-tailed bats (Waitaanga, Pureora, Waitotara, Rangataua, Kaimanawa, and Whirinaki), each is centred around a number of clusters of large colonial roost trees. Adjacent populations are separated by between 60 and 80 km. Densities of bats are generally high throughout forest within 5 or 10 km of the roost clusters and low in forest remote from them. Preliminary genetic analysis of a mitochondrial DNA marker has identified high levels of gene flow between local populations (unpub. data.). Thus, the lesser short-tailed bat populations in the central North Island may comprise a single metapopulation with high levels of dispersal between local populations. The distances between adjacent populations do not present serious obstacles to dispersal as the bats can fly at 60 km h^{-1} and routinely forage up to 10 km from their daytime roosts.

Population parameters have been determined for a range of microbats outside of New Zealand (Tuttle & Stevenson 1982; Ransome 1990; Altringham 1996). Sexual maturity is normally reached in one to two years. Typically preflight mortality is low (mean 3%, range 0–12%), but mortality during the early flight period can reach > 50%. Mortality during the first year of life is quite high for many species, but decreases quickly. If individuals survive the first year they have 40–80% chance of surviving to seven or eight years old. In Europe there are records of microbats surviving for more than 30 years in the wild.

Causes of decline

The lesser short-tailed bat is thought to have been widespread throughout pre-human New Zealand and to have suffered massive declines as a result of forest clearance following human settlement (e.g., Dwyer, 1962; Daniel, 1990a). The evidence for this is

persuasive but largely circumstantial. Most of the forest cleared since human colonisation was low-altitude forest on fertile land, the type of forest likely to contain the highest numbers of bats. The role of introduced mammals in the species decline is uncertain. Degradation of indigenous forest by introduced herbivorous mammals (cattle, sheep, goats, pigs, deer, possums and rodents) may have reduced bat numbers in some areas, but is unlikely to have led to their disappearance.

Threats

Predators

At least three native avian predators preyed on lesser short-tailed bats: the extinct laughing owl *Sceloglaux albifacies*, New Zealand falcon *Falco novaeseelandiae* and morepork *Ninox novaeseelandia*. Large numbers of bones found in laughing owl's midden deposits indicate that until kiore or Pacific rats *Rattus exulans* arrived in New Zealand *Mystacina* spp. was an important component of the laughing owl's diet (Worthy & Holdaway 1996). Lesser short-tailed bats were occasionally taken by New Zealand falcons, as small numbers of their bones have been found in falcon midden deposits (Worthy & Holdaway 1995). Moreporks have been observed hunting lesser short-tailed bats near colonial bat roosts (B.D. Lloyd pers. obs.). They appear to target juvenile bats learning to fly in the vicinity of maternity roosts.

The only introduced mammalian predator known to prey on short-tailed bats on the New Zealand mainland is the domestic cat *Felis catus*. Six, of 23, dead short-tailed bats handed in by the general public were caught by domestic cats (Daniel, 1990a), but short-tailed bat remains have not been recorded in diet studies on feral cats. During video surveillance of short-tailed bat roosts at Rangataua Forest, stoats *Mustela erminea* and ship rats were recorded visiting roosts though only stoats actually entered them (B.D. Lloyd pers. obs). No kills by either species were recorded.

Rats and other introduced mammalian predators have not had catastrophic impacts on lesser short-tailed bats in the central North Island. Large populations of the bats have persisted in their presence for over 100 years. Nevertheless, introduced predators could be responsible for the absence of bats from other mainland forests. The persistence of high densities of bats on Little Barrier Island and Codfish Island despite the presence of kiore indicates that they are not a serious threat to lesser short-tailed bats.

Lesser short-tailed bats exhibit a number of behaviours that may have evolved to minimise predation in or near their roosts, which is where bats are most vulnerable. Colonial summer roosts appear particularly vulnerable to predators as they are often noisy and smelly, with large entrances low to the ground. Yet, the results of long-term video surveillance, together with the persistence of major populations in the presence of predators, indicate predation is not a serious problem. There are a number of possible reasons for this. Short-tailed bats are extremely aggressive and if they attacked in large numbers they could injure any predators entering the roost. Large group sizes (i.e., up to 6000) in colonial roosts and frequent movements of groups between roost trees many kilometres reduce the probability of predation for any individual by predator confusion and satiety. Evening departures and morning returns over short periods during darkness minimise the risk of attack by diurnal or crepuscular predators. When departing from colonial roosts, the bats usually leave in small groups at random intervals, with individuals flying at high speed in different directions ("burst-emergence") which may confer an advantage against avian predators. On return to roosts, individuals usually make several high-speed exploratory approaches before landing. During winter roosting bats remain in torpor for long periods and are vulnerable to predators, but the entrance to winter roosts are usually too small for rats or stoats to enter.

It has been suggested that terrestrial and arboreal foraging by short-tailed bats may render them vulnerable to mammalian predators (Daniel & Williams 1984; Molloy 1995). Some may be caught while foraging on the ground or in trees, but they would not be easy prey. They are cryptic, fast moving, with acute hearing, and sense of smell, and can take to flight easily.

Impact of poison operations

A variety of poisons and bait types are distributed throughout New Zealand forests in the course of vertebrate pest control operations. Because lesser short-tailed bats are omnivorous and terrestrial they could be vulnerable to poisoning during these operations. Feeding trials with the carrot and grain-based baits commonly used with the toxin 1080 and second-generation anticoagulants showed that bats will not feed on them (Lloyd 1994). However, captive bats do feed avidly on jam bait, which is used with 1080, and one bat was found killed by a fruit-lured cyanide bait (Daniel 1990a).

The bats may also be at risk of secondary poisoning by consumption of arthropods that have fed on toxic baits. A short-tailed bat will receive the median lethal

dose (LD_{50}) of 1080 from as little as 0.04 g (0.7% of its daily food intake) of arthropods containing 57 $\mu g\ g^{-1}$ of 1080, the mean concentrations in arthropods collected from toxic baits after a possum control operation (Lloyd & McQueen 2000). The risk of secondary poisoning with second-generation anticoagulants, such as brodifacoum, may be higher, as they have a cumulative effect and are more persistent in the environment than 1080.

Despite evidence that individual bats may be killed during pest control operations, there is no indication that these operations have a serious impact on populations of lesser short-tailed bats. A possum control operation using aerial broadcast 1080 had no measurable impact on a bat population in Rangataua Forest (Lloyd & McQueen, 2002) and populations persist at Pureora and North Waitaanga despite extensive possum control operations with a variety of toxins and baits. The bat population on Codfish Island survived aerial broadcast brodifacoum used to eradicate kiore (Sedgeley & Webb, 1999).

Adaptations to the New Zealand environment

The wide range of foraging modes exhibited by lesser short-tailed bats is probably an adaptation to the unusual pre-human New Zealand environment, characterised by the absence of significant ground predators or nocturnal competitors. The wide range of foraging modes is facilitated by a number of morphological adaptations. Folding of the wing and tail membranes, the reduced propatagium (which increases the freedom of movement of the forelimb), the robust hind limbs, the basal talons and the wide range of movements of the femur, are all adaptations for terrestrial or arboreal foraging. The unspecialised wing morphology probably represents adaptation to the wide variety of flight strategies required for the species' diverse foraging modes. The tongue and teeth of short-tailed bats appear to be adapted for feeding on nectar and fruit as well as insects. The tongue's extensile nature and the brush of fine papillae on its tip are suited to feeding on nectar, while the rough transverse ridges on the tongue may help in extracting juice from fruit. The dentition is essentially insectivorous; however, one upper premolar and a lower premolar and incisor have been lost, and there is a reduction in the size of remaining lower incisor. The resulting gap in the teeth at the front of the lower jaw facilitates use of the extensile tongue to feed on nectar.

Significance to the New Zealand environment

As the only extant species of an ancient endemic New Zealand family, lesser short-tailed bats provide a significant contribution to New Zealand's biodiversity. They also pollinate a number of indigenous forest plant species, including kiekie *Freycinetia bauriana*, wood rose *Dactylanthus taylorii*, pohutakawa and rata *Metrosideros* spp., rewarewa *Knightia excelsa*, and the perching lillies *Collospermum hastatum* and *C. microspermum*. None of these species is completely dependent on lesser short-tailed bats for pollination, but bats are often more effective pollinators than birds. It has been suggested that *F. bauriana* and *D. taylorii* are adapted for pollination by short-tailed bats (Lord 1991; Ecroyd 1995). Lesser short-tailed bats disperse seeds of *C. microspermum*, *C. hastatum* and *F. bauriana* in their droppings and adhering to fur and wing membranes.

Conservation Status

Various conservation ranking systems list *M. tuberculata* as threatened (Bell 1986), vulnerable (Groombridge 1993) and a species of highest conservation priority, i.e., Category A, (Molloy & Davis 1994). Baillie & Groomridge (1996) rank *M. tuberculata* as: "VU 42 A2c, C2a". VU is defined as: "Not critically Endangered or Endangered but is facing a high risk of extinction in the medium term future on the basis of criteria A2c and C2a". Criterion A2c is: "A reduction of at least 20%, projected or suspected to be met within the next ten years or three generations, whichever is the longer, based on a decline in area of occupancy, extent of occurrence and or quality of habitat." Criterion C2a is: "Population estimated to be less than 10 000 mature individuals and a continuing decline, observed, projected, or inferred, in numbers of mature individuals and population structure in the form of severely fragmented (i.e., no subpopulation estimated to contain more than 1000 mature individuals)." This ranking requires revision as recent work indicates the overall population is in excess of 30 000 individuals and several populations contain more than 1000 bats. There is insufficient information to predict population trends. Although there is no good reason to suspect a future significant reduction in the overall population, it would be prudent to institute a programme to monitor population trends.

GREATER SHORT-TAILED BAT

Mystacina robusta Dwyer, 1962

Synonym *Mystacina tuberculata robusta* Dwyer, 1962

Measurements

Daniel (1979, 1990a) estimated the body mass of adult greater short-tailed bats as 25–35 g by extrapolating the regression of body mass against forearm length for lesser short-tailed bats. A similar regression, using data from 227 non-breeding adult lesser short-tailed bats captured in central North Island during evening departures (authors unpub. data), indicates a mean body mass (with 95% confidence intervals) for greater short-tailed bats of between 14.19 ± 0.174 g (for forearm lengths of 45.3 mm) to 14.82 ± 0.174 g (for forearm lengths of 47.5 mm).

Plate 2.10 Greater short-tailed bat, *Mystacina robusta*, July 1965 Big South Cape Island (photograph Don Merton)

Variation

Worthy *et al.* (1996) examined size variation in the greater short-tailed bat by comparing recent specimens from Big South Cape and Solomon Islands with fossil remains, mainly of Holocene age, from throughout New Zealand. There was significant clinal variation, with a 10–20% size increase in size from the most southern specimens to the most northerly. They theorised that this variation developed in response to two selection pressures: large size in the north to facilitate exploitation of the abundant macro-invertebrates; and small size in the south to reduce energy expenditure during arousal from torpor.

Distribution

Recent fossil remains (i.e., < 20 000 years old) have been found in sites at Waitomo, Hawke's Bay, and Wairarapa in the North Island, and north-west Nelson, Westland, Canterbury, and Central Otago in the South Island (Worthy & Holdaway 1994a; Clark *et al.* 1996; Daniel 1990a; Worthy *et al.* 1996). No specimens have been collected from New Zealand's three main islands since European arrival, *c.* 200 years ago. By that time the species was probably restricted to islands off the coast of Stewart Island (Daniel & Williams 1984; Daniel 1990a). There are recent reports of bats on Putauhina Island, one of the neighbouring southern Titi Islands, (Hunter 1999; O'Donnell 1999) close to the last known location of greater short-tailed bats.

Habitat

There are differences in the relative proportions of greater and lesser short-tailed bats in fossil deposits in different parts of New Zealand (Worthy & Holdaway 1996). Greater short-tailed bats are more abundant in deposits from Canterbury, whereas lesser short-tailed bats are more abundant in deposits from north-west Nelson. These differences may reflect differences in the habitat preferences of the two species, but could also be a result of the small sample sizes, or reflect biases in the depositional processes at the different sites.

Food

Reports that *Mystacina* are carnivorous and scavenge vertebrate carcasses (Daniel 1976, 1990a) stem from Stead's (1936) observations of greater short-tailed bats on Solomon Island. Seven bats were held in captivity for a few days. On the first night the only food provided was the skinned carcass of a diving petrel: some flesh was consumed on the first night but none subsequently. Stead (1936) also tentatively suggested, without evidence, that bats may have been responsible for partly eaten bellbird nestlings in a nest he found. Dwyer (1960) reported unsubstantiated observations that short-tailed bats on the Titi Islands had been observed chewing fat and meat off plucked mutton-birds *Puffinus griseus* hung out overnight.

Social organisation and behaviour

Flight

Norberg & Rayner (1987) reviewed the influence of microbats' wing morphology on flight performance. They concluded that, because greater short-tailed bats had higher wing loading and aspect ratio (9.2 and 20.2 N m^{-2}) than lesser short-tailed bats (7 and 12.3 N m^{-2}), their flight would have been faster, with more rapid turns (i.e., more agile), but requiring larger turning radii (i.e., less manoeuvrable). This estimate of wing loading for greater short-tailed bats was based on a body mass estimate of 24.5 g. Using the revised body mass estimate of 14.5 g, the wing loading for greater short-tailed bats reduces to12.2 N m^{-2}, almost identical to that of the lesser short-tailed bat. Thus, flight performance of the two species was probably quite similar.

Echolocation calls

There is a linear relationship between the frequency of maximum intensity of the echolocation calls of insectivorous microbats and their forearm length (Bogdanowicz *et al.* 1999). According to this relationship, the frequency of maximum intensity for greater short-tailed bats should be 1 to 2 kHz lower than that of lesser short-tailed bats, i.e., 26–27 kHz.

Roosting

The concentrations of greater short-tailed bats' remains in some limestone caves in the Waitomo area indicate they roosted in caves (Worthy & Holdaway 1994a). They probably also roosted in tree cavities but evidence for this would not persist.

Torpor and hibernation

Greater short-tailed bats used torpor; Stead (1936) describes removing cold and sluggish (i.e., torpid) bats from a day roost on Solomon Island during early summer. Like other temperate-zone microbats, they almost certainly also used seasonal hibernation, with occasional flights during the winter months (Daniel, 1990a).

Threats

Laughing owl *Sceloglaux albifacies*, morepork *Ninox novaeseelandia* and falcon *Falco novaeseelandiae* all preyed on greater short-tailed bats. Large numbers of remains of greater short-tailed bats were found in laughing owl middens in north-west Nelson and Canterbury (Worthy & Holdaway 1994b, 1995, 1996; Worthy *et al.* 1996). Small numbers of remains were found in falcon deposits in North Canterbury (Worthy & Holdaway 1995).

Fossil evidence indicates that greater short-tailed bats declined markedly on the New Zealand mainland following the arrival of kiore or Pacific rat *Rattus exulans*, brought to New Zealand by early Polynesian migrants. There is a close association between the disappearance of greater short-tailed bat and the appearance of kiore in laughing owl middens (Holdaway, pers. comm.) and it seems probable that kiore wiped out greater short-tailed bats on the mainland shortly after their arrival (Daniel & Williams 1984; Daniel 1990a). There is no evidence that greater short-tailed bats were more terrestrial than lesser short-tailed bats, but the larger size of the greater short-tailed bats would make them vulnerable to predation in their hibernacula, as crevices accessible to them would also be accessible to kiore.

Conservation status

Greater short-tailed bats are assumed to be extinct, as there have been no confirmed sighting since 1967 (e.g., Daniel & Williams 1984; Daniel 1990a; Baillie & Groomridge

1996). There is a remote chance that the species persists on one of the privately owned islands off the south-west coast of Stewart Island.

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Chapter 3:

The intraspecific phylogeny of the New Zealand short-tailed bat *Mystacina tuberculata* inferred from multiple mitochondrial gene sequences

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ABSTRACT

An intraspecific phylogeny was established for the New Zealand short-tailed bat *Mystacina tuberculata* using a 2878 bp sequence alignment from multiple mitochondrial genes (control region, ND2, 12S rRNA, 16S rRNA, and tRNA). The inferred phylogeny comprised six lineages with estimated divergences extending back between 0.93 and 0.68 My to the middle Pleistocene. The lineages do not correspond to the existing subspecific taxonomy. Although multiple lineages occur sympatrically in many populations, the lineages are geographically structured. This structure must have persisted despite repeated cycles of range expansion and contraction in response to climatic oscillations and catastrophic volcanic eruptions. The distribution of lineages among populations in the central North Island indicates a hybrid zone was formed by simultaneous colonisation from single-lineage source populations inhabiting remote forest refugia. The observed pattern is not typical of microbats, which because of their high mobility generally exhibit low levels of genetic differentiation and geographic structure over continental ranges. Although lineages of *M. tuberculata* occur sympatrically in many populations genetic distances between them are sufficiently large to suggest that they may be considered as evolutionary significant units (ESU) or taxonomic subspecies. Confirmation requires further study using a recombining biparental marker.

INTRODUCTION

The endangered lesser short-tailed bat *Mystacina tuberculata* is a moderate sized (14 g) noctilionoid microbat endemic to New Zealand (Lloyd, 2001). The species is forest dwelling, with populations occurring only in extensive stands of tall old-growth forest. It was widespread throughout the forest that dominated pre-human New Zealand, but has suffered massive declines as a consequence of extensive deforestation since human settlement began about 1000 years ago. Of the three subspecies *M. t. aupourica, rhyacobia* and *tuberculata* (Hill and Daniel, 1985), only *M. t. rhyacobia* remain in good numbers on mainland New Zealand (Lloyd, 2001). Seven populations of *M. t. rhyacobia* are known to persist within remnants of the almost continuous tract of old growth forest (Fig. 3.1), which covered central North Island until deforestation during the period 1890 to 1980 (McGlone, 1988). Populations in the central North Island are large, each containing several thousand bats. Although adjacent populations are separated by distances of as little as 60 km, field investigations indicate there is little movement between populations (Lloyd, 2001). Radiotagged bats remained within about 10 km of colonial roost trees clustered in the core of the population's home range. Short-tailed bats forage in a variety of habitats, including forest margins, but they are rarely found far from forested areas, and dispersal over long distances outside of forest has not been documented. In the contemporary fragmented landscape, deforested areas between populations may hinder dispersal and have deleterious demographic and genetic consequences for the long-term viability of the populations (Lande, 1988).

The primary objective of this study was to estimate levels of dispersal between central North Island populations using molecular markers. These estimates are required to assess the impact of recent forest fragmentation and interpret data on population trends and conservation management outcomes. Control region sequences were initially chosen for the analysis as they have been widely used to examine intraspecific phylogenies and investigate population dispersal patterns in many mammal species including bats (e.g., Worthington Wilmur et al., 1994; Palumbi, 1996; Wilkinson and Fleming, 1996; Petri et al., 1997; Ingman et al., 2000). Preliminary phylogenetic analysis of control region sequences showed high levels of variation between and within populations, but the inferred phylogeny was poorly resolved. Nodal support was generally low throughout the phylogeny and sequences from central North Island

individuals were paraphyletic to sequences from the other two subspecies, originally included as outgroups. Although the control region is suited to phylogeographic analyses over microevolutionary timescales of tens of thousands of years (Avise, 2000), over longer timescales high levels of homoplasy can confound distance estimation and phylogenetic inference (Tamura and Nei, 1993; Ingman et al., 2000). It seems likely that divergence times between lineages of *M. tuberculata* exceed the working timescale for control region sequences.

Estimating phylogenies using sequences from a number of non-contiguous mitochondrial genes improves the probability of recovering an accurate phylogeny (Cummings et al., 1995; Otto et al., 1996). I therefore chose to proceed using mtDNA sequences from three regions outside the control region, both to establish a robust phylogeny and to confirm the occurrence of homoplasy among control region sequences. The regions selected for further sequencing were: the ribosomal RNA subunit genes 12S rRNA and 16S rRNA; and the entire protein-coding gene NADH (nicotinamide adenine dinucleotide dehydrogenase) subunit two (i.e., ND2). These regions were selected both for ease of sequencing and because, together with the control region sequence, they spanned the range of substitution rates in mammalian mtDNA (Lopez et al., 1997; Pesole et al., 1999). A geographically and genetically representative subset of samples for sequencing was chosen using a phylogenetic tree estimated from control region sequences. Partitions derived from the entire sequence of the four mtDNA regions were analysed to establish a robust intraspecific phylogeny for *M. tuberculata* over its entire range. Reasons for the failure of analyses using control region sequences were investigated. Dates for major divergence events within the phylogeny were estimated. Finally, the origin of the phylogeny, and the geographic distribution of lineages within it, were considered in relation to New Zealand's palaeoecology. Detailed phylogeographic analyses to estimate gene flow between populations and elucidate recent population history are described elsewhere (Lloyd, submitted).

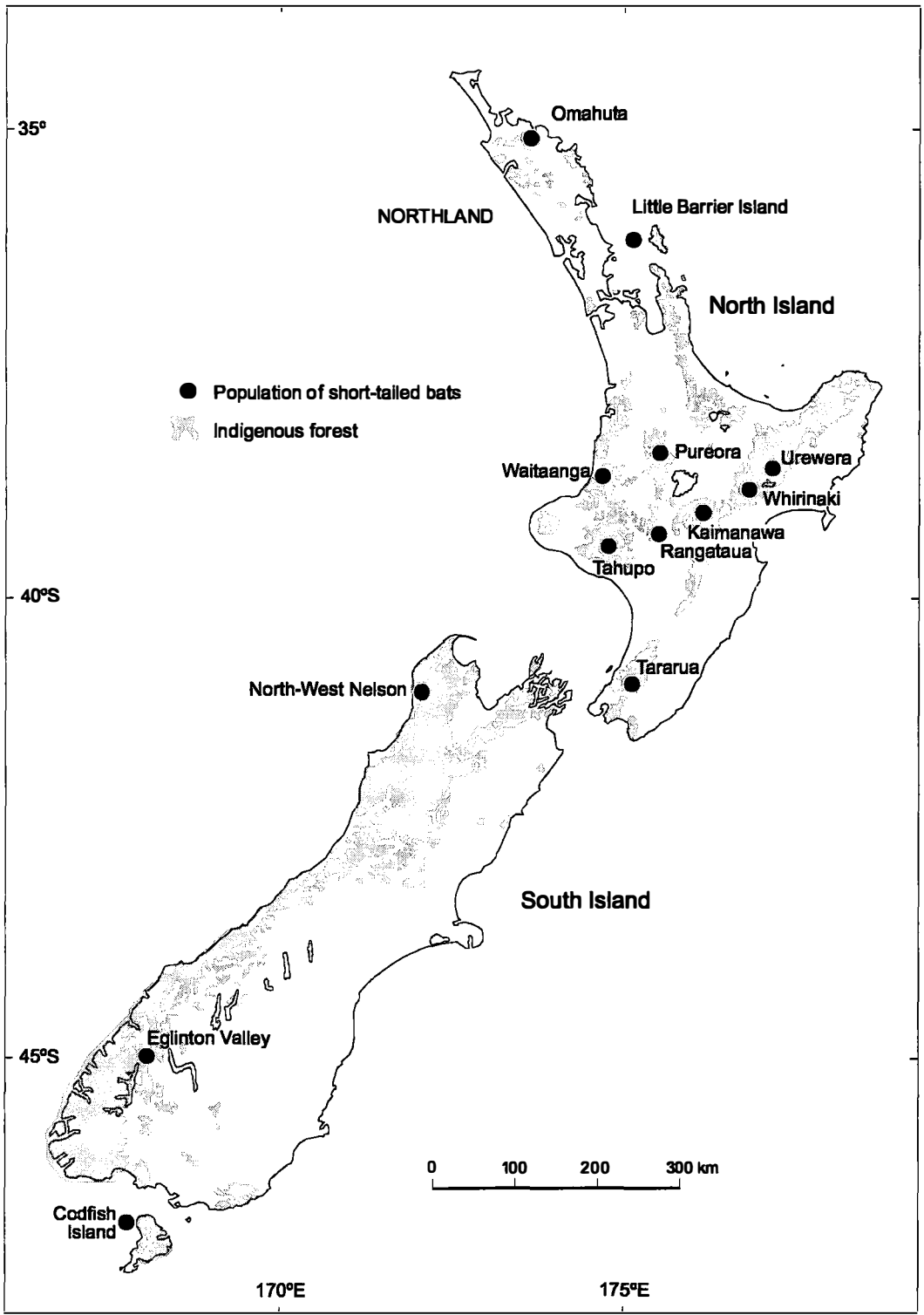

Figure 3.1 Location of known populations of *M. tuberculata* (from Lloyd, 2001)

MATERIALS AND METHODS

Sample collection

Samples were collected from thirteen populations distributed throughout the species range (Fig. 3.1): Little Barrier Island (LBI) and Omahuta (Om) in Northland (NO); Urewera (Ur), Whirinaki (Wh), Kaimanawa (Ka), Pureora (Pu), Waitaanga (Wa), Rangataua (Ra), and Tahupo (Ta) in central North Island (CNI); Tararua (Tr) in southern North Island (SNI); and north-west Nelson, Eglinton Valley (Eg), and Codfish Island (Co) in South Island (SI). Bats were caught in either mist nets, or a harp-trap placed at a colonial roost entrance, and biopsy samples taken from the extended wing membrane with a 3 mm "Stiefel" biopsy-punch. Two samples were taken from each bat, one from each wing, and immediately placed into liquid nitrogen or, when liquid nitrogen was not available, 70% ethyl alcohol. Samples originally placed in liquid nitrogen were transferred to a cryo-freezer at -80 °C for long-term storage, while samples in alcohol were stored at 4 °C.

Plate 3.1 Taking a biopsy sample from the extended wing membrane

Extraction, amplification and sequencing of mtDNA

Before DNA extraction, samples stored in alcohol were rinsed in ultrapure water. DNA was extracted using DNeasy™ Tissue Kits (Qiagen). Four regions of mitochondrial DNA (mtDNA) were amplified and sequenced: the 5′ end of control region (5′CR) with adjacent tRNA, parts of 12S rRNA and 16S rRNA, and the entire ND2 gene with adjacent tRNA. Sequences of the 5′CR fragment were obtained for 232 samples. The neighbour-joining (NJ) tree estimated from these sequences was then used to select a geographically and genetic representative subset of 28 samples for amplification and sequencing of the other three regions. Amplification reactions were performed in 40 µl volumes, containing: 8 µl of the genomic DNA eluate, 1xPCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 250 pM of each primer (Table 3.1), and 2 to 4 units of *Taq* DNA polymerase. The thermal cycle profile used in amplification was: initial denaturation at 94 °C for 2 min, followed by 35 amplification cycles of: denaturation at 94 °C for 30 s, annealing for 1 min, extension at 72 °C for 1 min; and then a final extension period at 72 °C for 4 min. Annealing temperatures for the four fragments (5′CR, 12S and 16S rRNA, and ND2) were respectively: 56 °C, 56 °C, 50 °C, and 57 °C. Amplification products obtained using the primer pair L-Pro15975(18) and H-CSB-D(20) were gel purified (QIAquick® Gel Extraction Kit; Qiagen) to remove non-specific amplification products. All other amplification products were purified using purification columns (QIAquick® PCR Purification Kit; Qiagen). Sequences were obtained for both strands with sequencing reactions undertaken using the flanking primers from the original amplifications. Additional sequencing reactions with internal primers (see Table 3.1) were required to sequence the entire ND2 fragment. ABI dGPT kits (Perkin-Elmer) were used for sequencing reactions for internal regions of the ND2 fragment, all other sequencing reactions were undertaken using ABI Big-Dye kits (Perkin-Elmer). Sequences were generated with an automated DNA sequencer (ABI Prism Autosequencer; Perkin-Elmer).

Table 3.1 Primers used for amplification and sequencing of mtDNA from *M. tuberculata*

Primer	Primer sequence	Source
5′CR		
L-Pro15975(18)	5′-ACCTTCAGCACCCAAAGC-3′	Based on Wilkinson
H-CSB-D(20)	5′-CCTGAAGTAAGAACCAGATG-3′	& Chapman (1991)
L-Pro15975(20)	5′-CCACCTTCAGCACCCAAAGC-3′	
H-Bat15958R	5′-AAAAGATACCAGAGGCATGACAC-3′	This lab
12S rRNA		
BatL35	5′-AAATGCCTAGATGAGTCGTATGACT-3′	
H-1861R	5′-TCGATTATAGAACAGGCTCCTC-3′	
16S rRNA		
16S-Fwd	5′-TTACCAAAAACATCACCTCTAGC-3′	
16S-Rev	5′-CGGTCTGAACTCAGATCACGTA-3′	
ND2		
L-Met3841	5′-GGTCAGCTAAATAAGCTATCGGG-3′	This study
H-Asn5149	5′-GGAGAAGTAGATTGAAGCCAGTTGT-3′	
HS5356FND2[a]	5′-GGAGGATGAGGAGGACTAAA-3′	This lab
H-ND2mid[a]	5′-GAGCTTTGAAGGCTCTTGGTCT-3′	This study

[a] For internal sequencing of ND2

Phylogenetic analysis

Sequences were edited and aligned using Sequencher™ version 4.1 software (Gene Code Corporation, Ann Arbor, MI). Alignments obtained initially with the large gap option were revised manually and then trimmed to a minimum readable length. The identities of the amplified sequences were confirmed by aligning and comparing consensus sequences with registered sequences for the entire mitochondrial genome of *Artibeus jamaicensis* (AF061340; Pumo et al., 1998). This is the closest relative of *Mystacina* with sequence available for the entire mitochondrial genome. The consensus sequences for 5′CR, 12S and 16S rRNA fragments, were also aligned and compared with registered sequences for *M. tuberculata* and *Noctilio* spp. (AF263222 to AF263224;

Van den Bussche and Hoofer, 2000). The internal structure of the control region, as described by Douzery and Randi (1997), was determined by multiple alignment with published mammalian control region sequences. The reading frame for the ND2 gene was identified in Sequencher™ and the amino acid sequences aligned with, and compared to, registered amino acid sequence for *A. jamaicensis*.

5'CR sequences.—All unique haplotypes for the 5'CR fragment were identified by examining pairwise differences among sequences. A phylogenetic tree was estimated for unique haplotypes by neighbour-joining (NJ) analysis in MEGA version 2.1 (Kumar et al., 2001) using the Tamura and Nei (1993) substitution model (TN93). Pairwise deletions were used for handling gaps and missing data. Clades containing only haplotypes from one of the four main geographic regions (NO, CNI, SNI, and SI) were identified using the software package Tree Explorer 2.12 (Tamura, 1999). The tree was used to select a geographically and genetically representative subsample for further sequencing to obtain a multiple gene data set.

Multiple gene sequences.—Sequences of four amplification fragments (5'CR, 12S and 16S rRNA, and ND2) were obtained for individuals in the selected subsample. The alignment of multiple gene sequences was divided into two partitions for analyses: CR, control region sequences, and Ex-CR, the rest of the sequence excluding the control region. A third partition Ex-CR_{Slow} was created from the Ex-CR partition by excluding ND2 third codon sites. Phylogenetic analyses of the multiple gene data set were undertaken using PAUP* 4.0b8 (Swofford, 1998), unless otherwise stated.

Sequences from different mitochondrial genes are not independent because they share the same genealogical history, but incongruence may arise from different selective pressure or substitution rates. The incongruence length difference (ILD), or partition homogeneity, test (Farris et al., 1995) was therefore used to test for incongruence between CR and the Ex-CR partitions. The test was implemented using parsimony informative sites only, 1000 homogeneity replicates, and 10 random-addition tree searches per replicate.

Phylogenetic analyses were undertaken on the partitions separately and on the combined data, using both NJ analysis and tree building using heuristic searches with

three optimality criteria: maximum parsimony (MP), minimum evolution (ME), and maximum likelihood (ML). For MP analyses, to conserve phylogenetic information present in the distribution of indels, the first site in an alignment gap was considered a fifth base and subsequent sites were converted to missing to reduce the influence of long gaps. ME and MP trees were found using heuristic searches, with stepwise addition employing 10 random-addition replicates to obtain starting trees, and tree-bisection reconnection (TBR) branch swapping. Where two or more trees estimated from an analysis shared the same minimum tree length, an Adams consensus tree was calculated.

The best models of sequence evolution for ML analyses of the partitions and the combined data set were selected using likelihood ratio tests (LRT) performed in a hierarchical manner (Frati et al., 1997; Posada, 2001). NJ trees, estimated under the general time-reversible (GTR) model of evolution (Yang, 1994), were used as base trees to estimate the parameters and the likelihood of models for the partitions and combined data. Three substitution models and several rate-heterogeneity models were tested. The substitution models were: Felsenstein, (1981) (F81), Hasegawa et al. (1985) (HKY85), and GTR. Two classes of rate-heterogeneity models were tested, gamma distribution with invariant sites ($I + \Gamma$) with either or both parameters (Gu et al., 1995) and site-specific rates models (SSR) with varying numbers of rate partitions. The two classes of rate heterogeneity models ($I + \Gamma$ and SSR) are not nested and therefore can not be tested against one another. Because of the extreme variations in substitution rates present in the data, eight rate categories were used to define the gamma distribution. Sequence partitions for the SSR model were identified a priori according to the functional properties of the sites. The sequence partitions were pooled into progressively smaller number of rate classes, using both the percentages of variable sites in each partition and relative substitution rates estimated for the NJ trees. Parameter estimates for the substitution and rate heterogeneity models for ML tree searches were obtained from the data using NJ trees. All ML tree searches were undertaken with empirical base frequencies and using heuristic searches with starting trees obtained by stepwise addition using the ASIS procedure, followed by TBR branch swapping.

Nodal support for trees were estimated by the non-parametric bootstrap method (Felsenstein, 1985) with 1000 pseudo-replicates for NJ, ME, and MP trees and 100 pseudo-replicates for ML trees. Both ME and MP bootstrapping were undertaken using heuristic searches with the simple stepwise sequence addition procedure. Topological

congruence of trees obtained with different partitions and analytic methods were quantified using the symmetric-difference tree comparison metric (Penny and Hendy, 1985).

Spectral analysis (Hendy and Penny, 1993), implemented in Spectrum 2.3.0, was used to visualise support for all splits in the data. Hadamard spectra were calculated for the partitions and combined data under the TN93 model of sequence evolution. Because Spectrum 2.3.0 can only accommodate 18 sequences, analyses were undertaken on representative subsets containing 18 sequences. Trivial splits and splits with support values less than 10^{-4} were excluded from the displayed spectrum.

The extent of homoplasy in the partitions and the combined data set was assessed both with standard homoplasy indices (Nei and Kumar, 2000) and by estimating the proportion of homoplasic substitutions (*PHS*) using ancestral character-state reconstruction under the likelihood criterion. The standard homplasy indices, the homoplasy index (*HI*), the overall retention index (*RI*), and the overall rescaled index (*RC*), were all estimated during MP analyses. Assessing the extent of homoplasy using ancestral character-state reconstruction involved assigning character-states for all informative sites to each node of the ML + I + Γ tree from the combined data set. Substitutions at each node were scored as either homoplasic (i.e., including reversals or parallel substitutions) or not homoplasic, and the proportion of informative sites with homoplasic substitutions calculated.

The Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) was used to compare the topologies of two hypothesised trees with the consensus tree obtained during this study. The hypothesised trees are Hill and Daniel's (1985) sub-specific taxonomy and a simple geographic tree with sequences grouped into three geographically defined groups: Northland (NO), central and southern North Island (CNI and SNI) and South Island (SI). The tree descriptions of the hypothesised trees are (No, CNI, (SNI, SI)) for Hill and Daniel's (1985) taxonomy and (NO, (CNI, SNI), SI) for the simple geographic tree. One-tailed SH tests were implemented under HKY85, with log-likelihood scores estimated using a fully optimised model, and 1000 bootstrap replicates to generate the test distribution.

Lineage divergence times and geographic distribution

Divergence times.–To estimate divergence dates among lineages, trees were first estimated unrooted and the root subsequently located using both midpoint rooting and the standard outgroup rooting method. Midpoint rooting depends on approximately equal rates of evolution in different lineages, which seems a reasonable assumption over the relatively short time scale being considered in this study. In contrast outgroup rooting is problematic due to difficulties arising from the use of distant outgroups to root trees (Hendy and Penny, 1989). *Mystacina*'s closest extant relatives are south American members of the Noctilionoidea, which diverged from *Mystacina* some time between 35 and 68 My ago (Pierson et al., 1986; Kirsch et al., 1998; Kennedy et al., 1999; Van den Bussche and Hoofer, 2000). Outgroup rooting was attempted using published mitochondrial DNA sequences for three south American noctilonoids: ND2 and 12S and 16S rRNA from *Artibeus jamaicensis* (Pumo et al., 1998), 12S and 16S rRNA from *Noctilio leporinus* and *N. albiventris* (Van den Bussche and Hoofer, 2000).

The molecular clock hypothesis was tested for data partitions and the combined data using the log likelihood ratio test (Nei and Kumar, 2000). Tests were undertaken for ML analyses under the HKY85 with the best rate-heterogeneity models. The log likelihood ratio test statistic δ was calculated as $\delta = 2(\log L_{\text{no clock}} - \log L_{\text{clock}})$. The test statistic is distributed as χ^2 with $(n - 2)$ degrees of freedom, where n is the number of sequences. When the molecular clock hypothesis was rejected the most divergent sequences were removed from the data and the test repeated. When the molecular clock hypothesis was accepted ML analysis were undertaken assuming a molecular clock, with divergent sequences removed where necessary.

Times of divergence between lineages were calculated from HKY85 corrected distances on the estimated trees using mean nucleotide substitution rate estimates for different mtDNA regions from six pairs of closely related mammal species (Pesole et al., 1999). Rate estimates for CR-Left (1.94% My^{-1}), CR-Centre (0.38% My^{-1}), 12S rRNA (0.34% My^{-1}), 16S rRNA (0.49% My^{-1}), and tRNA (0.34% My^{-1}) were used without modification. Estimates of the substitution rates for the three ND2 codon sites (0.33, 0.21 and 2.23% My^{-1}) were obtained from rate estimates for synonymous and non-synonymous sites by assuming that 96%, 100%, and 30% of substitutions in first, second, and third sites, respectively, are non-synonymous (Ridley, 1996). The

nucleotide substitution rate for partitions and the combined data set was calculated by weighting the rate contribution of the various regions according to their relative length. Because substitution rate heterogeneity (between lineages, time scales, and mtDNA sites) is pronounced for the mammalian control region (Parsons et al., 1997; Pesole et al., 1999), the CR substitution rate was also calculated from the more reliable Ex-CR rate estimate using relative substitution rates computed for the *Mystacina* combined data set under ML + SSR.

Divergence times were estimated independently for the three partitions, the combined data, and combined 12S and 16S rRNA sequences. Homologous 12S and 16S rRNA sequences from *Noctilio leporinus* and *N. albiventris* (two of *Mystacina*'s closest relatives) were used to estimate the divergence time for the two *Noctilio* species for comparison with Lewis-Oritt et al. (2001) estimate of 1.1 to 2.75 My ago. This estimate is considered reliable as it was obtained using cytochrome *b*, which has been shown to evolve in a time-dependent fashion within phyllostomid bats (Lewis-Oritt et al., 2001).

Geographic distribution.–Each of the 5′CR sequences was assigned to the principal lineage that neighboring multiple gene samples belonged to on the NJ tree of 5′CR haplotypes. The geographic distribution of the lineages was then tabulated and mapped. The null hypothesis "no difference in the frequencies of lineages among CNI and SNI populations" was tested using the chi-square goodness of fit test.

RESULTS

Control region sequences

5′CR sequences were obtained for 232 samples collected from 12 of the 13 known populations (see Fig. 3.1 and Table 3.2). Unfortunately, despite intensive field effort, only one sample was obtained from Omahuta in mainland Northland. The 5′CR sequence encompassed the 3′-end of tRNA Proline (40 bp), the entire CR left peripheral domain (CR-Left) (365bp), and the 5′ end of the CR central domain (CR-Centre) (108 bp), including conserved sequence blocks (CSB) -F (27 bp), and -E (38 bp), and part of CSB-D (9 bp). There was no significant difference ($P = 1.000$) in base composition among sequences for either all sites or parsimony informative sites.

However, there was a slight bias in base frequencies towards adenine and thymine (A: 30.0%; C: 22.7%; G: 15.4%; T: 31.8%) typical of mammalian mtDNA control region sequences. The final alignment was 513 bp long, with an average sequence length of 506.1, because of insertion-deletion events (indels). Single-bp indels occurred at one site in tRNA Proline, five sites in CR-Left, and one site in CR-Centre. A number of multiple-bp indels were present in the CR-Left. Twenty-eight CNI sequences exhibited five distinct multiple-bp deletions in the region 61–78 of CR-Left (three 13-bp and two 2-bp deletions). One hundred and fifty (29.2%) of the total 513 characters were variable and 128 (25.0%) parsimony informative. Although nucleotide substitutions occurred along the length of the sequence, there were several 15 to 38 bp regions without substitutions corresponding to extended termination associated sequences (ETAS) in the CR-Left and CSB-F, -E and -D in CR-Centre. The highest rates of nucleotide substitution occurred in CR-Left at sites 60–80, 100–110, and 340–355. The transition:transversion ratio was 15.9 for all pairwise sequence comparisons, with slightly higher values for comparisons between populations (10.5–29.7; mean 17.5), than for comparisons within populations (8.7–24.9; mean 15.2). Nucleotide diversity (mean pairwise distance calculated using TN93) was 4.27%, with higher values for between-populations comparisons (2.4%–5.4%, mean 4.3%) than for within-population comparisons (2.0%–3.9%; mean 2.9%). For comparisons with other studies, nucleotide diversity was also calculated for CR-Left. Nucleotide diversity for CR-Left sequences was 5.3%, with higher values for between-populations comparisons (3.2%–6.5%, mean 5.3%) than for within-population comparisons (2.4%–5.0% mean 3.6%).

Table 3.2 Distribution of samples and haplotypes among populations

	Population												
	LBI	Om	Ur	Wh	Ka	Pu	Wa	Ra	Ta	Tr	Eg	Co	Total
5′CR samples	30	1	6	27	23	23	18	39	25	6	10	24	232
5′CR haplotypes	11	1	5	24	12	7	17	33	15	4	7	12	147[a]
Multigene samples	4	1	2	1	2	2	0	4	1	2	4	5	28

[a] Total exceeds sum of populations because one haplotype occurs in two populations

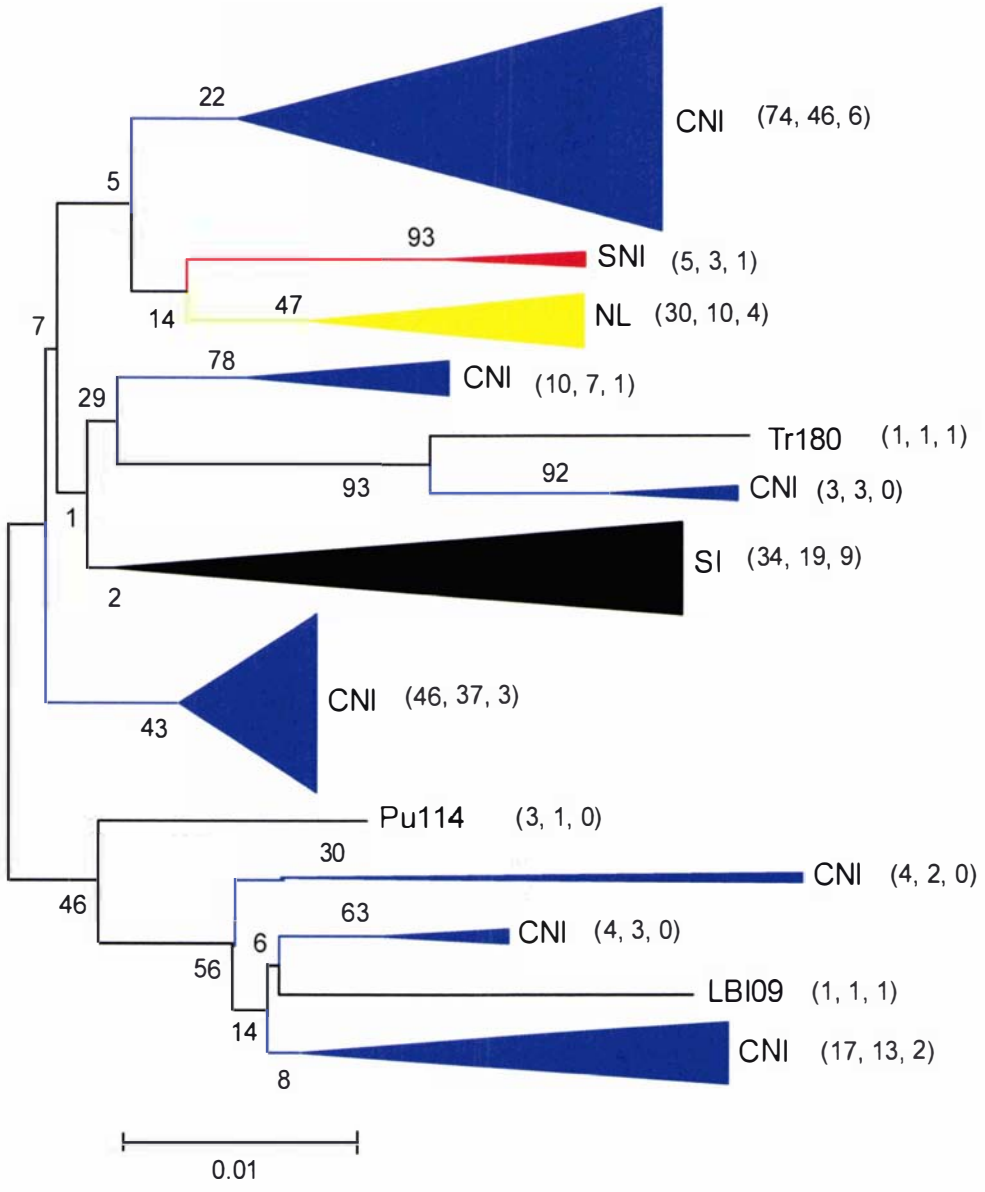

Figure 3.2 NJ tree of 147 5′CR haplotypes with geographical defined clades compressed. Bootstrap test values are shown for remaining nodes. Numbers in brackets are the number of 5′CR sequences and haplotypes, and multigene samples for each clade.

One hundred and forty-seven unique haplotypes were identified. One haplotype was found in two populations (Wh and Ta), the remainder were restricted to single populations. The NJ tree estimated from the 147 haplotypes comprised ten geographically defined clades and three haplotypes not belonging to geographically defined clades (Fig. 3.2). Polyphyletic and paraphyletic relationships are apparent on the NJ tree, with haplotypes from different geographic areas intermingled. Low bootstrap and interior branch test values throughout the tree indicate that the tree was poorly

resolved. On a consensus tree of 1000 replicate bootstrap trees, only 58 of 113 nodes scored more than 50%. A representative subset of twenty-eight samples for further sequencing was selected from among these clades as shown on Fig. 3.2. The subset included samples from eleven of the twelve sampled populations (see Table 3.2).

Multiple gene sequence characteristics

Further sequencing of the sub-set of 28 samples provided a 2878 bp sequence alignment from four amplification fragments: 5′CR (512 bp), 12S rRNA (597 bp), 16S rRNA (565 bp), and ND2 (1204 bp). The alignment included entire sequences for CR-Left, ND2, tRNA Tryptophan, and tRNA Alanine, as well as part sequences for CR-Centre, 12S rRNA, 16S rRNA, tRNA Proline, and tRNA Asparagine. Indels were present in tRNA Proline, CR-Left, and 16S rRNA sequences. Four SI samples had single bp insertions in both tRNA Proline and CR-Left. Other indels in CR-Left were a 13 bp deletion in one CNI sample, and different single bp insertions present in one CNI sample and one Northland sample. Six samples (five CNI and one SNI) had a 3 bp insertion in 16S rRNA. Differences in the indels present in 5′CR sequences of the original sample and the subsample account for the difference in lengths between the two 5′CR alignments (513 bp cf. 512 bp).

The complete alignment of multiple gene sequences was partitioned into nine functional partitions, identified a priori according to their functional properties: CR-Left, CR-Centre excluding CSB (CRCexCSB), CSB, 12S rRNA, 16S rRNA, ND2 Site1, ND2 Site2, ND2 Site3, and tRNA. The 12S rRNA and16S rRNA partitions are the 3′ end of the respective genes. ND2 Site1, ND2 Site2, and ND2 Site3 are respectively the first, second, and third codon sites of the ND2 gene. The tRNA partition comprises tRNA Tryptophan and tRNA Alanine together with parts of tRNA Proline and tRNA Asparagine. These functional partitions were used to define partitions both for data analysis and for the site-specific rates model used in ML analyses. There were no significant differences ($P = 1.000$) in base composition among sequences for all sites or for parsimony informative sites within the entire sequence or any of the functional partitions. However, there were significant differences ($P < 0.001$) in base compositions among the nine partitions. Base composition biases against G were marked in CR-Left (14%) and the three ND2 codon sites (14%, 10%, and 5% respectively). The first and third codon sites of ND2 had high proportions of A (43%

and 47%), whereas third codon sites had a high proportion of T (44%). Among the nine functional partitions CR-Left, CRCex-CSB, and ND Site3 exhibited the greatest variation with highest proportions of variable and informative sites and highest relative substitution rates (Table 3.3).

Table 3.3 Summary of mtDNA variation among functional partitions and data partitions used during analysis of the multiple gene sequences. Relative substitution rates are calculated using NJ tree for the combined data under maximum likelihood criteria using HKY85 + SSR_9.

		Number of sites (%)			Relative
		Total	Variable	Informative	rate
Functional partitions					
CR	CR-Left	365	99 (27)	67 (18)	4.04
	CRCexCSB	33	9 (27)	4 (12)	3.26
	CSBs	74	5 (7)	5 (7)	1.00
Ex-CR	12SrRNA	597	24 (4)	9 (2)	0.32
	16SrRNA	565	17 (3)	13 (2)	0.24
	ND2Site1	349	12 (3)	7 (2)	0.37
	ND2Site2	349	3 (1)	2 (1)	0.08
	ND2Site3	349	69 (20)	34 (10)	1.96
	tRNA	197	6 (3)	4 (2)	0.26
Partitions used in analyses					
	CR	472	113 (24)	76 (16)	
	Ex-CR	2406	131 (5)	69 (3)	
	$Ex\text{-}CR_{Slow}$	2057	62 (3)	35 (2)	
	Combined	2878	244 (9)	145 (5)	

Three partitions were created for analysis of the multiple gene sequence: CR, Ex-CR, and $Ex\text{-}CR_{Slow}$. CR is a 472 bp sequence encompassing the control region. Ex-CR is a 2406 bp sequence excluding the control region, but including 12S rRNA, 16S

rRNA, ND2, and tRNA sequences. Ex-CR_{Slow} is a 2057 bp sequence created from Ex-CR by excluding the fast evolving ND2 Site3. The combined data (i.e., the entire alignment of multiple gene sequences) contained 244 (8.5%) variable sites and 145 (5.0%) parsimony informative sites. Both variable and parsimony informative sites were divided approximately equally between CR and Ex-CR partitions (see Table 3.3). Removal of ND2 third codon sites from Ex-CR to create Ex-CR_{Slow} more than halved the number of variable and parsimony informative sites. Relative substitution rates of 3.58 and 0.49 were calculated for CR and Ex-CR partitions respectively using the combined data under maximum likelihood criteria with an HKY85 + SSR_2 model. Transition:transversion ratios calculated from NJ trees under maximum likelihood criteria using the HKY85 model were: 31.0:1 for the combined data set, 30.8:1 for CR, 31.4:1 for Ex-CR, and 37.0:1 for Ex-CR_{Slow}. There were 27 unique haplotypes for both the combined data set and the CR partition. The number of unique haplotypes declined to 23 for Ex-Cr, and 22 for Ex-CR_{Slow}. The mean and maximum uncorrected pairwise distances between haplotypes were 4.53% and 7.68% for CR, 0.88% and 1.88% for Ex-CR, 0.51% and 0.88% for Ex-CR_{Slow} and 1.46% and 2.20% for the combined data.

Phylogenetic analysis of multiple gene sequences

Results of ILD tests results showed no significant incongruence between either CR and Ex-CR ($P = 0.734$), or CR and Ex-CR_{slow} ($P = 1.000$). Likelihood ratio tests indicated that HKY85 is the best substitution model for ML analyses of the partitions and the combined data, but the best rate-heterogeneity models differed. Best-fit among the I + Γ class of rate-heterogeneity models were Γ for the CR partition, I for Ex-Cr, with or without the third codon sites, and I + Γ for the combined data set. Best-fit SSR models were SSR_2 for CR, SSR_3 for Ex-Cr, SSR_2 for Ex-CR_{Slow}, and SSR_4 for the combined data set. Although SSR and I + Γ classes of models can not be tested against one another, considerably smaller log likelihood values obtained with the SSR models for Ex-CR (4288 cf. 4333) and the combined data (6152 cf. 6185), indicated SSR models may be a better fit for these data. In contrast, larger log likelihood values obtained with SSR models for CR (1811 cf. 1723) and Ex-CR_{Slow} (3351 cf. 3332) indicated I + Γ models may be better than SSR models for these data.

Table 3.4 Symmetric-difference tree comparison metrics for topologies estimated from CR, Ex-Cr, and combined data using different methods. Shaded values are below the median (10.5).

		CR					Ex-CR					Combined				
		MP	ME	NJ	ML		MP	ME	NJ	I		MP	ME	NJ	ML	
					Γ	SSR					SSR				I+Γ	SSR
CR	MP	-														
	ME	12	-													
	NJ	12	8	-												
	ML+Γ	12	14	14	-											
	ML+SSR	14	10	16	12	-										
Ex-CR	MP	13	17	17	17	17	-									
	ME	16	20	20	22	22	5	-								
	NJ	16	20	20	22	22	5	2	-							
	ML+I	11	17	17	17	17	2	5	5	-						
	MLS	11	17	17	17	17	2	5	5	0	-					
Comb.	MP	11	13	15	19	17	6	7	7	4	4	-				
	ME	10	14	14	18	18	9	6	8	7	7	5	-			
	NJ	10	14	14	18	18	9	6	8	7	7	5	0	-		
	ML I+Γ	10	14	12	16	18	7	8	8	5	5	3	6	6	-	
	ML+SSR	11	13	15	19	17	6	7	7	4	4	0	5	5	3	-

Tree topologies estimated from both Ex-CR and the combined data set using five different phylogenetic methods (MP, NJ, ME, ML + I + Γ, and ML + SSR) were generally congruent, with low values for the symmetric-difference metric for comparisons of topologies estimated using different methods from the same data (means 3.6 for Ex-CR, and 3.8 for the combined data), and from the two data sets (mean 6.5) (Table 3.4). Removal of third codon sites from Ex-CR to create $\text{Ex-CR}_{\text{Slow}}$ caused slight increases in symmetric-difference metrics both within $\text{Ex-CR}_{\text{Slow}}$ (mean 5.4) and for comparisons with the combined data (mean 8.1). In contrast, topologies estimated from CR using different phylogenetic methods vary markedly. Symmetric-differences for tree

comparisons within CR (mean 12.4) and between CR and the other data sets (means 17.6 and 14.7 for Ex-CR and the combined data respectively) were considerably higher than for Ex-CR and the combined data (see Table 3.4).

Analyses of Ex-CR, Ex-CR_{Slow} and the combined data consistently recovered six monophyletic clades: NL, NE, NW, SN, S1 and S2 (Fig. 3.3). In contrast, analyses of the CR partition produced inconsistent results, with only NL, NW, and S2 consistently recovered as monophyletic clades (see Fig. 3.3). NE was monophyletic in all analyses of CR except MP, SN was only monophyletic in MP analysis of CR, and S1 was not monophyletic in any analysis. Bootstrap support for all six clades was strong for both Ex-CR (81%–100%) and the combined data (69%–100%) (Table 3.5). Removal of the ND2 third codon sites from Ex-CR reduced bootstrap support for all clades, in particular, support for the SN clade dropped to 49%. Only NW and S2 received bootstrap support from the CR data. Spectral analyses of the data partitions separately, and together, showed similar patterns of support for the six clades (Fig. 3.4). CR data provided little, or no support, and high levels of conflict for all clades. In contrast, Ex-CR data provided high levels of support and no conflict for all six clades. Removal of third codon sites reduced support slightly, but also reduced levels of conflict for all clades, especially SN. The combined data provided high levels of support and low levels of conflict for all clades.

Table 3.5 Bootstrap support values for the six principal monophyletic clades. Values are average values for MP, NJ, ME, and ML analyses.

	Clade					
Partition	NL	NW	NE	SN	S1	S2
CR	36	66	29	7	0	93
Ex-CR	100	96	92	81	92	100
Ex-CR_{Slow}	87	82	67	49	82	98
Combined	99	99	91	69	81	100

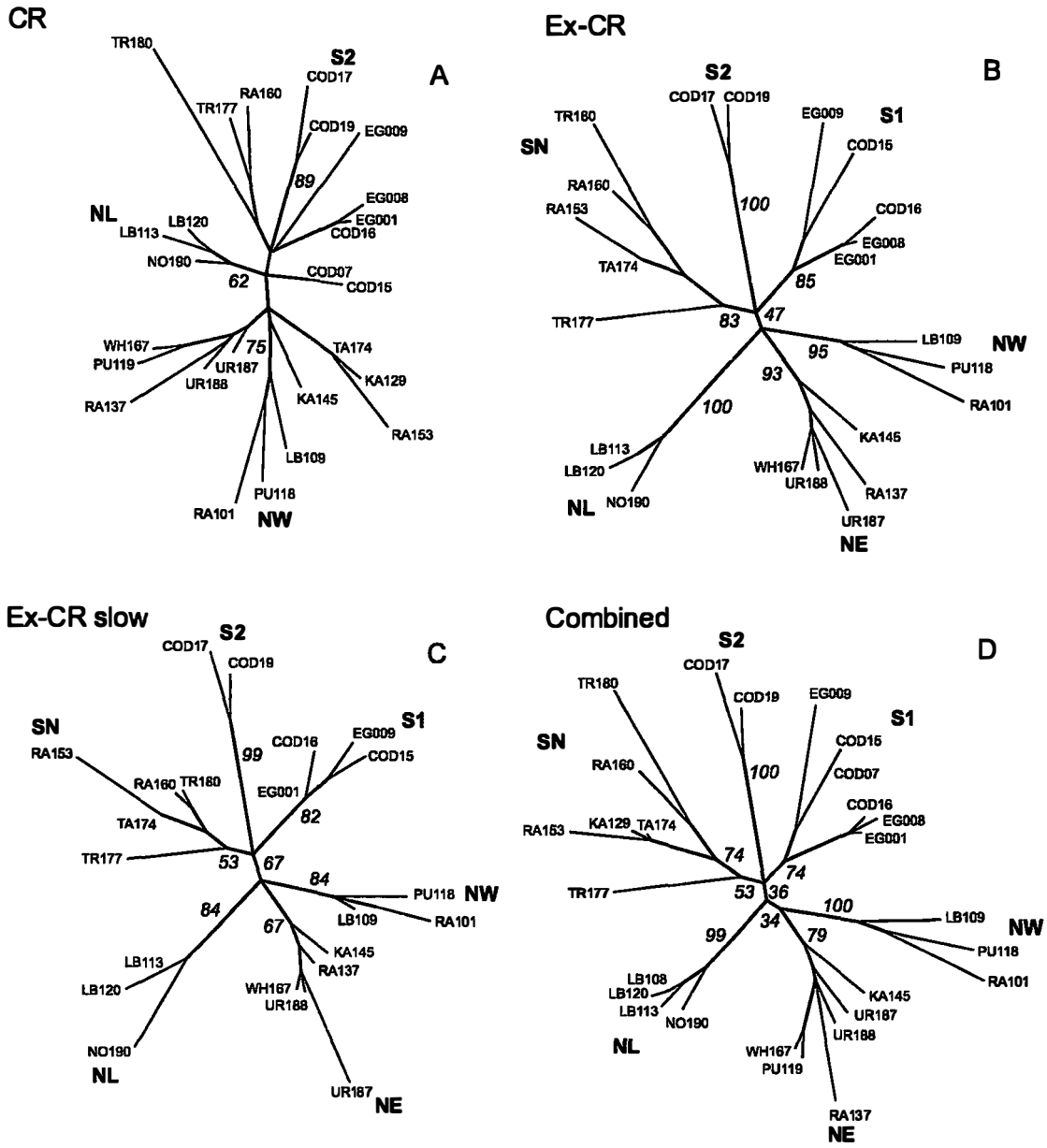

Figure 3.3 ML trees for the three partitions: CR, Ex-Cr, Ex-CR_{Slow}, and the combined data, with bootstrap values for nodes defining the six major lineages

The only consistent phylogenetic signal describing relationships between the six principal monophyletic clades was for separation between northern (NL, NE, NW) and southern (SN, S1 and S2) clades. This split was supported by all analyses of both the combined data and Ex-CR, with and without third codon sites. Bootstrap support was strongest (73%) in Ex-CR_{Slow} and declined to 51% for Ex-CR and 48% for the combined data. Spectral analyses of Ex-CR_{Slow} showed moderate support for, and no conflict with, the split between northern and southern clades (see Fig. 3.4). Lower levels of support for the split were accompanied by some conflict in spectral analyses of both combined data, and Ex-CR.

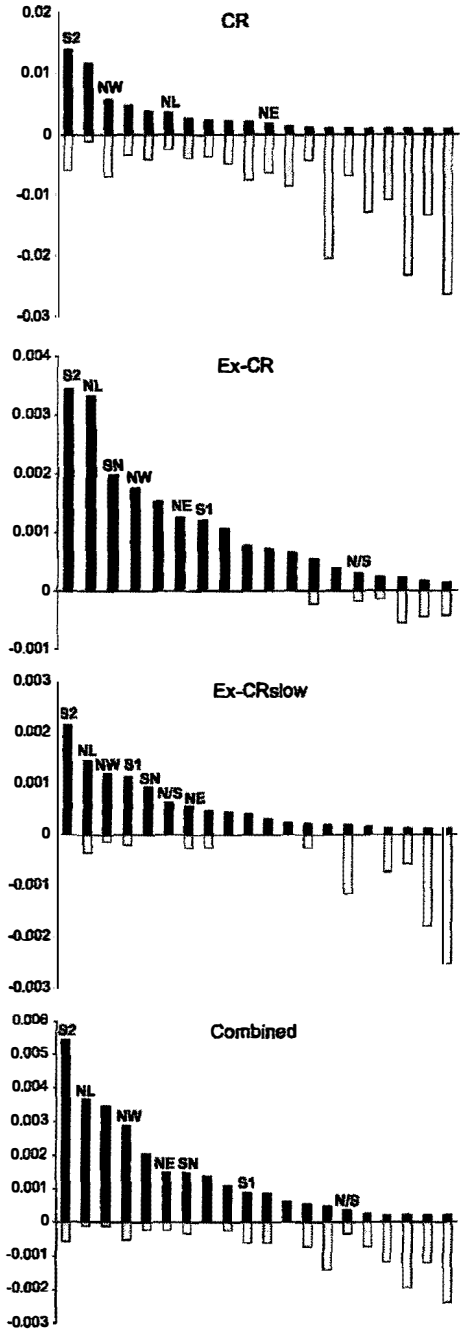

Figure 3.4 The results of spectral analyses of the three partitions CR, Ex-Cr, Ex-CR_{Slow}, and combined data. Splits identifying lineages and the split between northern and southern lineages (N/S) are labeled. Values above and below the x-axis are support and conflict values repectively.

Within both the northern and southern groups of clades relationships between clades were poorly resolved and best described as trifurcations. Among the northern clades contradictory splits were recovered with different data and methods. The split ((NW, NE), NL) appeared in most topologies (NJ, MP, MLG, MLSS) from combined

data and some (NJ, ME) from Ex-CR_{Slow}. The other possible split ((NL, NE), NW) appeared in topologies from combined data (ME) and Ex-CR (MP, ME, NJ). Bootstrap support for the two contradictory splits between northern clades were similar (mean 42%, range 0–69%; mean 39%, range 0–75%). There was little support for, and considerable conflict with, any of the possible splits between the three clades in the southern group. The consensus topology exhibited a separation between northern and southern groups of clades and trifurcations between the clades within each of these groups, i.e., ((NL, NE, NW) (SN, S1, S2)). The result of SH test comparing this topology with alternative topologies based on Hill and Daniel's (1985) proposed taxonomy and a simple geographic partitioning showed no support for the alternative topologies ($P < 0.0001$).

All measures of homoplasy (Table 3.6) showed much higher levels of homoplasy in CR than Ex-CR partitions and intermediate levels for the combined data. There was very little difference between homoplasy levels in Ex-CR and Ex-CR_{Slow}. The proportion of informative sites with homoplasic substitutions (*PHS*) obtained by ancestral character-state reconstruction was over twice as high in CR (72%) than the Ex-CR partitions (32.8 and 32.4%). The frequency distributions of homoplasic substitutions in the two partitions were also very different. Few sites in the Ex-CR partitions had more than one homoplasic substitution (6% and 8.8%) and none had more than two. In contrast, there were up to six homoplasic substitutions at a single site in CR, with 37.3% of sites having more than one and 18.7% more than two homoplasic substitutions.

Table 3.6 Measures of homoplasy for the partitions and combined data

	CR	Ex-CR	Ex-CR_{Slow}	Combined
HI	0.58	0.27	0.27	0.48
RI	0.58	0.87	0.88	0.71
RC	0.30	0.73	0.73	0.46
PHS (%)	72.0	32.8	32.4	53.5

Divergence times

Not unexpectedly, given the 35 to 68 million years divergence time between the outgroup and *Mystacina*, outgroup rooting provided inconsistent results, with radically different locations for the roots on trees estimated with different phylogenetic methods and partitions. Midpoint rooting of all trees estimated from Ex-CR_{Slow} consistently placed the root on the edge between the northern and southern clades. In contrast, trees estimated from Ex-CR are all rooted on the edge between the SN and the other clades, a short or non-existent edge. The location of midpoint roots on trees estimated from the combined data varied. ML and MP trees rooted on the edge between the northern and southern groups, whereas NJ and ME trees rooted on the edge between the SN and other clades. Removal of the single most divergent sequence from the SN clade shifted the root on all trees to the edge between the northern and southern groups. Midpoint rooting was used for estimating divergence times.

Using the log likelihood ratio test, the molecular clock hypothesis was accepted ($P > 0.05$) for Ex-CR and CR under both rate-heterogeneity models, and for Ex-CR_{slow} under the HKY85 + SSR_2 model. The hypothesis was initially rejected ($P \leq 0.05$) for Ex-CR_{slow} under the HKY85 + I model, but following removal of the single most divergent sequence the hypothesis was accepted. The molecular clock hypothesis was also initially rejected for the combined data under both rate-heterogeneity models. Following removal of the most divergent sequence from the combined data set the molecular hypothesis was accepted for HKY85 + I + Γ, but rejected for HKY85 + SSR_4.

Nucleotide substitution rates estimates calculated from Pesole et al. (1999) directly were 1.59% per My for CR, 0.63% per My for Ex-CR, 0.36% per My for Ex-CR_{Slow} and 0.79% per My for the combined data. Modified substitution rate estimates calculated from the Ex-CR rate estimate and relative substitution rates in the combined data, were 4.60% per My for CR, and 1.28% per My for the combined data. Using unmodified substitution rates the root divergence times estimated from CR and the combined data were very different: 3.040 and 1.332 My respectively. In contrast using the modified CR substitution rate (estimated from the Ex-CR rate and relative substitution rates estimated from the data) the root divergence time estimated from CR and the combined data were similar: 1.051 and 0.818 My respectively. The same pattern was present in estimates of divergence times between principal clades or lineages. Using the unmodified CR substitution rate the estimated divergence times between

lineages were 1.906 to 2.407 My for CR, and 1.106 to 1.251 My for the combined data. Whereas using the modified CR substitution rate the estimated divergence times between lineages were 0.659 to 0.832 My for CR, and 0.679 to 0.768 My for the combined data. This improved agreement between divergence times estimated from CR and combined data achieved with the modified CR substitution rate estimate indicates that the modified estimate is a better estimate of the true CR substitution rate. Divergence times from combined data were therefore estimated using a substitution rate estimate based on the modified CR substitution rate. Divergence times were estimated independently for the three partitions using the best model(s) for each partition (Table 3.7). Estimates for the root divergence among the lineages range from 0.818 to 0.931 My. Estimates for divergence among the northern and southern lineages were slightly more recent: from 0.723 to 0.841 My for the northern lineages and from 0.679 to 0.825 My for the southern lineages.

Table 3.7 Divergence times (My) for lineages of *M. tuberculata*

	Partition				
	Ex-CR	$Ex\text{-}CR_{Slow}$		Combined	Mean
	HK85+I	HK85 + I	HK85 + SS_2	HK85 + I + Γ	
Root	0.886	0.920	0.931	0.818	0.889
Northern	0.768	0.841	0.723	0.768	0.775
Southern	0.825	0.732	0.807	0.679	0.761

Using a substitution rate estimate of 0.41% My^{-1} and HKY85 + I, the root divergence time estimated for the combined 12S and 16SrRNA sequences of *M. tuberculata* was 1.080 My. This is only slightly higher than estimates from Ex-CR partitions and the combined data. Reassuringly, using this same method with 12S and 16SrRNA sequences from the two *Noctilio* species the estimated divergence time for the two species (2.591 My) was within the range of Lewis-Oritt et al.'s (2001) estimate (1.1–2.75 My).

Pairwise differences between lineages

A number of studies have used sequences from the left domain of the control region or cytochrome *b* to investigate interspecific divergences in mammalian taxa. As substitution rates for cytochrome *b* and ND2 in mammals are similar (Lopez et al., 1997; Pesole et al., 1999) divergences between taxa using the two genes should be comparable. To facilitate comparisons with these studies nucleotide divergences for the six lineages (i.e., principal clades) of *M. tuberculata* were calculated for both CR-Left and ND2. Pairwise distances for the 27 haplotypes were calculated using the HKY85 model of nucleotide substitution. The mean of pairwise distance between the 27 CR-Left haplotypes was 5.6% (max 9.8%), with between-lineage distances (4.5%–7.8%, mean 6.1%) nearly twice the magnitude of within-lineage distances (1.9%–5.2%, mean 3.6%). The mean of pairwise distances between all ND2 sequences was 1.30% (0–2.16%), with between-lineage distances (1.23%–1.81%, mean 1.46%) more than twice the magnitude of within-lineage distances (0.16%–0.94%, mean 0.62%).

Geographic distribution

The lineages are geographically structured with each lineages having a restricted range (Fig. 3.5). NL is only found in Northland. S1 and S2 are only found in South Island, with S2 restricted to Codfish Island, where it is sympatric with S1. The three lineages NW, NE, and SN occur sympatrically in three CNI populations (Ra, Pu, and Ka), but outside of these populations, the three lineages have separate ranges. NW occurs sympatrically with NL on Little Barrier Island. NE is found in north-eastern North Island and SN in southern North Island. The null hypothesis of equal frequencies of lineages among central and southern North Island populations was tested using the chi-square goodness of fit test. Cell sizes were increased by pooling Urewera with Whirinaki in the north-east, and Tahupo with Tararua in the south. The null hypothesis was rejected ($P < 0.001$, $\chi^2 = 111.6$, DF = 10). The null hypothesis of equal frequencies of lineages was also rejected ($P < 0.001$, $\chi^2 = 51.5$, DF = 6) for the four central populations (Pu, Wa, Ra, and Ka).

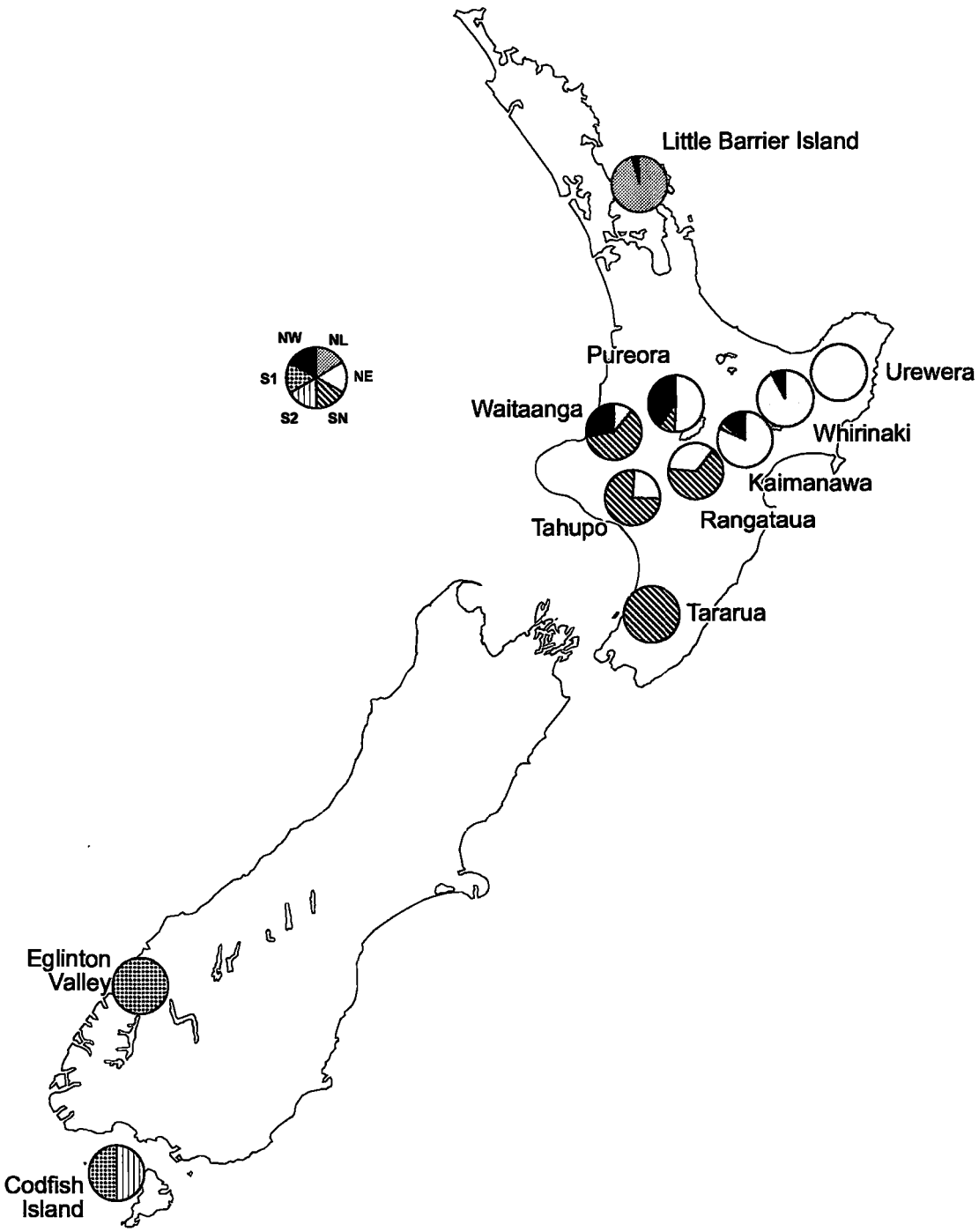

Figure 3.5 Geographic distribution of *M. tuberculata* lineages

DISCUSSION

Phylogenies estimated from control region and multiple gene sequences

Control region sequences alone do not provide a useful signal for elucidating phylogenetic relationships within *M. tuberculata*. There were large symmetric differences among phylogenies recovered from control region sequences and low nodal support using either bootstrap or interior branch tests. There were also high levels of conflict for all signals in spectral analyses. Over the timescale of intraspecific divergence in *M. tuberculata*, heterogeneity of substitution rates within CR results in a high level of multiple homoplasic substitutions at some sites. These homoplasic substitutions confound the recovery of an accurate phylogeny. In contrast, the results of all analyses other than CR sequence were generally concordant, with high levels of nodal support and little conflict in spectral analyses. The multiple mtDNA gene sequences provided strong support for the existence of six mtDNA lineages or matrilines, within *M. tuberculata*. Relationships among the lineages were poorly resolved. Although there was some indication of a basal bifurcation separating northern and southern lineages, support for this topology was weak. A conservative interpretation is that the basal node is a polytomy between the six lineages. Difficulty in resolving the relationships among the lineages probably reflects their rapid divergence rather than inadequate sequence data as the sequenced regions were selected to span a wide range of substitution rates. The range of estimated divergence dates for the lineages (0.93 to 0.68 My BP), during the middle Pleistocene, is consistent with observations in other mammals: 72% of inferred intraspecific lineage separations date to the Pleistocene (Avise, 2000).

Comparison with other bat species

Sequence divergence levels between the six mtDNA lineages of *M. tuberculata* (4.5%–7.8% for CR-Left, and 1.2%–1.8% for ND2) are similar to, or greater than, those reported for bat species ranging over continents (i.e., 3 % or less for the control region and < 2% for cytochrome *b*) (e.g., Webb and Tidemann, 1996; Petit and Mayer, 1999). In the absence of geographic barriers, most bat species have low levels of genetic differentiation and little geographic structure over large geographical ranges (2000 to 3000 km). This pattern is typical of highly mobile species, and presumed to result from

active or recent gene flow among populations at a continental scale, with long distance seasonal migrations playing an important role.

Intraspecific geographic structure in the absence of geographic barriers, as seen in *M. tuberculata*, has only been reported for three bat species: *Sturnira lilium*, *Carollia perspicillata* and *Macroderma gigas*. *S. lilium* and *C. perspicillata* were the only two of eighteen South American noctilionoid species studied by Ditchfield (2000) that exhibited geographic structure of cytochrome *b* lineages. Lineage divergences over the 3000 km ranges of the two species (4.0–4.5% and 2.5% respectively) are greater than for *M. tuberculata*. Geographic structure is presumed to be a result of Pleistocene forest fragmentation, as both species are restricted to forest habitats. Control region sequence divergence (2.3–6.1%, mean 4.5%) between populations of *M. gigas* (Worthington Wilmur et al., 1994) spread over 2300 km in northern Australia are slightly lower than found in *M. tuberculata* (3.2%–6.5%, mean 5.3%) over considerably smaller distances. Levels of divergence within populations of *M. gigas* (0.0–2.13%; mean 0.68%) are much lower than within populations of *M. tuberculata* (2.4%–5.0% mean 3.6%). Geographic structuring within *M. gigas* is presumed to be a result of strong female philopatry.

Sympatric clades similar to those found in *M. tuberculata* have only been reported from one bat species, the noctilionoid bat *Leptonycteris curasoae yerbabuenae* (Wilkinson and Fleming, 1996). In this case two clades with 3% sequence divergence in CR (cf. 4.5%–7.8% for *M. tuberculata*) occur sympatrically throughout the taxon's 3000 km range. The sympatric lineages are presumed to be a consequence of long-range seasonal migration having increased the effective population size and hence the lineage sorting time.

Hypotheses concerning the origin and maintenance of phylogenetic structure

The estimated range of dates for lineage divergence in *M. tuberculata* corresponds to the onset of both severe climatic oscillations (0.85 My BP) (Stevens et al., 1995) and catastrophic volcanic eruptions from the Taupo Volcanic Zone in the centre of CNI (0.75 My BP) (Healy, 1992; Stevens et al., 1995). Both caused large fluctuations in the extent of forest habitat suitable for *M. tuberculata* throughout the late Pleistocene up until the time of human settlement. During warmer interglacials and interstadia forest covered 85% to 90% of New Zealand (McGlone et al. 1993, 2001). In contrast, at

glacial maxima continuous forest cover was limited to Northland and the north-west North Island, with only scattered fragments of forest in favourable microclimates persisting elsewhere (McGlone et al., 1993) (Fig. 3.6). Catastrophic pyroclastic eruptions from rhyolitic volcanoes in the Taupo volcanic zone repeatedly destroyed forest across large areas of the central North Island (Healy, 1992; Stevens et al., 1995). In two of the most recent large-scale eruptions 20 000 and 1800 years ago all land surfaces in the central volcanic region, a 20 000 km^2 area within central North Island, were destroyed by pyroclastic flows. Ash deposits more than 100 mm deep damaged forest ecosystems over much wider areas. Such episodes of extensive habitat destruction by vulcanism were followed by rapid regeneration within 200 to 300 years (McGlone et al., 1996). Divergence among the lineages probably arose following contractions of forest habitat in response to either climatic fluctuation (SI and SNI) or catastrophic volcanic eruptions (CNI). Such range contractions would have fragmented the original population into sub-populations inhabiting isolated forest refugia.

The persistence of phylogenetic and geographic structure despite repeated cycles of range expansion and contraction since divergence is surprising as North and South Islands were connected throughout most of the late Pleistocene up until 12 000 years ago (Stevens et al., 1995). Thus, during periods when forest was most extensive the only significant barrier to dispersal throughout New Zealand was the Southern Alps (an axial mountain chain in the South Island). I propose the species phylogenetic structure was maintained within populations inhabiting refugia despite intermittent widespread lineage mixing elsewhere. Since the middle Pleistocene central regions of the North Island and most of the South Island have probably functioned as ephemeral population sinks with repeated cycles of extinction and recolonisation from a small number of persistent source populations inhabiting isolated refugia. Long-term genetic isolation and the retention of ancient matrilines in the source populations is a consequence of resistance to inward gene flow into established colonies arising from the species social behaviour. Strong female philopatry is common in other colonial microbat species (e.g., Wilkinson and Chapman, 1991; Worthington Wilmur et al., 1994; Burland et al., 1999; Kerth et al., 2000). Alternatively, resistance to inward gene flow could be a consequence of selection pressure, with ecological differences maintaining lineage separation, but given the level of lineage mixing in a broad range of forest types throughout New Zealand this seems improbable. The persistence of phylogeographic structure despite repeated cycles of expansion and contraction has been reported

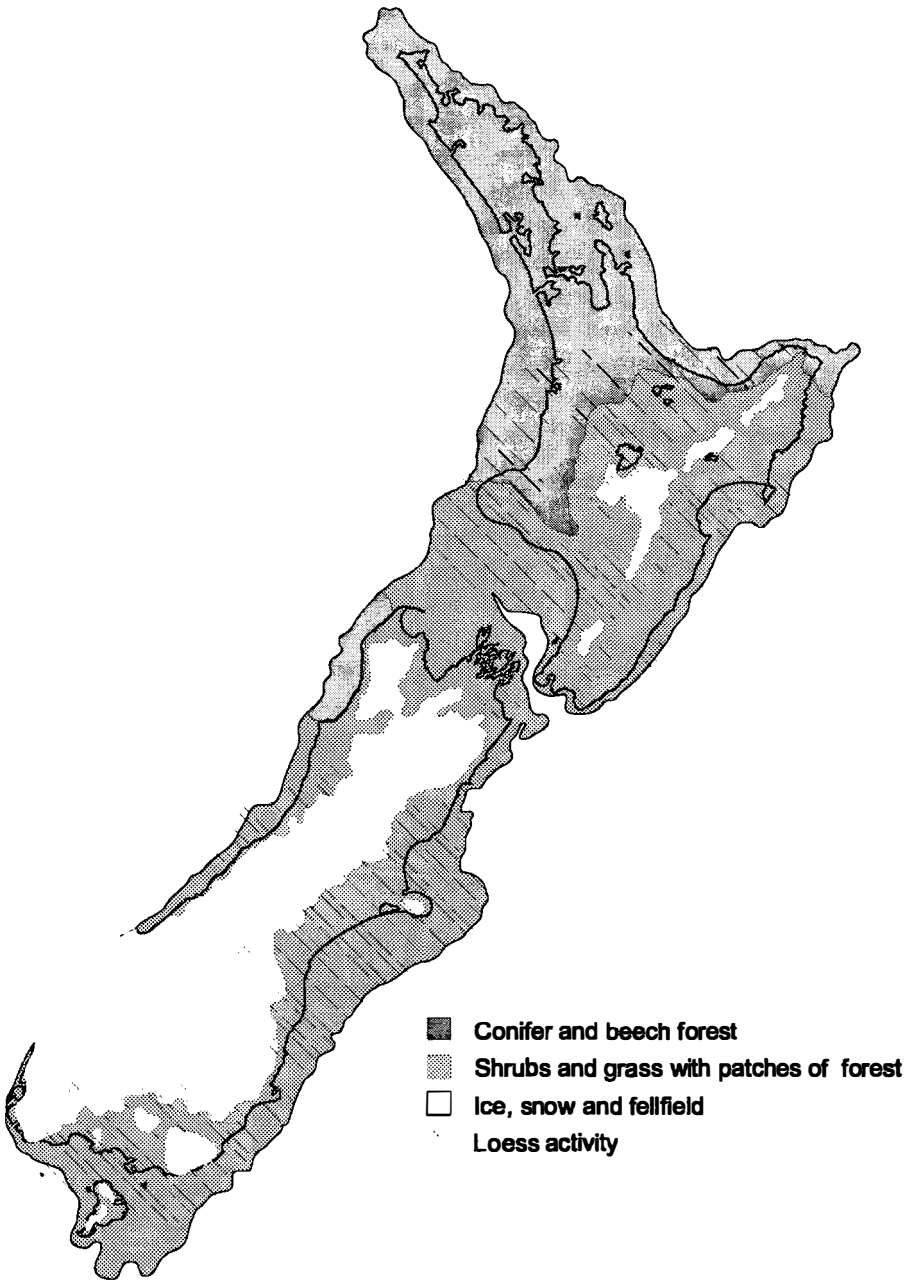

Figure 3.6 Forest cover in New Zealand during the last glacial maximum 20 000 years ago (from McKinnon et al., 1997)

previously for northern hemisphere taxa with ranges severely affected by Pleistocene climate oscillations (Neigel and Ball, 1993; Stewart and Lister, 2001). Computer simulations of lineage dispersal patterns (Neigel and Ball, 1993) indicate phylogeographic distributions can be remarkably resilient to repeated cycles of range contraction and expansion.

Hypotheses concerning current distributions

Current distribution of lineages probably reflects recent colonisation events accompanying reforestation following either climate warming 14 000 to 12 000 years ago, or catastrophic volcanic eruptions 1800 years ago. The occurrence of sympatric lineages in central North Island populations is consistent with simultaneous colonisation of the central region from single-lineage source populations inhabiting refugia in the south, north-east, and north-west of the North Island. Thus, the central North Island may be a hybrid zone (Hewitt, 2001) where lineages colonising from three different refugia meet. Relicts of the source populations persist in the Tararuas to the south and Urewera to the north-east. In the north-west extensive forest clearance since human settlement has rendered the persistence of short-tailed bat populations unlikely.

The occurrence of two lineages in South Island indicates that populations of lineages survived the last glacial maxima in separate refugia. Southern South Island populations then originated as a result of rapid southward expansion from refugia following rapid post-glacial range reforestation of the South Island between 10 000 and 9000 years ago (McGlone, 1988; McGlone et al., 1993; Stevens et al., 1995). The refugia would have been within central New Zealand (either southern North Island or northern South Island) as the severity of the climate in southern South Island during glacial periods makes southern South Island refugia for *M. tuberculata* improbable. Alternatively, the lineages may be survivors of sweepstake selection during recent rapid post-glacial range expansion from a more diverse, now extinct, population.

Comparison with other New Zealand phylogenetic studies

In the only comparable phylogenetic study of a New Zealand forest-dwelling vertebrate, allozymes and cytochrome *b* sequence were used to examine population structure of brown kiwi *Apteryx australis* (Baker et al., 1995). Estimated dates for basal divergences among clades of brown kiwi and *M. tuberculata* are concordant (0.9 My ago for brown kiwi and 0.93–0.82 My ago for *M. tuberculata*). The hypothesised southern South Island origin for brown kiwi (Baker et al., 1995), with divergences occurring within the South Island during northward dispersal, does not allow for the severity of the impact of glacial maxima on the South Island. The hypothesis proposed for *M. tuberculata*'s intraspecific phylogeny (repeated colonisation from refugia located in central New Zealand) seems a more plausible hypothesis to account for brown kiwi phylogeny. With

this hypothesis, deep divergences within southern brown kiwi populations reflect long-term isolation of small populations of kiwi in glacial refugia and repeated recolonisations from different refugia, rather than a southern origin for brown kiwi as suggested.

Taxonomy

Ideally identification of the appropriate taxonomic level of an organism requires concordance of two or more independent sources of evidence (Avise, 2000). Because of the absence of recombination in mitochondrial DNA, different mitochondrial genes are not independent, they share the same genealogical history. In this study, genealogical inference is made from single locus mtDNA. Thus the estimated phylogeny describes the mitochondrial genealogy, not that of the whole organism, and can only identify likely candidates for taxonomic status. Estimates of sequence differences and divergence time among *M. tuberculata* lineages (see Tables 3.6 and 3.7) are similar or greater than reported between some New Zealand avian species. North Island and South Island brown kiwi *A. mantelli* and *A. australis* exhibit 1.8% sequence divergence in cytochrome *b* and diverged about 0.9 My ago (Baker et al., 1995). The New Caledonian parakeet *Cyanoramphus saisetti* and six New Zealand *Cyanoramphus* species have interspecific difference in control region sequence of 0.95% to 9.82% and diverged between 0.625 and 0.450 My ago (Boon et al., 2000). These species have been defined on the basis of their discrete phylogenetic histories (Baker et al., 1995). Although discrete phylogenetic histories is a necessary criterion for defining species, it is not sufficient because small founding populations can quickly achieve discrete phylogenetic histories. The six *M. tuberculata* lineages have discrete phylogenetic histories, but the magnitude of divergence in ND2 sequence between them (1.2%–1.8%) is considerably less than the range of divergence in cytochrome *b* sequences (3.6%–13.4%) reported for congeneric comparisons between reproductively and morphologically distinct noctilionoid bat species (Lewis-Oritt et al., 2001; Van den Bussche and Baker, 1993; Van den Bussche et al., 1993). The lineages are not candidates for species status.

The most commonly used intra-specific taxonomic levels are subspecies, and evolutionary significant units (ESU) (Ryder, 1986; Moritz, 1994; Mallet, 1995; Avise, 2000). Although the ESU concept was conceived to overcome the problematic use of

subspecies (Ryder, 1986), ESU and subspecies are effectively equivalent (Avise, 2000). An ESU is one of a set of conspecific populations with a distinct long-term evolutionary history mostly separate from other such units (Ryder, 1986). This is effectively the same as a taxonomic subspecies, defined as distinguishable groups of individuals distinct from species only in their tendency to produce intermediates in zones of overlap (Mallet, 1995). *M. tuberculata* has been divided into three subspecies (*M. t. tuberculata, rhyacobia* and *aupourica*) (Hill and Daniel, 1985). These subspecies are not readily distinguished in the hand and were originally defined on the basis of geographical occurrence and a suite of morphological characteristics. The high degree of overlap in the principal metric (forearm length), especially for *M. t. tuberculata* and *rhyacobia*, raises doubts about the validity of the taxonomy (Lloyd, 2001). The phylogeny estimated in this study is only partially congruent with Hill and Daniel's (1985) subspecific taxonomy. Although the geographic range of the NL lineage corresponds closely to *M. t. apourica*, the existence of five other equally distinct lineages indicates a finer subdivision than between *M. t. tuberculata* and *rhyacobia*. The division of lineages into northern and southern groups resembles the division between *M. t. tuberculata* and *rhyacobia*. However, the suggested geographic boundary for *M. t. tuberculata* and *rhyacobia*, between southern and central North Island does not correspond to any observed boundary between northern and southern lineages (see Fig. 3.5). Although the existence of sympatric lineages discourages the notion that the lineages have taxonomic significance, anthropogenic loss of the lineages' core ranges may give a distorted picture of the species phylogeography. All extant populations outside of Northland are probably in the peripheries of the historic ranges of the lineages. In central North Island populations with sympatric lineages are located within a hybrid zone where lineages met during range expansion. Subspecies and even species commonly occur sympatrically in hybrid zones with varying degrees of intermixing and interbreeding. I suggest that the six lineages of *M. tuberculata* identified in this study might well be considered as ESU's or taxonomic subspecies. Confirmation requires further study using a recombining biparental marker.

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Chapter 4:

The demographic history of the New Zealand short-tailed bat *Mystacina tuberculata* inferred from modified control region sequences

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Brian D. Lloyd

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ABSTRACT

Short-tailed bats *Mystacina tuberculata* were widespread throughout the forest that dominated pre-human New Zealand, but extensive deforestation has restricted them to scattered populations in forest fragments. In a previous study, the species intraspecific phylogeny was investigated using multiple mitochondrial gene sequences. Six sympatric phylogroups were identified with estimated divergences of 0.93–0.68 My ago. In this study, the phylogeographic structure and demographic history of the phylogroups were investigated using control region sequences modified by removing homoplasic sites. Phylogeographic structure was generally consistent with an isolation-by-distance dispersal model. Coalescent based analyses (mismatch distributions, skyline plots, lineage dispersal analysis, and nested clade analysis) indicated the three phylogroups found in central and southern North Island expanded before the last glacial maximum. It is presumed this occurred during interstadials when *Nothofagus* forest was most extensive. Genetic structure within a central North Island hybrid zone was consistent with range expansion from separate refugia following reforestation after catastrophic volcanic eruptions. Absence of evidence for expansion since the last glacial maximum is presumed to reflect either limitations of the analytic methods or errors in substitution rate estimates. As dispersal has been dominated by episodic range expansion during infrequent periods of extensive reforestation, recent forest fragmentation will probably not have serious consequences on haplotypic differentiation.

INTRODUCTION

New Zealand's lesser short-tailed bat *Mystacina tuberculata* is one of only two bat species remaining in New Zealand. Populations of short-tailed bats are restricted to extensive stands of tall old-growth forest, because they require numerous large cavities in the main trunk of mature trees for their colonial roosts (Lloyd 2001). Despite being capable of strong flight, short-tailed bats are rarely found far from forest and long-distance dispersal has not been documented. The species was widespread throughout the forest that dominated pre-human New Zealand, however extensive deforestation since human settlement began about 1000 years ago has caused massive declines in the species abundance and range (Lloyd 2001). Seven large populations persist in the remnants of once continuous forest that extended across central North Island (see Fig. 3.1). Outside of central North Island, there are only four small isolated populations on the mainland, and two sizable populations on offshore islands. Fragmentation of previously widespread populations and massive reductions in range and abundance, as well as the continuing impact of recently naturalised taxa, all render the species vulnerable to further decline.

In a previous study (Lloyd, submitted), multiple mitochondrial gene sequences (control region, ND2, 12S rRNA, 16S rRNA, and tRNA) were used to investigate the species intraspecific phylogeny. Six lineages, or phylogroups, were identified with divergences ranging from 4.5% to 7.8% for CR-Left, and 1.2% to 1.8% for ND2. Estimated divergence dates among the phylogroups were between 0.93 and 0.68 My ago, during the middle Pleistocene. The estimated phylogeny did not correspond to the accepted intraspecific taxonomy of three subspecies (*M. t. aupourica, rhyacobia,* and *tuberculata*: Hill & Daniel 1985). Although phylogroups occurred sympatrically in many populations, there was evidence of well-defined phylogeographic structure not typical of bat species. Lloyd (submitted) suggested that divergence among the phylogroups of short-tailed bats arose when contractions of forest habitat, caused by either climatic fluctuation or catastrophic volcanic eruptions, fragmented the original population into sub-populations inhabiting isolated forest refugia. Hybrid zones, where phylogroups occur sympatrically, were formed by the recent range expansion from these refugia.

In this study, molecular genetic techniques were used to investigate phylogeographic structure to provide information for the development of long-term

strategies for the maintenance of the species. Detailed phylogeographic analyses were undertaken using control region sequences from 241 individuals collected from throughout the species' known range. Although control region sequences have been used to investigate intraspecific phylogeographic structure in many mammal species, multiple homoplasic substitutions at a number of nucleotide sites in the control region confound phylogenetic analysis of *M. tuberculata* (Lloyd, submitted). Therefore, control region sequences were modified by removing sites with more than one homoplasic substitution. This modification is analogous to the common practice of removing third codon sites from protein coding sequences. Results from the analyses were used to assess historical levels of dispersal between populations and the likely impact of recent forest fragmentation. Phylogeographic hypotheses proposed by Lloyd (submitted) were examined.

METHODS

Samples and sequence analysis

Wing biopsy samples were collected from the thirteen known populations distributed throughout the species range (see Fig. 3.1): Little Barrier Island (LBI), and Omahuta (Om) in Northland; Urewera (Ur), Whirinaki (Wh), Kaimanawa (Ka), Pureora (Pu), Waitaanga (Wa), Rangataua (Ra), and Tahupo (Ta) in central North Island; Tararua (Tr) in southern North Island; and north-west Nelson, Eglinton Valley (Eg) and Codfish Island (Co) in South Island. Samples from north-west Nelson and some samples from Omahuta were sequenced by Winnington & Lambert (1998). For all other samples, DNA was extracted and the 5′ end of mitochondrial control region (5′CR) amplified and sequenced as described in Lloyd (submitted). Sequences were edited and aligned using Sequencher™ version 4.1 software (Gene Code Corporation, Ann Arbor, MI). Sites within the 5′CR sequences exhibiting more than one homoplasic substitution were identified by ancestral character-state reconstruction of a ML tree estimated from a multiple gene data set (Lloyd, submitted). Sites exhibiting multiple homoplasic substitutions were excluded from the sequence alignments used for phylogeographic analyses. Unless stated otherwise, phylogenetic analyses were undertaken under the Tamura and Nei (1993) nucleotide substitution model with a gamma correction

(TN93 + Γ). The value of the gamma shape parameter (0.135) was estimated from a neighbour joining (NJ) tree in PAUP* 4.0b8 (Swofford 1998).

Overview of phylogeographic analyses

Phylogeographic structure throughout New Zealand was investigated both by testing the relationship between Wright's (1951) fixation index (F_{ST}) and geographical distances, and by examining the geographic distribution of the phylogroups. Population genetic structure of the central and southern North Island (CSN) populations was evaluated by hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992). The species demographic history in CSN was investigated using coalescent-based methods. The hypothesis of recent demographic expansion was tested using mismatch distribution analysis (Rogers & Harpending 1992; Harpending 1994; Rogers 1995) and Fu's F_S test of selective neutrality (Fu 1997). Skyline plots (Pybus *et al.* 2000; Strimmer & Pybus 2001) were used to obtain estimates of historical changes in the effective population size of females. The relationship between lineage age and dispersal was examined using lineage dispersal analysis (Neigel *et al.* 1991; Neigel & Ball 1993). Finally, nested clade analysis (Templeton 1998) was used to investigate the geographic distribution of genetic variation and infer which processes gave rise to it.

Analysis of F_{ST} values

F_{ST} values were estimated as 1 - (H_w/H_b) (Hudson *et al.* 1992), where H_w and H_b are the mean number of pairwise nucleotide differences between sequences from within populations and between populations respectively. Pairwise differences were estimated in PAUP. The relationship between F_{ST} values and geographical distances between populations throughout New Zealand was investigated by regression analyses using the software package SAS (1996).

Phylogroup assignment

All haplotypes were assigned to one of the phylogroups by estimating genealogies from alignments including sequences previously assigned to phylogroups by Lloyd (submitted) as markers. Genealogies were estimated for all sequences and for CSN sequences by both NJ and median joining (MJ) (Bandelt *et al.* 1999). Partial 5'CR sequences from Winnington & Lambert (1998) were assigned separately using a NJ tree estimated from an alignment including them with a subsample of sequences from this study. NJ trees were estimated in PAUP. MJ networks were estimated in the software package Network3.x (www.fluxus-engineering.com) using the reduced median (RM) algorithm (Bandelt *et al.* 1995) with sequences converted to binary form and transitions and transversions given weights of 10 and 20 respectively. To simplify MJ networks for display RM and MJ algorithms were applied sequentially.

Genetic diversity and AMOVA

Genetic diversity (i.e., number of haplotypes, gene diversity, and nucleotide diversity π) within population and phylogroups and AMOVA were estimated in ARLEQUIN (Schneider *et al.* 2000). AMOVA were used to compare five partitions (*a*–*e*) of the CSN sequences with partitions defined differently by either geographic location or phylogroup. The basic sampling units used were geographic population for partitions *a* to *c*, and sub-population defined by both phylogroup and geographic location for partitions *d* and *e*. In partition *a* populations (i.e., sampling units) were grouped into two geographic regions: northern (Ur, Wh, Ka, and Pu) and southern (Wa, Ra, Ta, and Tr). In partitions *b* and *c*, populations were grouped into three geographic regions. For partition *b* the groups were northern, (Ur, Wh, and Ka), central (Pu, Wa, and Ra) and southern (Ta and Tr). For partition *c* the groups were northern (Ur and Wh), central (Ka, Pu, Wa, and Ra) and southern (Ta and Tr). In partition *d* sub-populations (i.e., sampling units) were grouped into geographic populations, while in partition *e* sub-populations were grouped into phylogroups. Significance levels for AMOVA were obtained using non-parametric permutation methods (Schneider *et al.* 2000; Excoffier *et al.* 1992) with 1000 permutations.

Limitations to coalescent methods

Coalescent-based analyses were generally restricted to sequences from CSN because elsewhere, *M. tuberculata* persists in only a few small isolated populations which are not phylogenetically representative. The coalescent times within such populations are too short for accumulation of sufficient *de-novo* mutations (Avise 2000) meaning most variation will probably have arisen exogenously. Thus, samples from outside CSN are not suitable for coalescent-based analyses. In contrast, although the CSN meta-population has been fragmented by recent forest clearance, remaining populations are large and widely spread. Because phylogroups probably reflect the population structure at which coalescent processes operated during the species' evolutionary history, coalescent analyses were undertaken on the CSN phylogroups separately as well as together.

Estimating genealogies and parameters for coalescent analyses

Coalescent methods require estimates of genealogies, substitution rates and generation times. Genealogies were estimated by maximum likelihood (ML) in PAUP under the Hasegawa *et al.* (1985) substitution model using a gamma distribution and invariant sites (HKY85 + I + Γ) with a molecular clock enforced. HKY85 + I + Γ is a special case of TN93 + Γ and was used because TN93 + Γ is not available for ML estimation in PAUP. Separate trees were recovered for haplotypes belonging to the each of the three CSN phylogroups. Trees were rooted using members of other phylogroups as outgroups. The substitution rate per nucleotide site per year (μ) was calculated for each genealogy using root coalescence date estimates from Lloyd (submitted). Cohort generation time (Begon *et al.* 1990), calculated from the author's unpublished field data, was used as an estimate of generation time (g).

Mismatch distribution and Fu's F_S statistic

Mismatch analysis and Fu's F_S test of selective neutrality were implemented in ARLEQUIN. The significance of Fu's F_S statistic was tested by coalescent simulation with 5000 simulations. Significant large negative values are evidence for population expansion. Mismatch analyses were undertaken using TN93 without a gamma correction as implementation problems precluded use of TN93 + Γ. Expected

distributions were fitted to the observed mismatch distributions using a generalised least squares method to estimate the demographic expansion parameters τ, θ_0, and θ_1 (Schneider & Excoffier 1999). Parametric bootstrapping (Schneider *et al.* 2000) with 1000 pseudo replicates was used to obtain confidence intervals around the parameter estimates and to test whether the observed mismatch distributions fitted the sudden expansion model. Harpending's (1994) raggedness index (*HRag*) and the sum of the squared deviations (*SSD*) between observed and expected mismatch distributions were used as test statistics for fitting the distributions (Schneider *et al.* 2000). When distributions fitted the sudden expansion model, demographic parameters were calculated from the demographic expansion parameter estimates (Rogers & Harpending 1992; Rogers 1995). The time since expansion (t) and effective population size of females before and after expansion ($N_{F(e)0}$ and $N_{F(e)1}$) were computed using the relationships $\tau = 2ut$, $\theta_0 = 2uN_{F(e)0}$, and $\theta_1 = 2uN_{F(e)1}$, where u is the overall sequence divergence per generation. Values for u were calculated as $u = 2\mu kg$, where μ is the substitution rate per nucleotide site per year, k is the average sequence length and g is the generation time in years.

Skyline plots

Generalised skyline plots were estimated from ML trees for each of the three CSN phylogroups using the software GENIE v2.0 (http://evolve.zoo.ox.ac.uk/software) (Pybus 2001; Strimmer & Pybus 2001). Threshold values for grouping internode intervals were set to 10^{-4}. The plots provide estimates of $\hat{M}_i$, the harmonic mean of $N_{F(e)}(x)\mu g$ for time interval I, where $N_{F(e)}(x)$ is the effective female population size at time x, μ is the substitution rate per nucleotide site per year, and g is the generation time in years. Time is expressed as substitutions per nucleotide site. For display purposes, $\hat{M}_i$ was transformed to the harmonic mean of $N_{F(e)}$, and the time axis was transformed to chronological time (t) in ky from substitutions per nucleotide site (i.e., μt). Four different demographic models (constant-size, exponential growth, logistic growth, and expansion growth) were fitted to the data with parameters obtained using maximum likelihood estimation. Goodness-of-fits of the models were compared using one sample Kolomorogov-Smirnov tests to assess the difference between observed and expected distributions, and a likelihood-ratio test to compare nested demographic models (Pybus

et al. 2000). Significance values for the Kolomorogov-Smirnov tests were obtained by Monte-Carlo simulation of the null distribution using 500 simulations.

Lineage dispersal analysis

The relationship between lineage age and dispersal was investigated using methods based on a random-walk model of lineage dispersal (Neigel *et al.* 1991; Neigel & Ball 1993). The geographic dispersal of individuals belonging to lineages at all temporal depths in the ML trees of the CSN phylogroups was examined. Two dispersal parameters were estimated: the variance of the geographic positions of individuals belonging to a lineage (σ_H^2) and the standard single-generation dispersal distance (σ_F), estimated as $(\sigma_H^2/2G)^{½}$, where G is the number of generations to lineage coalescence. Lineage ages (in generation times) were calculated from distances on the ML tree (d) using the relationship $d/\mu kg$, where μ is the substitution rate per nucleotide site per year, k is the average sequence length, and g is the generation time in years. The longitudinal and latitudinal distances between the location of individuals belonging to a lineage and the geographic center of the lineage were used as a measure of geographic location. Distances were computed for individual lineages, but the variances of these distances were estimated for lineages grouped into age classes, with class-widths adjusted to include at least 5% of the observation in every age class.

Neigel & Ball (1993) described three stages of lineage dispersal which can be identified using this method. *Stage 1:* Dispersal is unconstrained and there is no equilibrium between dispersal and genetic drift. This stage is characterised by high positive linear correlations (> 0.7) between σ_H^2 and lineage age while σ_F is independent of lineage age. *Stage 2:* Dispersal continues, but is limited by geographic range. There is positive, but non-linear, correlation between σ_H^2 and lineage age while σ_F is negatively correlated with lineage age. *Stage 3:* Equilibrium between dispersal and genetic drift has been established. σ_H^2 is independent of lineage age while σ_F is negatively correlated with lineage age.

To identify departures from equilibrium between geographic dispersal and genetic drift, the data were tested for both geographic clustering and correlations between lineage age and dispersal (Neigel & Ball 1993). Geographic clustering was assessed by comparing σ_H^2 estimates with the distribution of estimates from 100 replicates of geographically randomised data. (In the geographically randomised data the

phylogenetic and geographic distributions of the original data were retained, but the locations of individuals were randomly exchanged.) Estimates of σ_H^2 from the original data falling below the fifth lowest estimate (i.e., $P < 0.05$) in the geographically randomised data were considered evidence of significant geographic clustering. Correlations between lineage age and dispersal (σ_H^2 and σ_F) were tested using Pearson's correlation coefficient (r^2) to detect linear correlation and Kendall's Tau (τ_K) to detect non-linear correlation. The significance of correlation values for σ_H^2 were further evaluated by comparison with distributions of correlation values estimated for 100 replicates of geographically randomised data. Analyses were undertaken using computer programs written in the software package SAS (1996).

Nested clade analysis

Nested clade analysis of CSN populations was undertaken using clades defined on a statistical parsimony cladogram (Templeton *et al.* 1992) of CSN sequences. Statistical parsimony was implemented in the software package TCS (Clement *et al.* 2000) with gaps set to missing and a 95% parsimony connection limit. Clades on the cladogram were nested using standard nesting rules (Templeton *et al.* 1987) and extensions to these rules for ambiguous linkages and stranded haplotypes (Templeton & Sing 1993). Nested clade analyses were implemented in the software package GeoDis (Posada *et al.* 2000). Clade distance D_c (the average distance of clade members from the clade centre) and nested clade distance D_n (the average distance of clade members from the centre of the clade it is nested in) were computed for clades within nesting clades that contain both genetic and geographic variation. When both interior and tip clades were present in a nesting clade the program also computed the average difference between D_c values for tip clades and interior clades $(I - T)_c$, and the average difference between D_n values for tip and interior clades $(I - T)_n$. The null hypothesis of no significant association between haplotypes and geographic structure was tested for all nesting clades containing both genetic and geographic variation using a permutation procedure with 1000 resamples. Where the null hypothesis was rejected inferences about population processes were made using inference keys (Templeton *et al.* 1995; Templeton 1998) to discriminate between restricted dispersal, range expansion, long distance colonisation, and allopatric fragmentation. Where allopatric fragmentation between lineages was inferred, the occurrence of secondary contact was investigated by examining the distributions of

average pairwise distances between geographical centres of clades present at sites at different levels (Templeton 2001). At the clade level associated with the fragmentation event, values for sites where secondary contact has occurred will be considerably larger than at other sites, whereas at lower clade levels values at all sites should be uniformly high.

The species' colonial behaviour and the impact of extensive forest clearance since human settlement complicated choices about sampling design in the inference-key. Where sampling sites were within continuous areas of unmodified habitat any bats in the intervening areas will have originated from one of the sampled colonies. Therefore, for inference purposes, bats were considered to be absent from unsampled areas between sampling sites within the two areas of continuous unmodified habitat (i.e., Ur, Wh, Ka, and Ra in the east, and Wa and Ta in the southwest). Bats are now absent from the large tracts of agriculturally modified habitat separating both these two areas, and Pureora and Tararua, but they were present less than 200 years ago. Therefore, for inference purposes, bats were considered as present in intervening agricultural areas.

RESULTS

5'CR sequences were obtained for 232 individuals from 12 of the 13 known short-tailed bat populations (see Fig. 3.1). Twenty-eight nucleotide sites exhibiting multiple homoplasic substitutions were excluded from the sequences. This reduced the alignment from 513 bp to 486 bp, the numbers of variable sites from 150 to 122, informative sites from 128 to 92, and uniquely defined haplotypes from 147 to 134. Incorporating nine partial sequences from Winnington & Lambert (1998) further reduced the alignment to 272 bp, which included 84 variable sites and 65 parsimony informative sites, and defined 116 haplotypes.

F_{ST} versus geographic distance

For pairwise comparisons among populations using the 272 bp alignment, two distinct trends were apparent in the plot of F_{ST} values versus geographic distance (Fig. 4.1). The dominant trend was a significant linear relationship between F_{ST} and distance

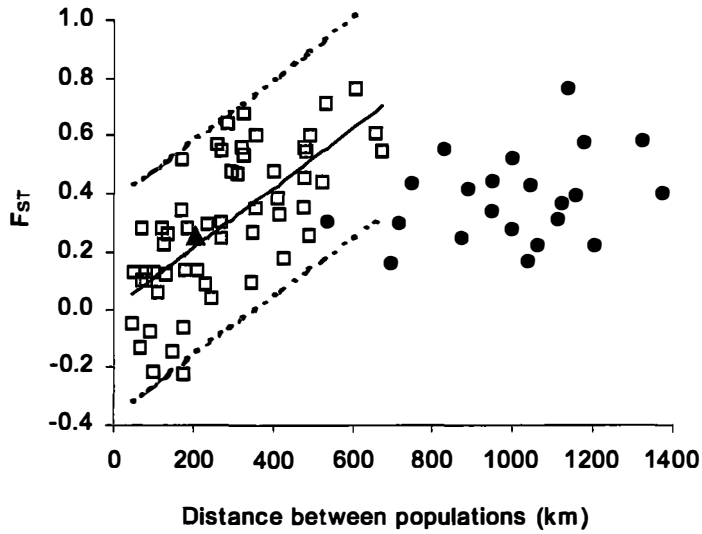


Figure 4.1 The relationship between F_{ST} and geographical distances among populations of *M. tuberculata*. Open squares and the regression line with 95% confidence intervals (dotted line) are for pairwise comparisons among northern populations. Dots are comparisons between northern and southern populations. The filled triangle is the comparison between the two southern populations.

($P = 0.0001$, $r^2 = 0.48$, and $\beta = 1 \times 10^{-3}$) for comparisons among populations from the North Island and northern South Island. The relationship did not hold ($P = 0.17$, $r^2 = 0.09$, $\beta = 0.2 \times 10^{-3}$) for comparisons between northern populations and the two populations from southern South Island (i.e., Co and Eg). A similar pattern was present among population comparisons using the 486 bp alignment. With these sequences there was a significant linear relationship for comparisons among northern populations ($P = 0.0001$, $r^2 = 0.80$, $\beta = 1.3 \times 10^{-3}$), but not for comparisons between northern and southern populations ($P = 0.69$, $r^2 = 0.01$, and $\beta = -6 \times 10^{-5}$). The linear relationship between F_{ST} and distance for northern population comparisons was consistent with the isolation-by-distance model, differentiation being a consequence of gene flow declining over distance. The departure from this relationship for comparisons between northern and southern populations indicated a lower level of genetic differentiation in southern South Island.

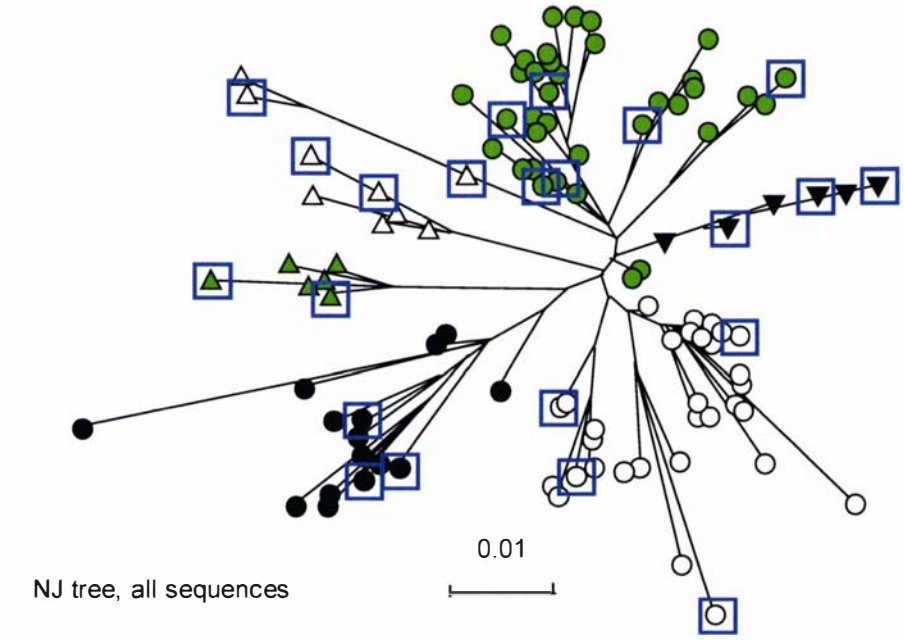
Phylogroup assignment

There was a high level of congruence between the topologies of NJ trees and MJ networks (Fig. 4.2) and no conflicts among the phylogroup assignments on the different genealogies. There was ambiguity over assignment of only 2 of 134 haplotypes from the 486 bp alignment, and none of the 116 haplotypes from the 272 bp alignment. The two ambiguous haplotypes were not included in a marked clade on the NJ tree of all haplotypes, but were included in marked clades or clusters on all other genealogies.

There was a marked reduction in nucleotide diversity within phylogroups (0.011–0.024) compared to the overall value (0.031) (Table 4.1).

Table 4.1 Diversity of modified 5′CR sequences in phylogroups of *M. tuberculata*

	Number of sequences	Number of haplotypes	Gene diversity	Nucleotide diversity (π)
Northland	30	9	0.77 ± 0.054	0.011 ± 0.006
CSN				
West	29	20	0.97 ± 0.016	0.024 ± 0.012
East	74	43	0.96 ± 0.013	0.020 ± 0.010
South	65	46	0.98 ± 0.006	0.015 ± 0.008
South Island				
SI1	22	10	0.90 ± 0.037	0.022 ± 0.012
SI2	12	6	0.88 ± 0.060	0.012 ± 0.007
All	232	134	0.99 ± 0.002	0.031 ± 0.015



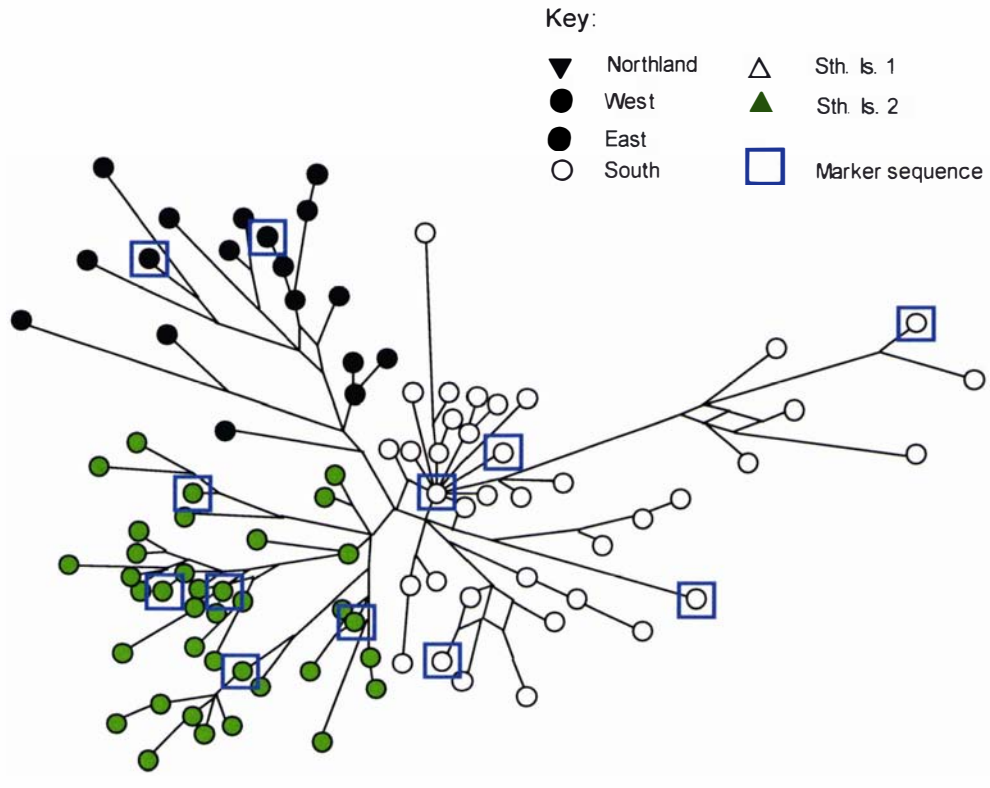


Figure 4.2 Distribution of phylogroups and marker sequences on NJ and MJ genealogies calculated from modified 5′CR haplotypes

Geographic distribution of phylogroups

Although haplotypes from two or more phylogroups occurred sympatrically in most populations, phylogroup distribution was geographically structured (Fig. 4.3). One phylogroup was restricted to Northland, where the only other haplotype was a single individual belonging to the western CSN phylogroup. In the rest of the North Island, most populations contained mixtures of haplotypes from the phylogroups, though western haplotypes were commonest in the north-west, while eastern and southern haplotypes dominated eastern and southern populations respectively. Populations in the centre (Ra, Wa, and Pu) contained moderate proportions of all three CSN phylogroups. Three phylogroups occurred in the South Island (SI). SI1 haplotypes were present in all three populations, whereas SI2 haplotypes were restricted to Codfish Island. One individual in the northern South Island population belonged to the southern North Island phylogroup.

Populations with the least mixing of phylogroups (i.e., LBI, Ta, Tr, and Ur) generally had lower nucleotide diversity values (0.013, 0.016, 0.014, and 0.011) than other populations (0.021–0.029). An exception to this pattern was the Eglinton population, which included only SI2 haplotypes, but had a relatively high nucleotide diversity (0.024).

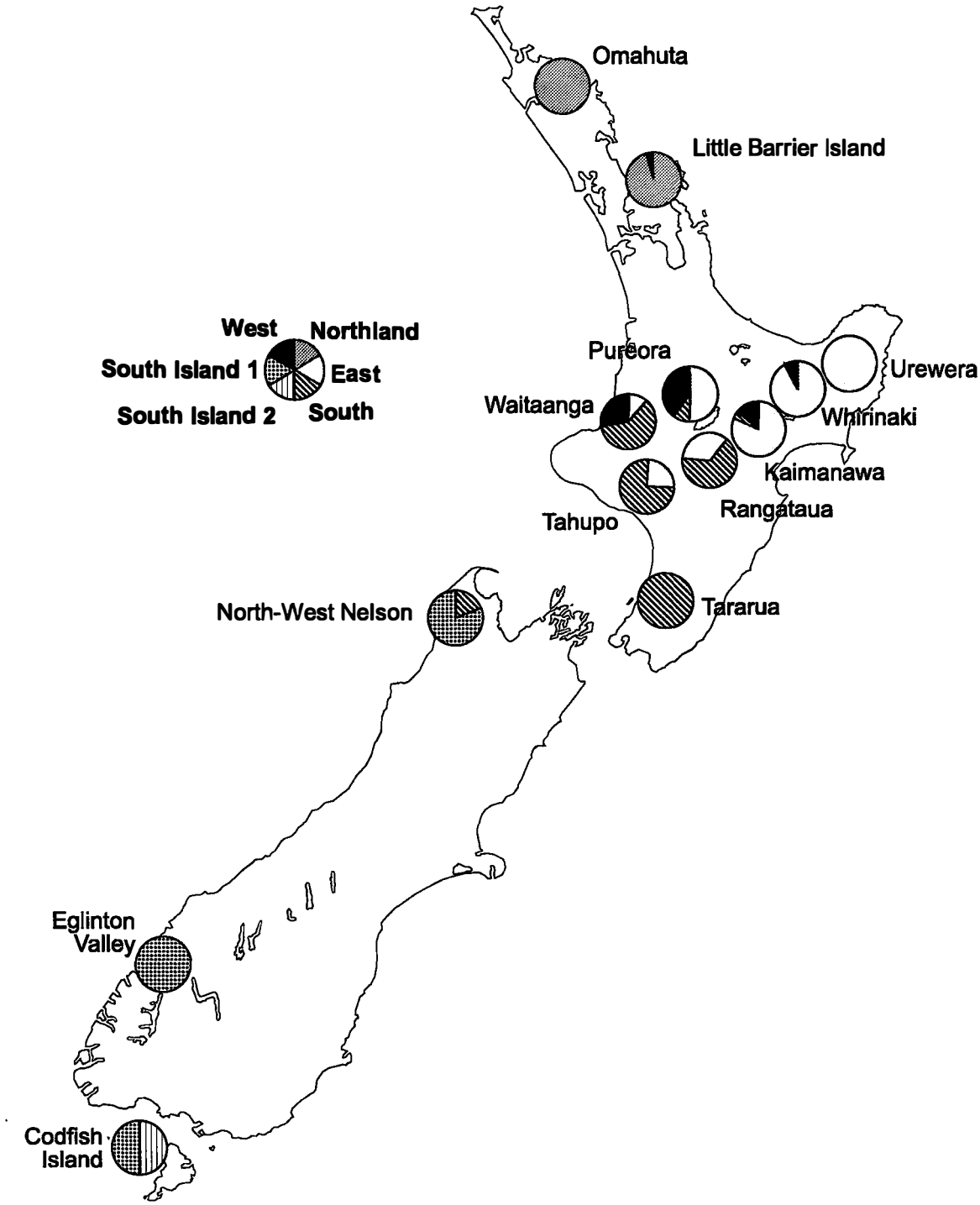


Figure 4.3 The geographic distribution of *M. tuberculata* phylogroups

AMOVA

Most of the diversity (75.4–79.4%) in partitions *a* to *c* was found within the basic sampling unit (i.e., geographic population) (Table 4.2). Differences among samples within groups (i.e., regions) were small (7.9%) for the two-region partition (*a*) and modest (12.9–15.2%) for the three-region partitions (*b* and *c*). There was a modest and significant ($P < 0.05$) difference (16.8%) among groups in the 2-region partition (*a*) whereas in the 3-region partitions (*b* and *c*) differences among groups were small and not significant. Variation within the basic sampling units (sub-populations) of partitions *d* and *e* was lower (45.4 and 52.7%) than in the sampling units of other partitions. In partition *d* variation among samples within groups (i.e., geographic populations) was high (72.3%) while variation among groups was negative (-25%) and not significant. In contrast in partition *e* variation among samples within groups (i.e., phylogroups) was moderate (13.6%) while variation among groups was high (41%) and significant ($P < 0.05$). The results indicated that CR sequence variation in the central North Island bats is better explained by phylogroup (i.e., partition *e*) than geographic location.

Table 4.2 Results of AMOVA showing percentage of variation at different hierarchical levels for five different partitions of modified 5′CR sequences from CSN

Sampling unit	Population			Sub-population	
Groups	N/S[1]	N/Cen/S[1]		Geographic	Phylogroup
Partition	*a*	*b*	*c*	*d*	*e*
Among groups	16.8*	7.9	5.4	-25.0	41.0*
Among samples, within group	7.9*	12.9*	15.2*	72.3*	13.6*
Within samples	75.4*	79.3*	79.4*	52.7*	45.4*

[1] Abbreviations for geographic regions: N - northern, Cen - central, and S - southern

* $P < 0.05$

General parameter estimates for coalescent methods

Phylogroup substitution rates (μ) estimated on the ML phylograms (Fig. 4.4) were 2.34, 1.64, and 1.76 % My^{-1}, for the western, eastern, and southern phylogroups respectively. Cohort generation time estimates ranged between 3.7 and 5.3 years. Because cohort generation time is generally greater than true generation time (Begon *et al.* 1990), 4 years was used as an approximation for generation time (g).

Figure 4.4 ML phylograms of modified 5'CR haplotypes for CSN phylogroups of *M. tuberculata*, with a molecular clock enforced

Mismatch distribution and Fu's F_S

Mismatch distributions for CSN phylogroups were unimodal (Fig. 4.5), with values of *HRag* and *SSD* consistent with the demographic-expansion model (Table 4.3). In contrast, mismatch distributions for Northland and South Island phylogroups were multimodal, and the demographic-expansion model was rejected ($P < 0.05$). The results of Fu's F_S test showed the same pattern: stasis was rejected for CSN phylogroups and accepted for Northland and South Island phylogroups.

The demographic parameter estimates from mismatch distribution parameters (Table 4.4) indicated population expansion for the three CSN phylogroups began during the last glacial period, before the last glacial maximum. The estimated patterns of expansion for western and southern phylogroup were similar. Expansion began 41

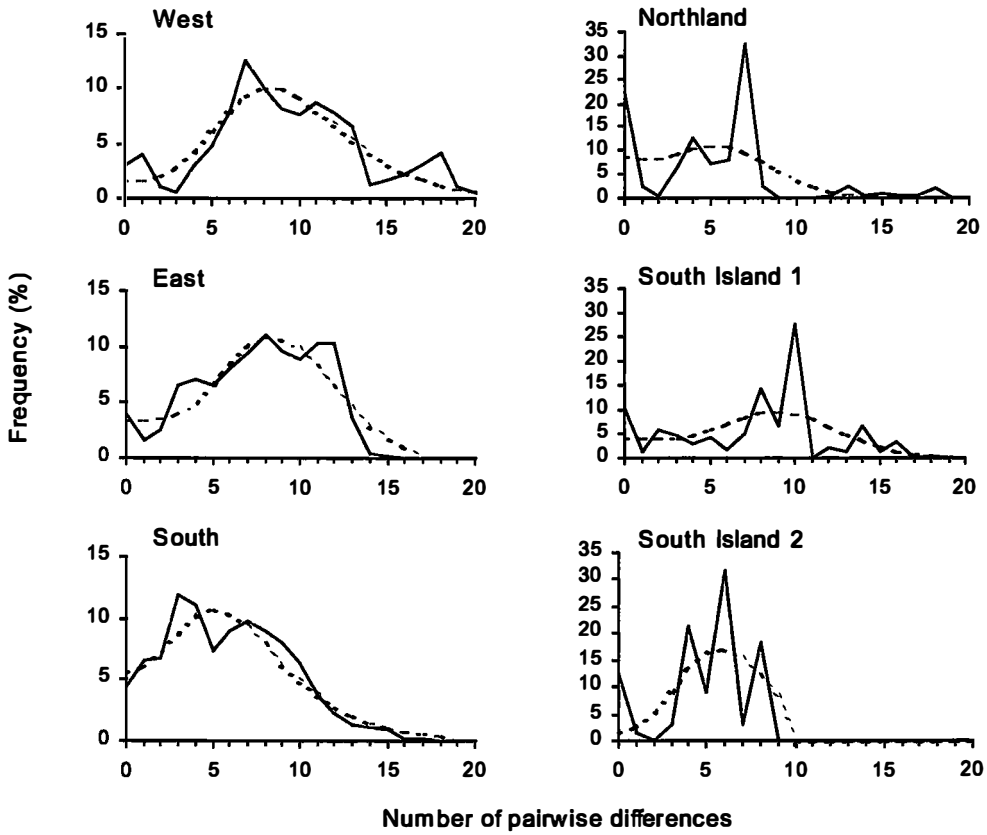


Figure 4.5 Mismatch distributions (solid line) for phylogroups of *M. tuberculata* with expected distributions (dotted lines) under the sudden demographic expansion model fitted to the observed distributions

and 32 ky ago, respectively, with initial moderately sized populations of 15 400 and 21 100 increasing 22-fold and 6-fold respectively to 341 000 and 130 000. Expansion in the eastern phylogroup followed a different course, with expansion beginning earlier (73 ky ago) from a small population of 100 individuals which increased by a factor of 1549 to 219 000, respectively. However, demographic parameters for population expansion estimated from mismatch distributions should be treated with caution, as the confidence intervals are wide and errors in μ and g are not incorporated into the intervals.

Although mismatch distributions for the CSN phylogroups fitted the model for population expansion, visual inspection of the distributions (see Fig. 4.5) indicated the distributions were possibly a composite of overlapping distributions. Minor peaks at large mismatch values, present in the western distribution, and steep slopes on the leading face of the distributions, present in both western and eastern distributions, may be evidence of bottlenecks (Rogers & Harpending 1992).

Table 4.3 Sample size and probability values for tests of the hypothesis of recent demographic expansion for phylogroups of *M. tuberculata*

Phylogroup	Sample size	$P(F_S)$	*P(HRag)*	*P(SSD)*
Northland	30	0.63	0.00	0.00
CSN				
West	28	0.07	0.59	0.40
East	74	0.00	0.63	0.43
South	65	0.00	0.94	0.74
Sth. Is				
SI1	22	0.69	0.01	0.01
SI2	12	0.70	0.02	0.02

Table 4.4 Expansion parameters and estimated demographic parameters with 95% confidence intervals for mismatch distribution of the CSN phylogroups

	τ	θ_0	θ_1	t (ky)	$N_{F(e)0} \times 10^3$	$N_{F(e)1} \times 10^3$
West	7.4 (4.2–15.1)	2.8 (0–9.1)	62 (26–6577)	40.6 (23–83)	15.4 (0–50)	341 (145–36299)
East	9.3 (5.2–13.4)	0.0 (0–3.3)	28 (18–1418)	73.3 (41– 106)	0.1 (0–26)	219 (144–11149)
South	4.6 (1.4–15.3)	2.9 (0–7.5)	18 (9–2473)	33.9 (11–112)	21.1 (0–55)	130 (68–18 138)

Skyline plots

Generalised skyline plots (Fig. 4.6) indicated that there have been large fluctuations in effective female population size during the history of each of the three phylogroups. For western and eastern phylogroups the fluctuations overlaid overall trends of increasing population size with maximum values (1.7 and 5.3 million) for the most recent (0–35 and 0–44 ky) estimates. For the southern phylogroup a peak value of 7.8 million

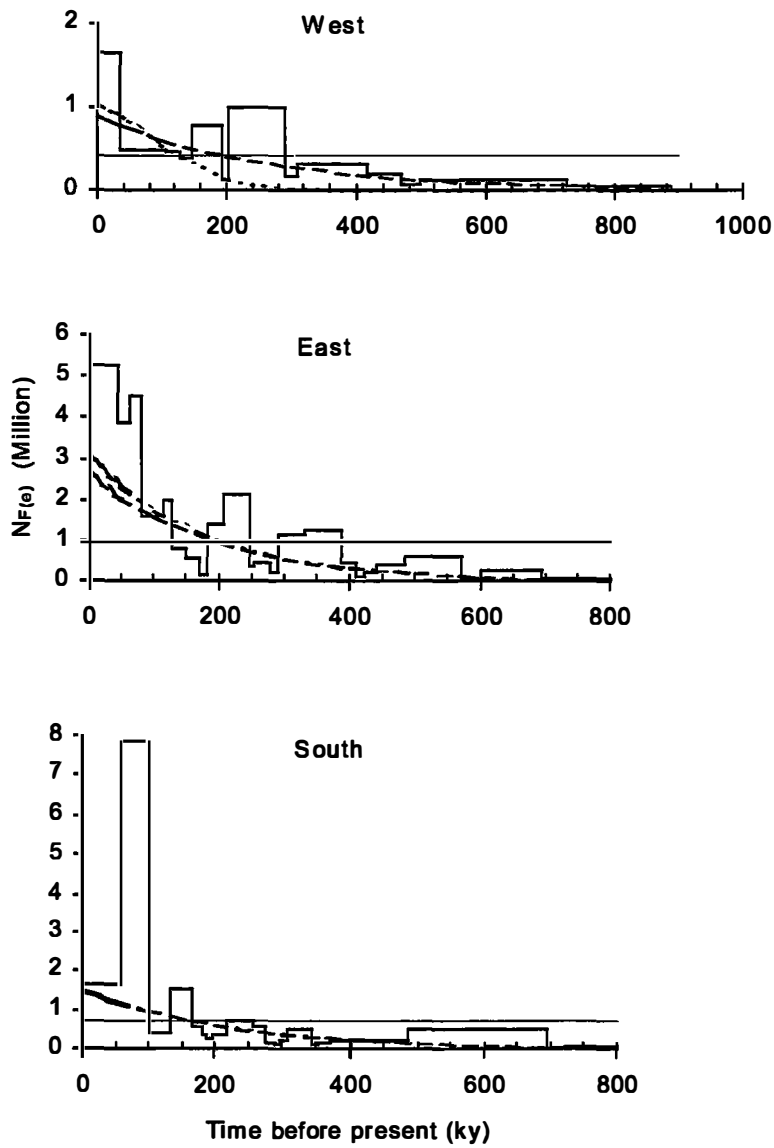


Figure 4.6 Generalised skyline plots for CSN phylogroups of *M. tuberculata*, with demographic models fitted to the data. Demographic models are constant-size (fine straight line), logistic growth (dotted line), exponential growth (dashed line) and expansion growth (broken line).

between 58 and 100 ky ago, was followed by a decline to 1.7 million for the most recent (0–58 ky) estimates. None of the demographic models fitted the data well, although likelihood-ratio test results indicated exponential growth models were the best fit for all three phylogroups. Implementation problems within Genie meant Kolomorogov-Smirnov test results were not obtained for the exponential model for the

south phylogroup and the expansion model for any phylogroup. Of the remaining models, exponential growth and logistic growth models were accepted for the west phylogroup ($P = 0.06$ and $P = 0.15$), while all other models were rejected ($P < 0.05$).

Comparing demographic inferences from mismatch distributions and skyline plots

Inferences about the timing and magnitude of recent demographic events from mismatch distributions and skyline plots are not directly comparable. Mismatch distributions provide estimates for a single hypothesised expansion, whereas skyline plots provide estimates of the harmonic mean of effective female-population size for a series of time intervals. Despite this, inferences about the times of expansion from the two methods were generally concordant for the three CSN phylogroups. The 95% confidence interval around *t*-estimates from mismatch distributions enclosed time-intervals in which population expansion was inferred on skyline plots. Although there were large discrepancies, post-expansion effective female-population sizes inferred from skyline plots were within the 95% confidence interval of mismatch distribution estimates. In contrast, pre-expansion effective female-population sizes inferred from skyline plots were all considerably higher than the upper 95% interval for estimates from mismatch distributions.

Inferences about demographic expansion from mismatch distributions should be interpreted with caution, as mismatch distributions analysis is based on a model of monotonic change in an unstructured population. Temporal and geographic variation in climatic, tectonic, and biotic factors render this model improbable for the long-term history of most wild animal populations. Mismatch distributions can be affected by other demographic events such as population bottlenecks and subdivision (Marjoram & Donnely 1994; Rogers 1997; Polanski *et al.* 1998). Although skyline plots incorporate phylogenetic structure and therefore should be more powerful than mismatch distribution analyses which do not (Felsenstein 1992; Pybus *et al.* 2000), their limitations are not well explored. There is considerable, but unquantified, error in estimates from skyline plots, as they include coalescent error and uncertainty over estimates of coalescent times inferred from a single tree (Strimmer & Pybus 2001), as well as errors in μ and g. The temporal resolution of generalised skyline plots is limited by the width of time intervals, which means demographic cycles within a time interval may leave no trace.

Lineage dispersal analysis

The results of lineage dispersal analyses showed departures from equilibrium between geographic dispersal and genetic drift in each of the CSN phylogroups and the combined data (Fig. 4.7 and Table 4.5). The pattern of departure from equilibrium differed among the groups.

Western phylogroup. Estimates of σ_H^2 were mostly within the non-significant range obtained using geographically randomised data. The few estimates outside the non-significant range were for the youngest, presumably localised, lineages. Correlation coefficients for lineage age versus longitudinal σ_H^2 were high and significant (i.e., exceed 95% of the values from randomised data), whereas values for lineage age versus latitudinal σ_H^2 were low and not significant. Values of r^2 for σ_F versus lineage age were low and positive for longitude, but large and negative for latitude. These results indicated lineage dispersal along the longitudinal axis was unconstrained and not at equilibrium with genetic drift (i.e., Stage 1), whereas dispersal along the latitudinal axis was limited and at equilibrium (i.e., Stage 3).

Eastern phylogroup. Longitudinal and latitudinal σ_H^2 estimates were, with a few exceptions for the oldest lineages, below the non-significant range. Correlation coefficients for lineage age versus σ_H^2 were high and significant, whereas both σ_F estimates were independent of lineage age. These results match the criteria for unconstrained lineage dispersal without equilibrium (i.e., Stage 1).

Southern phylogroup. Longitudinal σ_H^2 estimates were within the non-significant range, whereas latitudinal estimates were below the non-significant range, indicating clustering of lineages along the latitudinal axis. Correlation coefficients for lineage age versus longitudinal σ_H^2 were positive but moderate and not significant, whereas values for lineage age versus latitudinal σ_H^2 were high and significant. Values of r^2 for σ_F versus lineage age were large and negative for longitude, but moderate and positive for latitude. Removing sequences belonging to the geographically outlying Tararua population produced very different results. Both longitudinal and latitudinal σ_H^2 estimates became non-significant, indicating clustering was between Tararua and other lineages. Correlation coefficients for lineage age versus σ_H^2 were all positive but moderate and not significant, whereas both σ_F estimates showed strong negative correlations with lineage age. Thus, when the Tararua lineage was included in the

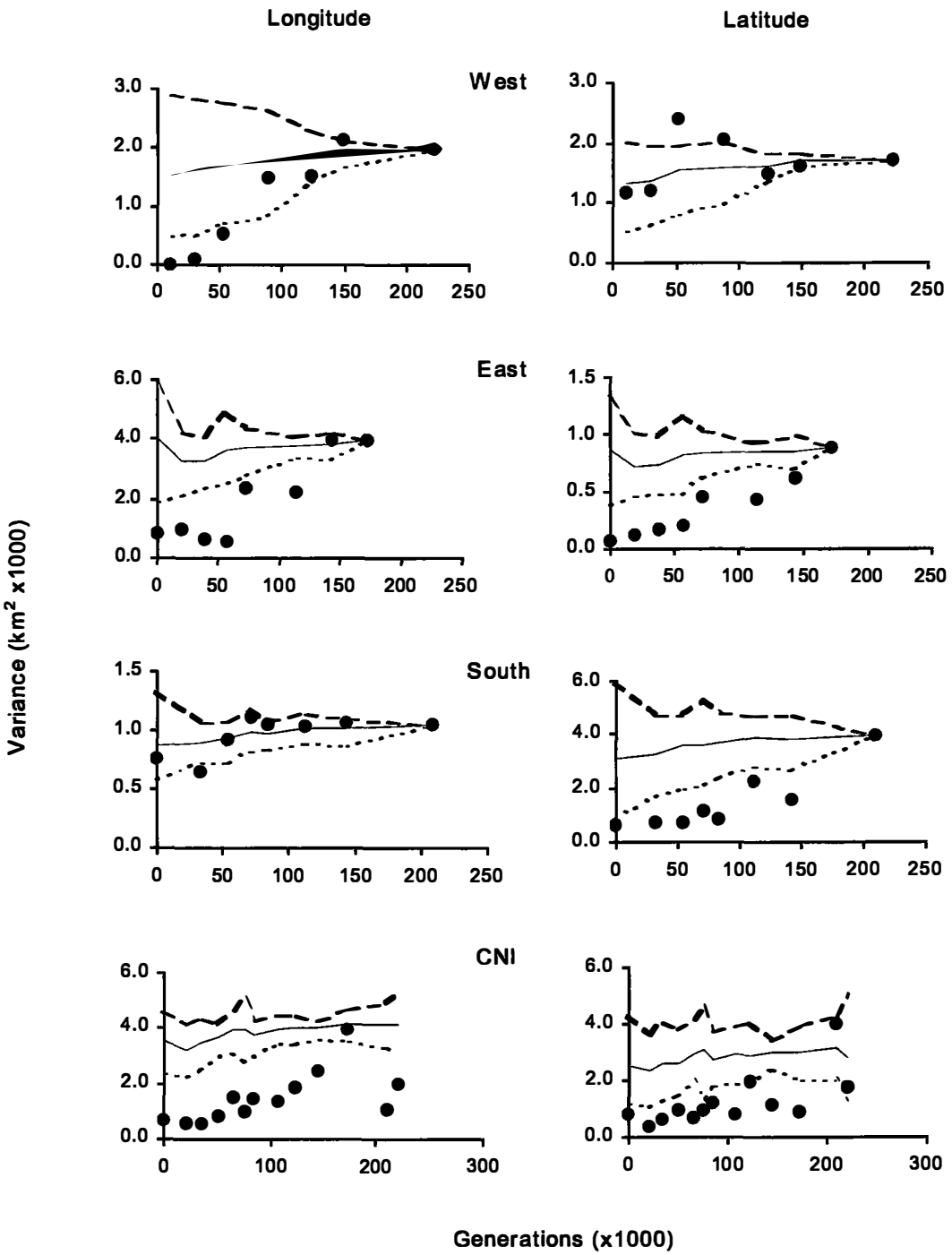


Figure 4.7 Patterns of lineage dispersal and age for CSN phylogroups, separately and together. Points are values of σ_H^2 for longitude (left) and latitude (right), estimated for lineage age classes. Lines are the mean, and the upper and lower (dotted) 95% limits for values of σ_H^2 from 100 replicates of geographically randomised data.

southern phylogroup latitudinal lineage dispersal and genetic drift for the phylogroup were strongly out of equilibrium, but when it was excluded dispersal approached equilibrium with genetic drift (i.e., Stage 3). This pattern is consistent with historical

disruption of gene flow partitioning the Tararua lineage from the rest of the southern phylogroup.

Combined CSN data. Estimates of σ_H^2 were, with a few exceptions for the older lineages, non-significant indicating lineage clustering. Correlation coefficients for lineage age versus σ_H^2 were all moderate, but values for longitudinal σ_H^2 were not significant, whereas values for latitudinal σ_H^2 were significant. Both σ_F estimates showed moderate negative correlation with lineage age. When the Tararua lineage was removed from the combined data correlation values for latitudinal σ_H^2 became not significant and correlation between latitudinal σ_F estimates and lineage took on a high negative value. Clustering of lineages in the CSN was probably a consequence of geographical partitioning of the three CSN phylogroups.

Table 4.5 Correlations of lineage age versus σ_H^2 and lineage age versus σ_F for CSN phylogroups

	Lineage age versus σ_H^2				Lineage age versus σ_F	
	Longitude		Latitude		r^2	
Phylogroup	r^2	(τ_K)	r^2	(τ_K)	Longitude	Latitude
West	0.91*	(0.91)*	0.18	(0.33)	0.58	-0.87
East	0.91*	(0.50)	0.96*	(0.93)*	-0.16	-0.16
South						
including Tr	0.66	(0.43)	0.89*	(0.79)*	-0.98	0.01
excluding Tr	0.72	(0.64)	0.62	(0.57)	-0.96	-0.91
Combined						
including Tr	0.63	(0.62)	0.69*	(0.56)*	-0.62	-0.39
excluding Tr	0.64	(0.62)	0.67	(0.51)	-0.63	-0.83

r^2 - Pearson correlation coefficient

τ_K - Kendall's Tau

* Value exceeds 95% of values for 100 replicates of geographically randomised data

Nested clade analysis

The statistical parsimony cladogram (Fig. 4.8) had a similar topology to the NJ and MJ genealogies (see Fig. 4.2), with haplotypes clustering into the same three phylogroups. Two haplotypes (one each from the southern and eastern phylogroups) were not incorporated into the cladogram. The null hypothesis of no association between haplotypes and geographic structure was rejected ($P < 0.05$) for nesting clades (Fig. 4.9) at all levels on the cladogram. Inferred causes for the observed geographical associations varied for different clades (Table 4.6) and locations (Fig. 4.10). Results for the oldest (most interior) nesting clade indicated that allopatric fragmentation gave rise to the observed distributions of the three phylogroups. Within the eastern phylogroup there was evidence of contiguous range expansion among the eastern sites (Ur, Wh, and Ka) at intermediate nesting levels (levels 2 to 4). Failures to discriminate between range expansion and restricted dispersal in nesting clades 4E1 and 3E2 may be evidence that range expansion within the eastern regions was accompanied, or followed, by dispersal from the eastern region to southern sites (Ra and Ta) and Pureora respectively. Indeed recent restricted dispersal or long distance colonisation by a single eastern haplotype from the east to Pureora was inferred from 1E07, nested within 3E2. The southern phylogroup showed evidence of contiguous range expansion at the earliest level. The Tararua clade separated from other clades soon after original expansion (4S2) of the phylogroup. Absence of geographically intermediate populations because of forest clearance means it was not possible to distinguish between allopatric fragmentation and isolation by distance resulting from restricted dispersal. There was some evidence (1S08) of recent restricted dispersal of southern haplotypes between southern and western sites (Ra, Ta, Pu, and Wa). Conclusions about the western phylogroup were limited by the loss of the phylgroup's core range as a result of extensive forest clearance in northwest CSN. Dispersal of clades nested in 4W1 between widely spaced sites (Wh, Ka, Pu, Wa, and Ra) may be a result of either restricted dispersal or allopatric fragmentation. At a lower (more recent) clade level, dispersal of clades nested in 3W5 provided evidence of contiguous range expansion between Waitaanga and Pureora.

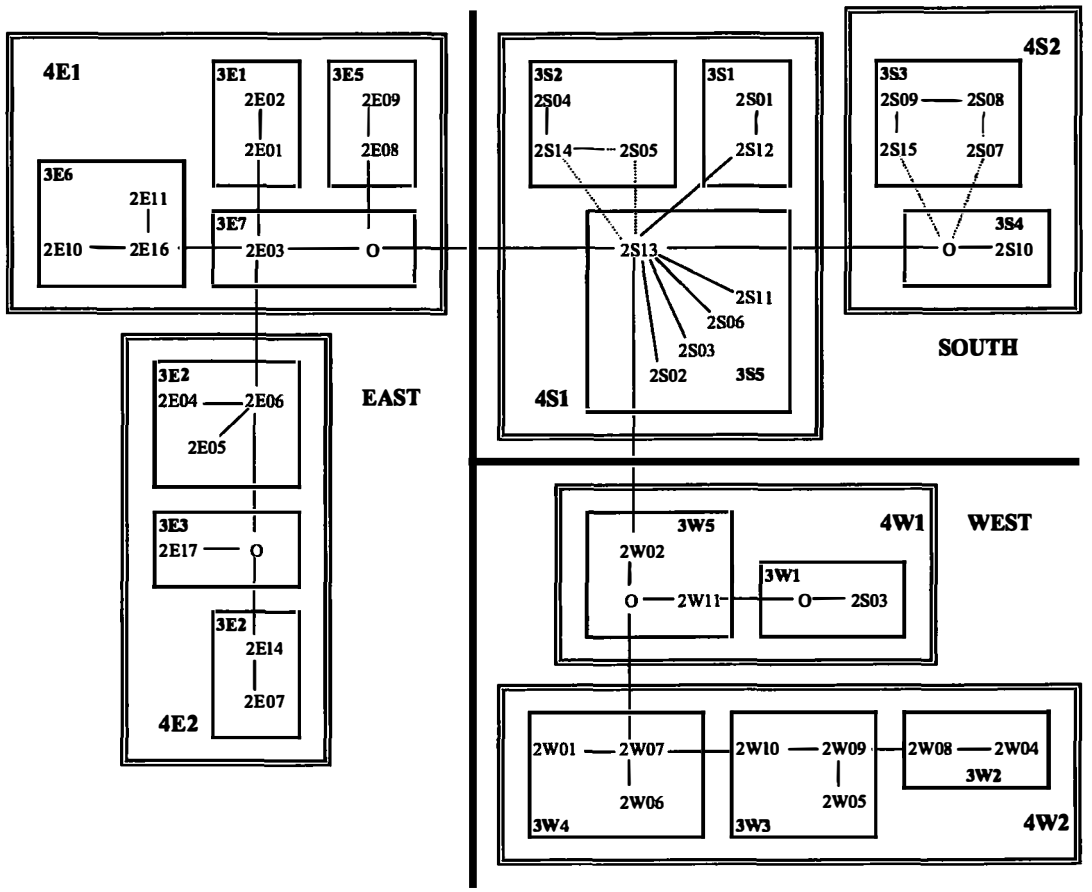


Figure 4.8 Statistical parsimony cladogram of modified 5'CR haplotypes for *M. tuberculata* from CSN showing nested clade structure. Solid lines indicate unambiguous linkages between level 2 clades whereas dashed lines indicate ambiguous linkages. 'O's indicate intermediate clades not found in the sample. Boxes with fine lines enclose nested level 2 clades. Boxes with double lines enclose nested level 3 clades. Heavy lines enclose nested level 4 clades and separate the phylogroups.

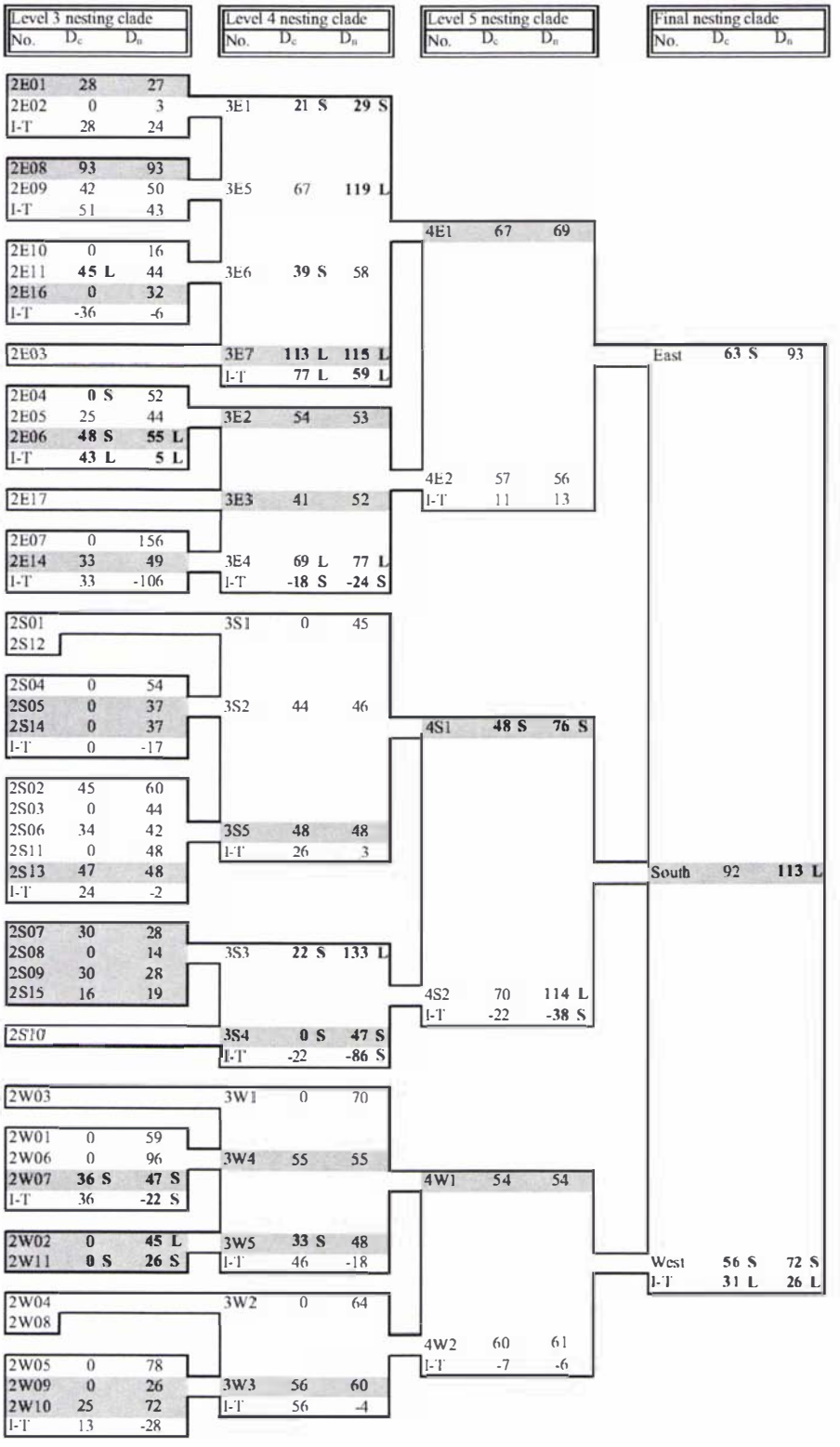


Figure 4.9 Results of nested clade analysis of modified 5′CR haplotypes for *M. tuberculata* from CSN. Interior clades are shaded. Within each nesting clade, the clade distance D_c and nested clade distance D_n follow the number or name of each clade. Where both interior and tip clades are present in a nesting clade the average difference between interior and tip clades for both distance measures is given in the row labled I-T. Significantly small or large values for distance measures are in bold and indicated by '**S**' or '**L**', respectively.

Table 4.6 Inference chain for nested clade analysis

Nesting clade		Inference chain	Demographic inference
All CSN			
	Final	2n-3n-4y-9y-10y	Allopatric fragmentation
East			
	4E1	2y-3y-5n-6(<2)-7y	Either: Range expansion
			or: Restricted dispersal with long distance colonisation
	3E6	2n-11y-12n	Contiguous range expansion
	2E11	2n-11y-12y-13n-14y	Range expansion with long distance colonisation
	4E2	2n-11y-12n	Contiguous range expansion
	3E2	2y-3y-5n-6(<2)-7y	Either: Range expansion
			or: Restricted dispersal with long distance colonisation
	1E07	2y-3y-5n-6n-7n-8n	Either: Restricted dispersal causing isolation-by-distance
			or: Long distance colonisation
South			
	South	2n-11y-12n	Contiguous range expansion
	4S2	2y-3y-5y-15y-16n-18y	Either: Allopatric fragmentation
			or: Restricted dispersal causing isolation-by-distance
	2S13	2n-11n-17n	Inconclusive outcome
	1S08	2y-3n-4n	Restricted dispersal causing isolation-by-distance
West			
	4W1	2n-3n-4y-9y-10n	Either: Allopatric fragmentation
			or: Restricted dispersal causing isolation-by-distance
	3W4	2(Tip/Int?)	Inconclusive outcome
	3W5	2n-11y-12n	Contiguous range expansion

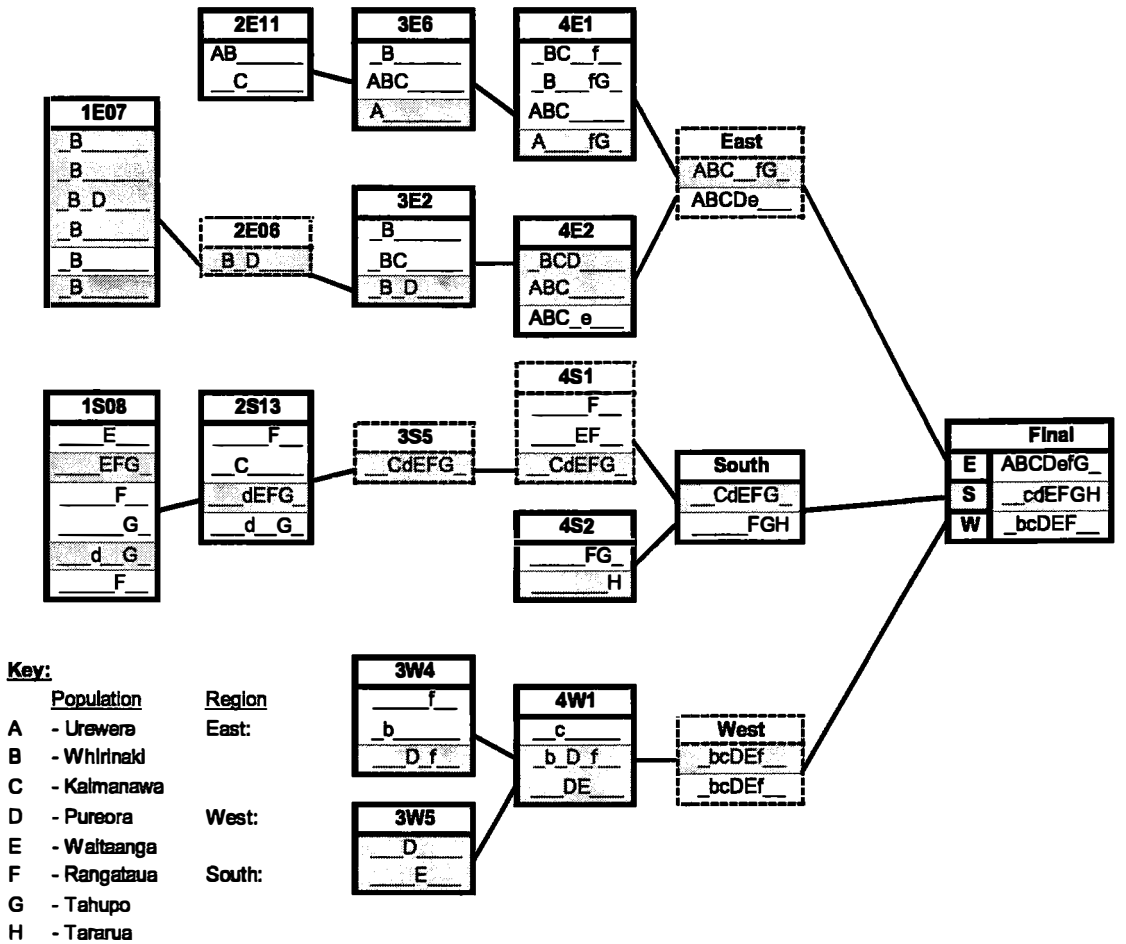


Figure 4.10 Geographic distribution of *M. tuberculata* clades within CSN. Solid lines enclose nesting clades exhibiting significant association between haplotypes and geographic structure. Interior clades are shaded. Dashed lines enclose non-significant clades. To conserve space only significant nesting clades and clades they are nested in are shown.

Despite the absence of extant populations throughout the probable historical centres of each of the three phylogroups' range, there was evidence of secondary contact following earlier allopatric fragmentation. The distributions of average pairwise distances between geographical centres of level 4 and level 5 clades among sites (Fig. 4.11) were consistent with the occurrence of secondary contact between the three phylogroups. At the fragmentation level (i.e., level 5) values for sites in the central zone

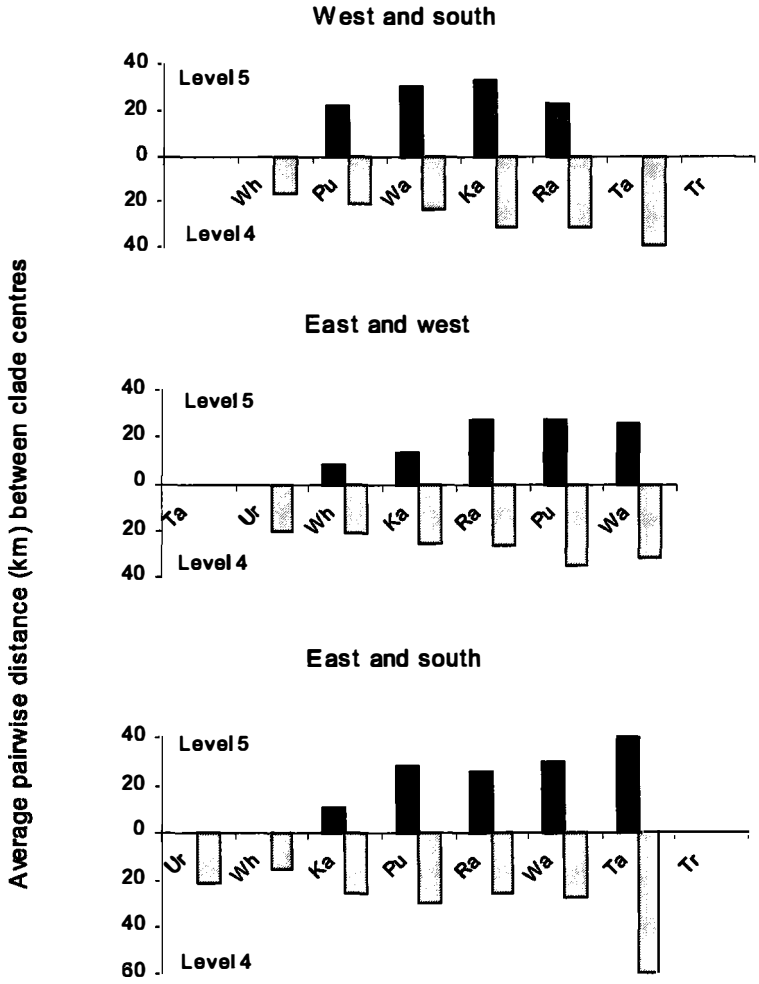


Figure 4.11 Evidence of secondary contact following allopatric fragmentation between CSN phylogroups. Comparisons of average pairwise distances between clade centres for all clades represented at each site.

were larger than peripheral sites, whereas at level 4 values were uniformly high except for Tararua. Low values for Tararua reflected a more recent fragmentation (inferred in clade 4S2) between Tararua lineages and other southern lineages. There was no evidence of secondary contact between Tararua lineages and other southern lineages, as pairwise distances for clade 4S2 and nested clades were low for all sites. Regions of secondary contact for all three pairs of phylogroups overlapped in the three central populations (Pu, Wa, and Ra), but extended further east (to Ka) for western and southern phylogroups, and further south (to Ta) for eastern and southern phylogroups.

DISCUSSION

Phylogeography within central and southern North Island

Results from this study support the intraspecific phylogeny and phylogeographc hypotheses proposed by Lloyd (submitted). The various analyses undertaken on CSN scqucnces provide insight into the separate demographic histories of the three CSN phylogroups. Nested clade and lineage dispersal analyses indicate the phylogroups originated from ancient allopatric fragmentation. Although the phylogroups coalesced between 0.93 and 0.68 My ago (Lloyd, submitted), large effective population size could mean population fragmentation was much more recent. Results from Fu's F_S test, mismatch distributions, and skyline plots provide evidence of population expansion for all three CSN phylogroups. Estimated dates for the expansion events are in the last glacial period, before the last glacial maximum (20 ky ago). It seems probable that populations expanded during the warmer interstadials within the glacial period. At these times, *Nothofagus* forest dominated central and southern North Island extending over a greater area than in the previous interglacials or the current post-glacial period (McGlone *et al.* 1996). Currently most mainland populations of short-tailed bats, outside of Northland, inhabit *Nothofagus* forest and almost all known colonial roosts are in mature specimens of *Nothofagus* spp. (Lloyd 2001).

Nested clade and lineage dispersal analyses provide evidence of eastward range expansion by the western phylogroup, and unconstrained range expansion by the eastern phylogroup into areas of central North Island subject to vulcanism. During the most recent eruptions, 30 and 1.8 ky ago, pyroclastic flows from the Taupo Volcanic Zone engulfed 20 000 km^2 of forests in the Central Volcanic Region (Fig. 4.12) (Stevens *et al.* 1995; McKinnon *et al.* 1997). Wind-borne tephra deposits devastated forest ecosystems over a much wider area to the north and east. Range expansion by the two phylogroups probably followed rapid reforestation after the most recent catastrophic eruptions. Sympatric phylogroups in a central North Island hybrid zone (see Figs. 4. 3 and 4.11) resulted from secondary contact between the two expanding phylogroups and the southern phylogroup (see Fig. 4.12).

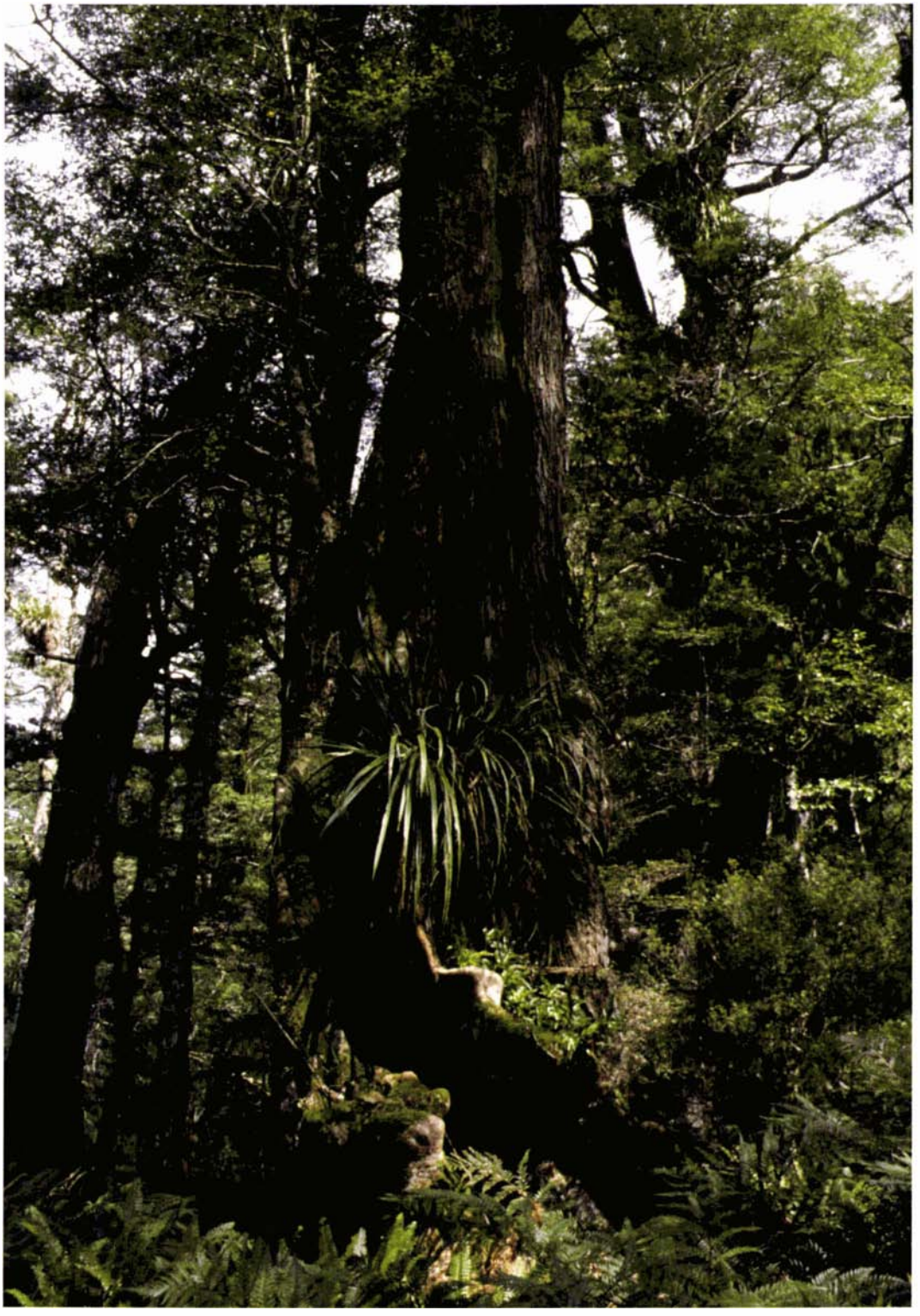


Plate 4.1 Old growth *Nothofagus* forest interior, including a mature *Nothofagus fusca* with a short-tailed bat colonial roost inside the cavity on the main trunk

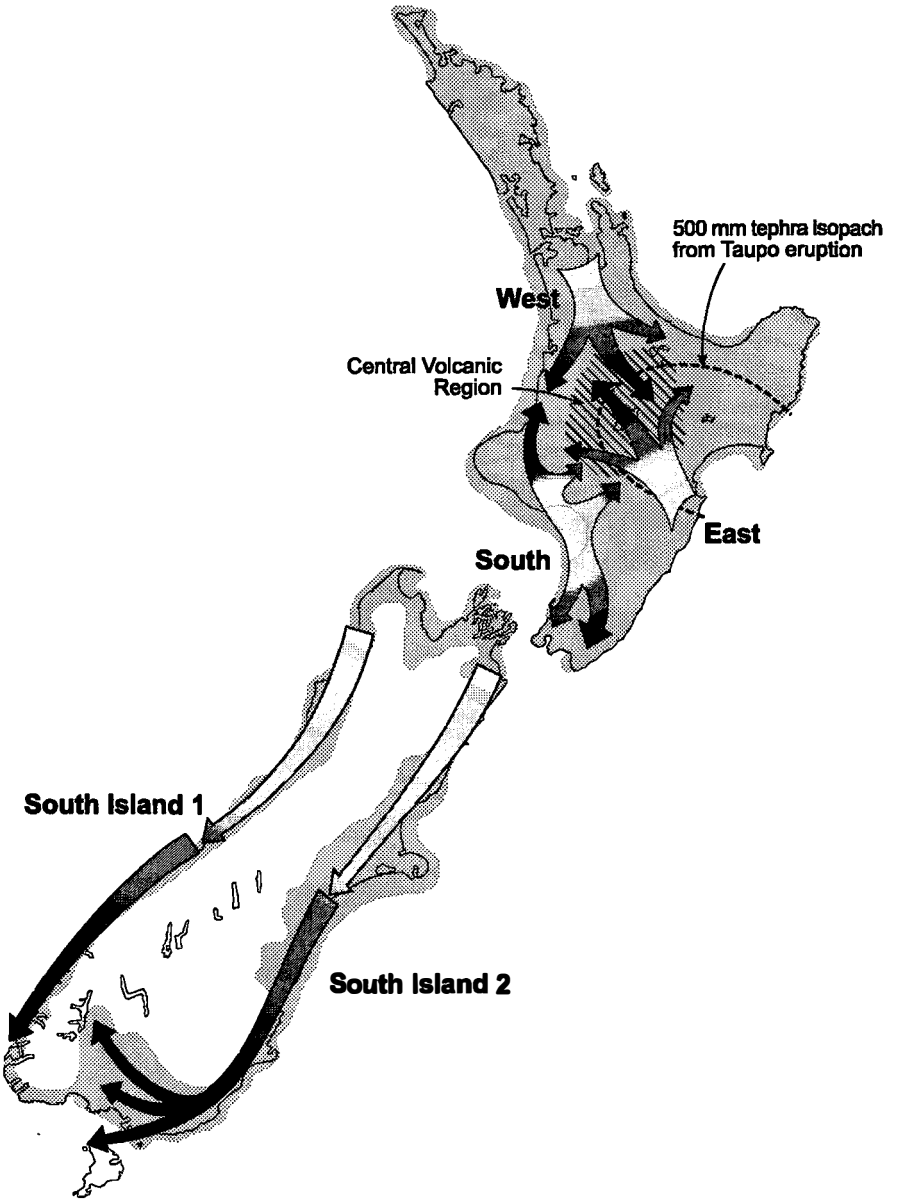


Figure 4.12 Hypothesised historical range expansion events for *M. tuberculata* overlaying post-glacial reforestation (9000 years ago) and areas affected by central North Island vulcanism. (Areas of forest and vulcanism are based on McKinnon *et al.*, 1997.)

Results from nested clade analyses indicate ancient contiguous range expansion by the southern phylogroup was followed by a fragmentation event that separated the Tararua lineage from other lineages in the phylogroup. The range expansion probably corresponds to the massive population expansion inferred from the skyline plot and mismatch distribution analysis, while the fragmentation event might correspond to the subsequent population decline inferred from the skyline plot. Expansion presumably occurred during glacial interstadials in response to the expanding *Nothofagus* forest, while decline was probably a result of contraction in *Nothofagus* forest area caused by cooling prior to the last glacial maximum. Marine inundation of central New Zealand would have restricted the area available for post-glacial re-expansion in southern North Island.

Reliabilty of estimates for the timing and magnitude of population expansion

Absence of evidence for more recent population expansion of the CSN phylogroups in response to either post-glacial warming or the most recent catastrophic volcanic eruptions probably reflects limitations of the analytic methods. Population expansions or bottlenecks following an initial expansion can not be detected by mismatch distribution analysis (Rogers 1995; Lavery *et al.* 1996), while the resolution of the skyline plots precludes discrimination between glacial and post-glacial events. The alternative explanation that population expansions were post-glacial and wrongly assigned to the glacial period because of errors in the CR substitution rates (μ), requires a five fold underestimation of μ. Although CR substitution rates are unreliable (Parsons *et al.* 1997), estimates of μ were derived from divergence dates calculated using the more reliable substitution rates for 12s and 16s rRNA, and ND2 (Lloyd, submitted).

To consider their biological feasibility, post-expansion effective female population size estimates for the CSN phylogroups were converted to estimates of total census size (N_T) for both sexes using an N_e/N_T ratio of 0.11 (the mean of 192 estimates of N_e/N_T from 102 species (Frankham 1995). Census population size estimates derived from mismatch distributions and skyline plots were 12.5 and 158 million bats respectively. During the glacial period, the CSN phylogroups ranged over an area of between 9 and 12 Mha. Thus, the population of 12.5 million bats, inferred from mismatch distributions, would have had a population density only slightly higher than

observed for the present Rangataua short-tailed bat population (1 bat ha^{-1}: unpublished data). This is within the range of population densities reported for other temperate bat species (0.1–1.1 bats ha^{-1}) (Ransome 1990). The population of 158 million short-tailed bats, inferred from skyline plots, would have had a population density of approximately 13 bats ha^{-1} and occasionally much greater, as skyline plot population estimates are harmonic means. Because there were few competitors and no mammalian predators, short-tailed bats might have achieved much higher densities before human settlement than currently. Indeed the species' wide foraging repertoire could have allowed densities to approach cumulative densities achieved by co-existing bat species. Nevertheless a density estimates of more than 13 bats ha^{-1} seem improbable as it is outside the range of cumulative densities reported for co-existing bat species (2.5–10.0 bats ha^{-1}) (Gaisler 1979; Heideman & Heaney 1989).

Phylogeography outside central and southern North Island

Information on phylogeographic structure outside of CSN was limited by the sparsity of populations. Results from mismatch distribution analysis and Fu's F_S test (see Fig. 4.5 and Table 4.3) do not support population expansion of either Northland or South Island phylogroups. These results should be viewed cautiously, as coalescent-based analyses of sequences from isolated local populations are mostly uninformative (Avise 2000). A constant sized bat population seems plausible for Northland, which had not undergone periods of extensive deforestation before human settlement, but is implausible for the South Island, where Pleistocene climate oscillations caused massive fluctuations in forest cover (McGlone *et al.* 1993).

Departure from isolation-by-distance dispersal in the southern South Island is consistent with southern populations being a product of rapid southward range expansion from refugia in northern South Island following post-glacial reforestation of the South Island 10–9 ky ago (McGlone *et al.* 1993). Vicariance does not provide an adequate alternative explanation as North and South Islands were a continuous landmass throughout most of the late Pleistocene. Separation only occurred about 12 ky ago, as a consequence of post-glacial rising sea level (Stevens *et al.* 1995). Other explanations such as ongoing high levels of dispersal between populations, lack of resolution of the markers, or balancing selection on the markers (Bossart & Prowell 1998) are not tenable given the observed differentiation among northern populations.

Range saturation also seems unlikely, as there are higher F_{ST} values among northern populations and the distribution of pairwise differences is not asymptotic.

The geographical distribution of the two South Island phylogroups (see Fig. 4.3) indicates the South Island phylogroups were probably separated by the island's axial mountain range. Post-glacial range expansion would have followed the path of initial forest expansion along coastal corridors, with SI1 in the west and SI2 in the east (see Fig. 4.12). In this model the Codfish Island population, where SI1 and SI2 are sympatric, is a remnant of a hybrid zone formed at the southern end of the axial mountain range. The alternative that SI2 is a local southern phylogroup, which persisted in the area through the last glacial maximum, is contradicted by both the severity of the glacial maximum in southern South Island and the lack of differentiation from northern populations.

Population decline

The total population of short-tailed bats in CSN is currently less than 40 000 (Lloyd 2001 and unpublished data). The most conservative estimate for the pre-human CSN population (12.5 million) indicates a decline from the pre-human carrying capacity by a factor of more than three hundred. There are no estimates for pre-human population sizes in Northland and South Island, but the small current populations (about 5000 in each area) and the more severe impacts of human settlement indicate declines in these areas may have been greater.

Decline since human arrival is a consequence of both widespread deforestation and the impact of introduced alien taxa. Forest cover has been reduced from 78% to 23% of New Zealand land area (King 1990). The most productive forest ecosystems (e.g., at low altitude, in sheltered microclimates, and on fertile soils) have all been converted to agrosystems, leaving only montane or sub-montane forests in most mainland areas. Productive areas converted to agrosystems probably included the historical refugia from recurrent climatic cooling and vulcanism. Twenty alien mammal species have established in New Zealand forests (King 1990) and affect short-tailed bats either directly by predation and competition, or indirectly by transforming the remnant forest ecosystems.

Conservation

Although the total New Zealand population of approximately 50 000 short-tailed bats is substantial compared to other endangered New Zealand taxa, the species' colonial and cryptic behaviour combine to make it uniquely vulnerable. Colonial roosts containing more than 5000 bats could allow a single event to cause a significant reduction in the overall population. The species' cryptic, nocturnal behaviour and deep-forest home range mean that in the absence of effective monitoring programme serious declines could pass unnoticed.

Historically there have been low levels of inter-population female dispersal interspersed with episodes of large-scale dispersal in response to forest expansion recurring at intervals of many thousand years. Given this history, recent forest fragmentation and contemporary low levels of dispersal are unlikely to have serious consequences for haplotypic differentiation. Ongoing low levels of dispersal indicate that observed short-term population fluctuations are probably not the result of large-scale dispersal or migration as previously suggested (Lloyd 2001). Thus, census methods can be used to assess long term population trends and monitor responses to management activity.

The core ranges of all known populations are now within forest areas afforded full legal protection which should provide refuge from further anthropogenic deforestation as long as the present conservation-oriented ethos persist. It should be noted that many of the areas have been protected for less than 30 years. Whether mainland populations can survive the impacts of naturalised alien taxa without management intervention is unclear. The species' long-term future depends on its ability to adapt to both human modified landscapes and ecological pressure from naturalised mammals. Maximising genetic diversity, and hence adaptive diversity and evolutionary potential, may therefore be crucial to the species' long-term survival. This can be achieved by increasing the number and geographic range of populations by translocations to new areas. Translocation sites should include forests managed for alien predators and competitors, and forest restored on fertile lowland sites. The phylogeographic pattern observed in this study should guide selection of propagules for translocation.

Polyphyletic and paraphyletic relationships among existing populations preclude conservation management based on evolutionary significant units, defined as reciprocal monophyletic units (Moritz 1994). Although the six phylogroups are the component

evolutionary lineages of the species, their sympatric distribution renders them an unsuitable choice for conservation management units. However, gene flow between populations is sufficiently low that all populations are functionally independent and can be considered the basic management units for conservation (Moritz 1994; Avise 2000). Using Faith's (1992) Phylogenetic Distance measure, any one of the populations in the central North Island hybrid zone contains more evolutionary diversity than all Northland and South Island populations combined. Thus, although conservation of genetically "pure" populations is usually afforded higher conservation priority than hybrid populations (Allendorf *et al.* 2001), conserving populations within this hybrid zone is the most cost-effective method for retaining diversity within short-tailed bats.

I hope that the phylogeographic pattern observed in this study will stimulate comparable studies of other widespread forest-dwelling taxa in New Zealand. Comparisons between results from different taxa should provide further insight into the region's biogeographic history and identify evolutionary distinct communities and areas containing the greatest biological (as opposed to species) diversity (Moritz & Faith 1998).

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Appendix 1:

An assessment of the probability of secondary poisoning of forest insectivores following an aerial 1080 possum control operation

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ABSTRACT

Assays for the toxin sodium monofluoroacetate (compound 1080) were undertaken on arthropods collected from toxic baits after a brushtail possum (*Trichosorus vulpecula*) control operation in *Nothofagus* forest in central North Island, New Zealand. The 1080 concentrations measured (mean 57 $\mu g\ g^{-1}$, max 130 $\mu g\ g^{-1}$) are considerably higher than those reported by other researchers who collected arthropods randomly after control operations. These data, together with published information on sensitivities to 1080, as well as diet and consumption rates, were used to calculate the median lethal doses of arthropods that have fed on 1080 baits for a number of vertebrate insectivores found in *Nothofagus* forest. The results indicate small insectivores that feed on, or close to, the ground (e.g., tomtit *Petroica macrocephala*, robin *P. australis*, hedge sparrow *Prunella modularis*, and the short-tailed bat *Mystacina tuberculata*) may be vulnerable to secondary poisoning. For instance, a tomtit will receive the median lethal dose of 1080 from 1.32 g (i.e., 14.7% of its daily food intake) of arthropods containing 57 $\mu g\ g^{-1}$ of 1080. Because of their greater sensitivity to 1080 poisoning, bats are at much greater risk; a short-tailed bat will receive the median lethal dose of 1080 from as little as 0.04 g (0.7% of its daily food intake) of arthropods containing 57 $\mu g\ g^{-1}$ of 1080.

INTRODUCTION

The brushtail possum (*Trichosurus vulpecula* Kerr) was introduced into New Zealand from Australia at the end of the nineteenth century. It is now a serious pest throughout New Zealand, having detrimental impacts both on agriculture, through spreading tuberculosis and destroying crops, and on conservation values, through the destruction of natural biological communities (Department of Conservation, 1994; Livingston, 1994). Since the 1970s the principle method for control of possum numbers in forest areas has been poisoning using aerial broadcast carrot or grain-based baits impregnated with the toxin sodium monofluoroacetate (1080). The method was observed to cause mortality in non-target wildlife from early on in its use (Harrison, 1978). Since then considerable research effort has been expended in monitoring the severity of non-target mortality. The results of this effort, and similar work overseas, have been reviewed extensively (e.g., Spurr, 1979, 1991, 1994; McIlroy, 1992; Eisler, 1995; Spurr and Powlesland, 1997).

Poisoning of non-target species may be primary, by direct consumption of baits, or secondary, by consumption of animals that have fed on toxic baits. Secondary poisoning of carnivores and scavengers, is well documented (McIlroy and Gifford, 1992; Eisler, 1995; Gillies and Pierce, 1999; Murphy *et al.*, 1999). The analogous process of secondary poisoning of insectivores mediated via arthropods has been suggested by a number of authors (Spurr, 1979; McIlroy, 1984; Hegdal *et al.*, 1986; Notman, 1989; Lloyd, 1994), but has not been verified. (The term insectivore is used to describe animals that consume any terrestrial invertebrates, not just insects.) Although mortality of many insectivorous birds has been documented following the use of 1080 both in New Zealand (e.g., Harrison, 1978; Spurr, 1991; Powlesland *et al.*, 1999), and overseas (Hegdal *et al.*, 1986; McIlroy and Gifford, 1992), it has been assumed to be by primary poisoning (Spurr, 1991).

A wide range of invertebrate groups, particularly arthropods, have been reported as feeding on baits used in 1080 operations both in New Zealand (McIntyre, 1987; Notman, 1989; Hutcheson, 1989; Eason *et al.*, 1993; Eisler, 1995) and overseas (Marsh, 1968; Eisler, 1995). Recent studies (Spurr and Drew, 1999; Sherley *et al.*, 1999; S.M. McQueen and B.D. Lloyd, *unpubl.*) provide detailed evidence of leaf litter arthropods feeding on the baits in indigenous New Zealand forests. The results of trials on the

persistence of 1080 in arthropods led Eason *et al.* (1993), and Booth and Wickstrom (1999) to conclude that the risk of secondary poisoning of insectivorous birds is negligible.

This paper presents the concentrations of 1080 in forest arthropods collected after a possum control operation. These data are used to quantify the risk of secondary poisoning of a number of forest dwelling insectivores by calculating the lethal doses of arthropods that have fed on 1080 baits as a percentage of the insectivores' daily food consumption.

METHODS

Study area

This study was undertaken within Karioi Rahui/Sanctuary, in the south-east of Rangataua Conservation Area. The area is part of a 10 000 ha continuous tract of mature old growth *Nothofagus* forest on the southern slopes of Mt. Ruapehu, central North Island, New Zealand (39°26' S 175°32' E, 700–750 m a.s.l.). Forest canopy in the study area is dominated by *Nothofagus fusca* (Hook. f.), *N. menziesii* (Hook. f.), and *Dacrydium cupressinum* (Lamb.), but includes a variety of other podocarps and hardwoods.

Field methods

The work was undertaken during a large-scale possum control operation, by aerial broadcast of toxic 1080 baits on 30 August 1997. The toxic baits were Wanganui No. 7 pollard (i.e., grain based) baits from Pest Control Services, Wanganui, with a 0.15% toxic loading of 1080. The baits were dyed green and cinnamon lured. Individual bait mass ranged from 4–6 g. Baits were broadcast over a total of 2500 ha, with sowing rates of 5 kg/ha for 1200 ha and 3 kg ha^{-1} for 1300 ha. The study was undertaken in the centre of the area sown at 5 kg ha^{-1}.

Four parallel 150 m long transects were marked at right angles to an access track, using hip chain and compass. The transects were in pairs spaced more than 100 m apart with the individual transects in each pair 21 m apart. On the day of the aerial broadcast individual toxic baits were placed at 3 m intervals along all four transects (i.e., a total of 200 baits) to approximate the bait spacing for a 5 kg ha^{-1} sowing rate. The

baits were taken from the batch used for the large-scale possum control operation. Any aerial broadcast baits seen on, or close to, transects were removed to maintain the local bait density close to 5 kg ha^{-1}. A small aluminium reflector (10 x 30 mm) was pinned to the ground, close to each bait. All baits were inspected during the two hours after dark on the first four nights after the aerial operation and then on the sixth and eighth night after the operation. A "Petzl" head-lamp fitted with a standard 0.22 amp light bulb was used to illuminate the baits during inspection. Each arthropod more than 4 mm long found on a bait was placed in a dry empty vial. The arthropods were identified using a reference collection, inspected for any bait particles adhering to them and then placed in a -20°C freezer within three hours of collection.

Arthropods were also collected from non-toxic baits to determine average body mass of taxa frequently found feeding on baits. These arthropods were collected in the study area some weeks after the aerial broadcast. They were stored in a -20°C freezer before being weighed on a Mettler bench balance with a 0.01 g resolution. Larger individuals were weighed separately, whereas individuals of smaller species were pooled and weighed together.

Twenty pitfall traps were placed at 15 m intervals along a transect line through the study area. Each trap consisted of a plastic cup (60 mm diameter and 70 mm deep) set into the ground and covered with a 150 mm diameter plastic plate raised *c.* 30 mm above the cup mouth. To prevent 1080 leaching out of the arthropods, the pitfall traps did not contain collection fluid. Arthropods were collected from the pitfall traps daily for the ten days after the aerial broadcast operation, identified using a reference collection, and then stored in a -20 °C freezer.

A random sample of ten baits was taken from the batch of baits used for the control operation before broadcast. Further samples of ten baits were collected at random from within the operational area 5, 10, 17 and 32 days after broadcast. The baits were placed in individual plastic bags and stored in a -20 °C freezer.

Rainfall was measured using a tipping bucket rain gauge (RGD-01 from Monitor Sensors) with values logged at ten-minute intervals on a Datataker 500 datalogger. The rain gauge was sited in open farmland approximately 9 km west of the study area. The study-area and the site with the rain gauge have similar aspects and are at the same

altitude. Rainfall data from a number of sites around Mt. Ruapehu (Atkinson, 1981) indicate that the annual rainfall in the study area may be 15% lower than at the rain gauge.

Sodium monofluoroacetate (1080) concentrations

Concentrations of 1080 in baits and arthropod samples were measured by gas chromatography with flame ionisation detection (Hoogenboom and Rammell, 1987). The baits were assayed individually, but arthropods were pooled to provide sufficient material for assay. The limits of detection for the method were 5 $\mu g\ g^{-1}$ for the baits and 0.1 $\mu g\ g^{-1}$ for the arthropod samples. The precision of the assays varied according to the 1080 concentrations present, but averaged 5% for baits and 15% for arthropod samples. Recovery rates were 100% for baits and 81% for arthropod samples.

The weighted mean 1080 concentration of the arthropod samples ($\bar{c}_w$) was calculated as

$$\bar{c}_w = \sum_{i=1}^{n}(m_i c_i) \Big/ \sum_{i=1}^{n}(m_i)$$

where n is the number of samples, m_i is the mass of sample i, and c_i is the 1080 concentration of sample i. The standard error of the weighted mean was calculated as $\sqrt{s^2/n}$, where s^2 is the variance calculated as

$$\sum m_i(c_i - \bar{c}_w) \Big/ (n-1).$$

LD_{50} estimates of 1080 contaminated arthropods for forest insectivores

Seven species of vertebrate forest insectivores were considered vulnerable to secondary poisoning because they forage on, or close to, the ground, and consume a wide variety of arthropod species, including some that feed on baits (Daniel, 1976; Moeed and Fitzgerald, 1982; Arkins, 1996; Heather and Robertson, 1996). The seven species are: robin (*Petroica australis* Sparrman), tomtit (*P. macrocephala* Gmelin), hedge sparrow (*Prunella modularis* Hartert), kingfisher (*Halcyon sancta* Gmelin), blackbird (*Turdus merula* L.), morepork (*Ninox novaeseelandiae* Lesson), and the short-tailed bat (*Mystacina tuberculata* Gray). Published median lethal dose (LD_{50}) estimates for 1080 were used to obtain best LD_{50} estimates for the seven species.

McIlroy (1984) provides an LD_{50} estimate of 9.5 μg g^{-1} for the blackbird. There are no published LD_{50} estimates for the other six species, therefore mean estimates for related groups were used as the best available estimates. The value 6.85 μg g^{-1} was used for the five untested bird species. This is the mean LD_{50} for 31 species of non-Australian birds (579 individuals) from McIlroy (1986). LD_{50} values for Australian species were not included as some species have elevated tolerances to 1080 as a result of adaptation to foraging on 1080 bearing plants (McIlroy, 1986; Twigg *et al.*, 1988). Two values were used as estimates of the LD_{50} for the short-tailed bat. One value is 0.15 μg g^{-1}, the only published LD_{50} for any microbat, the American big brown bat (*Eptesicus fuscus* Beauvois) (Timm, 1983). The other value is 0.37 μg g^{-1}, the weighted mean LD_{50} for three groups of mammals comprising 50 species (1297 individuals) obtained from McIlroy (1986). The three groups are: eutherian carnivores (0.19 μg g^{-1}, 13 species, 130 individuals), eutherian herbivores (0.40 μg g^{-1}, 7 species, 201 individuals) and non-Australian rodents (0.44 μg g^{-1}, 30 species, 966 individuals). The mass of arthropods containing LD_{50}s of 1080 was calculated for each of the seven vulnerable species. LD_{50} estimates of 1080-contaminated arthropods are for three different concentrations of 1080 that encompass a range of the reported values for 1080-contaminated arthropods after aerial possum control operations. The three concentrations are the mean and maximum concentrations found in this study (57 μg g^{-1} and 130 μg g^{-1}, respectively) and the mean 1080 concentration (12 μg g^{-1}) in tree weta collected two days after a 1080 operation (Eason *et al.*, 1993).

Daily food consumption was calculated for each of the seven species as FMR/ME (g per day), where FMR is field metabolic rate (kilojoules per day), and ME is the metabolisable energy in food (kilojoules per gram of dry matter) (Nagy, 1987). FMR estimates were calculated using the allometric equation $FMR = am^b$ (Nagy, 1987), where m is body mass (g), and a and b are parameter estimates. Values for the parameter estimates were $a = 8.88$ and $b = 0.749$ for passerine birds (Nagy, 1987), and $a = 4.63$ and $b = 0.762$ for eutherian mammals (not using torpor) (Nagy, 1994). Body mass estimates for the bird species are from Heather and Robertson (1996) and the body mass estimate for the short-tailed bat is from the authors' personal observations. ME estimates for arthropods are 18.7 kJ g^{-1}, when consumed by mammals, and 18.0 kJ g^{-1}, when consumed by birds (Nagy, 1987). The water content estimate for adult arthropods

(67%) is the average of published values for ten species (Redford and Dorea, 1984; Barker *et al.*, 1998). The LD_{50}s of arthropods containing 1080 were then expressed as percentages of the estimated daily food consumption for each species.

RESULTS

Arthropod collections and 1080 concentrations

Four of the 200 toxic baits on the transects went missing on the first two nights, presumably having been eaten by possums or rodents. The rest of the baits remained in place and intact until the end of the eight-day monitoring period. Seventy individual arthropods, belonging to 20 taxa, were collected from toxic baits, and 28 individuals,

Plate 5.1 Cave weta, *Gymnoplectra tuarti*, feeding on pollard bait

belonging to 9 taxa, were collected in pitfall traps (Table 5.1). The compositions of the arthropod collections taken from baits and pitfall traps were significantly different ($\chi^2 = 39.16$, d.f. = 3, $P < 0.001$). Samples from the baits contained mostly Insecta, while samples from pitfall traps contained mostly Malacostraca (Table 5.2).

The weighted mean 1080 concentration of the 8 samples of arthropods found feeding on toxic baits was 56.8 μg g^{-1} (S.E. = 25.60 μg g^{-1}), the maximum sample concentration 130 μg g^{-1}, and the minimum sample concentration 14 μg g^{-1} (Table 5.3). Concentrations of 1080 in arthropod collections from the toxic baits did not consistently increase or decrease during the 8-day monitoring period (Table 5.3). The peak value of 130 μg g^{-1}, for two cave weta (*Gymnoplectron tuarti* Richards) collected on the fourth night, is likely to be the result of the small sample size rather than a real peak in concentrations of 1080 in arthropods feeding on baits at that time.

LD_{50} estimates of 1080 contaminated arthropods for the six avian species range from 6.4% to 163.8 % of their daily food requirements (Table 5.4). The short-tailed bat could receive an LD_{50} from as little as 0.3% of its daily food intake. A sample of four spiders (two collected from toxic baits, and two from pitfalls traps) had a 1080 concentration of 14 μg g^{-1}. The sample of 26 arthropods (mass 1.5 g) collected from pitfall traps (see Table 5.1) in the operational area during the ten days after the 1080 operation had a 1080 concentration of 0.8 μg g^{-1}.

1080 concentrations in baits

Mean concentrations of 1080 in the bait samples ($n = 10$) declined slowly during the first 17 days after the baits were broadcast (Figure 5.1). An increase in the cumulative rainfall during the period 17–32 days after broadcast, accelerated the decline in mean 1080 concentration.

Table 5.1 The numbers of arthropods collected from toxic baits and pitfall traps during a 1080 operation in Rangataua Forest, August–September 1997, and the mean body masses of some forest arthropods found feeding on non-toxic baits

Taxonomic group	Lowest taxonomic unit	No. of individuals		Body mass of individuals (g)
		Baits	Pitfall	
Cockroach (Blattaria)	Blattaria	2	0	
Weta (Orthoptera)	*Hemideina thoracica* White	1	0	4.7
	Gymnoplectron tuarti Richards	9	0	1.96
	Rhaphidophoridae	7	0	
	Neonotus sp.	1	0	
	Zealosandrus gracilis Salmon	2	0	
	Weta sp.	6	1	
Beetles & weevils (Coleoptera)	*Ctenognathus adamsi* Broun	2	1	
	Saphobius squamulosa Broun	11	0	0.01
	"weevil"	1	0	
Spiders (Araneae)	Araneae	2	2	
Harvestmen (Phalangidae)	*Nuncia* sp.	6	2	0.05
	Pristobunus sp.	5	0	
	Soerensenella rotara Phillips & Grimmet	1	0	0.06
	Pantopsalis sp.	1	0	
Millipedes (Diplopoda)	*Dimerogonus* sp.	3	0	0.59
	Icosidesmus sp.	1	2	0.15
	"grey millipedes"	0	2	
Centipedes (Chilopoda)	"orange centipedes"	4	1	
Slaters (Isopoda)	"slaters"	0	3	
Amphipod (Amphipoda)	Amphipods	5	14	
Total number of arthropods		70	28	
Total mass of arthropods		33.1 g	1.5 g	

Table 5.2 A comparison of the composition of arthropod collections from toxic baits and pitfall traps

Taxonomic Group	Baits	Pitfalls
Insecta (Insects)	42 (60%)	2 (7%)
Arachnida (Spiders & harvestmen)	15 (21%)	4 (14%)
Myriapoda (Millipedes & centipedes)	8 (11%)	5 (18%)
Malacostraca (Slaters & amphipods)	5 (7%)	17 (61%)
Total	70	28

Table 5.3 The 1080 concentrations of the eight samples of arthropods found feeding on baits. Details of the composition of the three collections of smaller arthropods (Collections numbers 1–3) are in Addendum 1.

Night after broadcast	Arthropods	*n*	Sample mass (g)	1080 conc. ($\mu g\ g^{-1}$)
1	*Hemideina thoracica*	1	4.7	66
1	*Gymnoplectron tuarti*	1	4.2	32
1	Collection No. 1	15	2.6	22
2 & 3	Collection No. 2	24	2.4	46
2 & 3	*G. tuarti*	3	3.4	53
4	*G. tuarti*	2	4.4	130
6	*G. tuarti*	3	7.3	60
6 & 8	Collection No. 3	19	3.6	14
	All Samples Combined	68	32.6	56.8

Table 5.4 Estimated amounts of 1080-contaminated arthropods required to provide a LD_{50} of 1080 for seven forest-dwelling insectivorous species at three different concentrations of 1080 contamination (12 μg g^{-1}, 57 μg g^{-1}, and 130 μg g^{-1})

	Body mass (g)	Food intake (g day^{-1})	LD_{50} of 1080[1] (μg g^{-1})	LD_{50} dose of arthropods g (%)		
				12 μg g^{-1}	57 μg g^{-1}	130 μg g^{-1}
Tomtit	11	9.01	6.85	6.28 (69.7)	1.32 (14.7)	0.58 (6.4)
Hedge sparrow	21	14.62	6.85	11.99 (82.0)	2.52 (17.3)	1.11 (7.6)
Robin	35	21.44	6.85	19.98 (93.2)	4.21 (19.6)	1.84 (8.6)
Kingfisher	65	34.08	6.85	37.10 (108.9)	7.81 (22.9)	3.43 (10.0)
Blackbird	90	43.49	9.5	71.25 (163.8)	15.0 (34.5)	6.58 (15.1)
Morepork	175	71.56	6.85	99.90 (139.6)	21.0 (29.4)	9.22 (12.9)
Short-tailed bat a[2]	14	5.60	0.15	0.18 (3.1)	0.04 (0.7)	0.02 (0.3)
Short-tailed bat b[3]	14	5.60	0.37	0.43 (7.7)	0.09 (1.6)	0.04 (0.7)

[1] LD_{50} estimates are given as both fresh weight (g), and % of the estimated daily food intake, in parentheses.

[2] Estimates for short-tailed bat a are based on a LD_{50} of 0.15 μg g^{-1}, the LD_{50} for the American big brown bat from Timm (1983).

[3] Estimates for short-tailed bat b are based on a LD_{50} of 0.37 μg g^{-1}, the mean LD_{50} for 50 mammal species from Mellroy (1986).

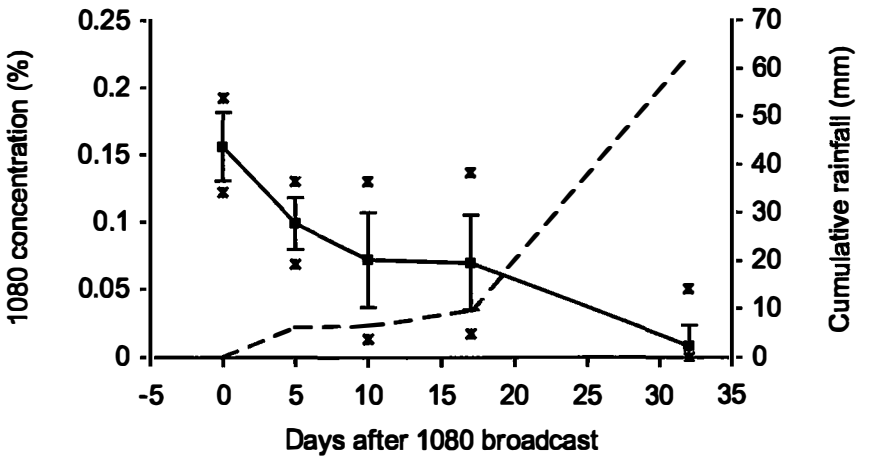

Figure 5.1 Changes in the mean percentage of 1080 in baits (n = 10) (solid line) and cumulative rainfall (dashed line) with time. Also shown for each mean 1080 concentration value are ± standard deviation (vertical line) and sample maximum and minimum values (crosses).

DISCUSSION

Although the results of this study do not prove secondary poisoning of forest insectivores occurs after aerial 1080 operations, they provide persuasive supportive evidence. Short-tailed bats are more vulnerable than birds to secondary poisoning because mammals are extremely sensitive to 1080. A short-tailed bat can consume an LD_{50} from a single *Nuncia* harvestman (mass 0.05 g) containing 130 μg g^{-1} of 1080, two *Nuncia* containing 57 μg g^{-1}, or a single *Dimerogonus* millipede (mass 0.59 g) containing 12 μg g^{-1}. The results for avian insectivores also indicate some cause for concern. The tomtit, which is the smallest of the avian species considered, is at most risk because it has the highest ratio for daily food intake to body mass. A tomtit could receive a lethal dose from a single *Dimerogonus* millipede (mass 0.59 g) containing 130 μg g^{-1} of 1080, a single average sized *Gymnoplectron tuarti* (White) weta (mass 1.96 g) containing 57 μg g^{-1} or two *Hemideina thoracica* (Richards) weta (mass 4.7 g) containing 12 μg g^{-1}. Thus, the high levels of mortality reported for tomtits after aerial 1080 operations (R.G. Powlesland, *pers. comm.*) could be caused by secondary poisoning.

During this study the risk of secondary poisoning probably remained high for a considerable period. The 1080 concentrations in arthropods collected from baits were high for the entire eight-day monitoring period and concentrations of 1080 in baits remained high for at least 17 days after application. As high concentrations of 1080 persist within arthropods for up to 4 days after ingestion of sub-lethal doses (Eason *et al.*, 1993; Booth and Wickstrom, 1999), it is probable that arthropods with moderate to high 1080 concentrations were present in the forest for at least 21 days after bait application.

With 1080-contaminated arthropods present for a 21-day period, insectivores may consume repeated sub-lethal doses of 1080. Consumption of repeated doses would be especially prevalent when a high proportion of arthropods is contaminated with low concentrations of 1080. In this situation median lethal concentrations (LC_{50}) may be a more appropriate statistic for assessing the impact of secondary poisoning than LD_{50}, which describe the impact of a single dose of toxin. Unfortunately there are relatively few LC_{50} estimates for 1080 in the literature. Repeated consumption of sub-lethal doses can have variable and sometimes adverse effects (Atzert, 1971; Eisler, 1995). They may increase an individuals tolerance to 1080, or alternatively they may accumulate to a lethal level (McIlroy, 1981). Repeated sub-lethal doses can also adversely affect reproduction, growth and behaviour (Eisler, 1995). Despite the sometimes adverse affects from repeated sub-lethal doses, 1080 is not generally considered a cumulative toxin, as it has a plasma half-life of between 1 and 14 hours in a range of mammal species (Eason, 1993; Gooneratne *et al.*, 1995). The risk of secondary poisoning following repeated sub-lethal doses would be far greater for persistent and cumulative toxins such as the second-generation anticoagulants (e.g., brodifacoum) which have tissue half-lives of months.

The mean 1080 concentration of the arthropods (56.8 $\mu g\ g^{-1}$) is higher than the mean concentrations (0–12 $\mu g\ g^{-1}$) reported in previous studies (Eason *et al.*, 1991; Pierce and Montgomery, 1992; Eason *et al.*, 1993). Although mean sample concentrations were low, individual sample concentrations as high as 46 $\mu g\ g^{-1}$, which approaches the sample mean in this study, were reported by Eason *et al.* (1993).

The difference between the concentrations of 1080 in arthropods collected from toxic baits in this study and the values reported in previous studies has probably arisen because in previous studies the arthropods were collected randomly (C.T. Eason, *pers.*

comm.), not from toxic baits. Only one of the mean concentrations reported in previous studies was higher than 2.1 $\mu g\ g^{-1}$. Thus, most sample mean concentrations are of a similar order to the mean 1080 concentration of arthropods collected from pitfall traps (0.8 $\mu g\ g^{-1}$) during this study. There was a significant difference between the species composition of samples of arthropods feeding on baits and collected in pitfall traps in this study, indicating many of the taxa collected from pitfall traps may never feed on baits. This is probably also the case for random collections. Among taxa that feed on baits many of the individuals collected may not have fed on baits, and the 1080 concentrations of those that have fed on baits may have declined between feeding and collection. The probability of random capture of individuals that have consumed high doses of 1080 may also be reduced, because these individuals are likely to die from 1080 poisoning before capture.

The risk of insectivores being affected by secondary poisoning will be strongly influenced by the behaviour of arthropods after they ingest 1080. There are three LD_{50} estimates available for arthropods, 91 $\mu g\ g^{-1}$ for tree weta (*Hemideina crassidens* Blanchard) (M. Wickstrom, *pers. comm.*), 42 $\mu g\ g^{-1}$ for striated ants (*Huberia striata* Smith) (Booth and Wickstrom, 1999) and 9 $\mu g\ g^{-1}$ for honey-bees (*Apis melifera* L.). [The LD_{50} estimate for honey-bees was calculated from the LD_{50} of 0.8 μg per honey-bee, reported by Palmer-Jones (1958), using a mean mass of 90 mg for honey-bees (n = 10) weighed by the author.] The three LD_{50} estimates for arthropods encompass the mean 1080 concentrations in arthropods collected from baits during this study. Thus, some of these arthropods may have ingested only sub-lethal doses of 1080, while others may have ingested lethal doses. Those arthropods that have consumed lethal doses may take between 1.5 h (Goodwin and Ten Houten, 1991) and 14 days (Hutcheson, 1989) to die, during this time they are available for predation by insectivores. The large variation in estimates of the time between ingestion and death for arthropods may be a consequence of faulty estimates, but it is consistent with the pattern in vertebrates. For most vertebrates the time between ingestion and death is between 1 h and 1 day (Eisler, 1995), but delays as long a 7 days occur in some species (McIlroy, 1981). Significant concentrations of 1080 persist in arthropods that have consumed sub-lethal doses for up to 4 days after ingestion (Eason *et al.*, 1993; Booth and Wickstrom, 1999). Arthropods that have consumed 1080 may be prone to predation by insectivores as their normal

behaviour patterns can be disrupted as a consequence of 1080 intoxication (McIntyre, 1987; Hutcheson, 1989; Goodwin and Ten Houten, 1991).

Errors in estimating lethal consumption levels may arise from a number of sources, including errors in the estimates of 1080 concentrations in the arthropods, errors in the original LD_{50} estimates, and errors in estimates of the daily food intake. The mean 1080 concentration in arthropods collected from the baits is likely to underestimate the actual concentrations for two reasons. Firstly, there will have been a decline in the 1080 during the delay between collection and freezing. Secondly, the arthropods were collected from the baits at random times during their feeding bouts, thus, on average they will have been collected half way through their feeding bouts.

Although the LD_{50} of a species for 1080 cannot be accurately predicted from data obtained on other closely related species (McIlroy, 1986; Calver *et al.*, 1989), the best available estimates of LD_{50} for species which have not been tested is the mean LD_{50} for related taxa (McIlroy, 1986). This may be true for most species but can be misleading for individual species, as some species have very different LD_{50} values to related taxa. For instance, the red-browed firetail *Neochmia temporalis* (Latham) has an LD_{50} of 0.63 $\mu g\ g^{-1}$, less than one tenth of the mean LD_{50} for other bird species (McIlroy, 1984). The estimates of lethal consumption levels in Table 5.4 are based on LD_{50} values typical of the species' wider taxonomic group, atypically low values for a species could increase its vulnerability considerably.

Published LD_{50} estimates are obtained using orally administered solutions of 1080 (McIlroy, 1981). Although oral toxicity of 1080 is independent of all the carriers tested (Atzert, 1971), 1080 in contaminated arthropods may not all be available for absorption. If this is the case LD_{50} of 1080-contaminated arthropods calculated from published LD_{50} estimates may underestimate the true values.

Nagy and Obst (1991) and Nagy (1994) concluded that the 95% confidence interval for estimates of daily food requirements obtained using the allometric equations is $\pm$ 40% of the predicted value. Within-species variation, resulting from seasonal and geographic variation in activity, is large enough to encompass most between-species variation. Estimates of daily food requirements are likely to underestimate the actual daily food requirements, as they do not include growth needs or periods of hyperphagy, such as pre-ovulation in birds or pre- and post-hibernation in bats. Direct observations confirm the estimated daily food intake for short-tailed bats. Captive short-tailed bats regularly consume 5–7 g of insects in a night (i.e., 36–50% of pre-feeding body mass)

as well as honey water (Daniel, 1979; B. Blanchard, *pers. comm.*; B.D. Lloyd and S.M. McQueen, *unpubl.*). This is close to the estimated daily food intake of 5.6 g (i.e., 40% of body mass). Further support for the estimated figures comes from Gould (1955) and Ransom (1990), who report that free-flying insectivorous microbats consumed 18–41% of their own pre-feeding weight within 30–70 min of emergence from their day roosts.

None of the seven species considered are exclusively insectivorous. Thus, the daily consumption of arthropods may be over-estimated when non-arthropod food-types are also taken. Morepork and kingfisher feed on small vertebrates (Heather and Robertson, 1996). Tomtit, robin, hedge sparrow and blackbird all supplement their arthropod diet with small fruits when available (Moeed and Fitzgerald, 1982; Heather and Robertson, 1996) and the short-tailed bat eats fruit, pollen and nectar when available (Daniel, 1979; Arkins, 1996). Generally there are few, or no, fruit or flowers available in late winter when possum control operations are commonly undertaken, therefore over-estimation of daily consumption of arthropod at this time of the year may be minor.

Continuous video surveillance of baits, undertaken in a concurrent study by the authors, showed that almost all bait consumption by arthropods was at night. Thus nocturnal insectivores (i.e., short-tailed bats and morepork) are at greater risk of secondary poisoning than diurnal insectivores. Nevertheless, diurnal insectivores may consume nocturnal arthropods that have fed on baits. They take nocturnal arthropods during the day by gleaning them from crevices and under bark and leaf litter. Some diurnal insectivores (e.g., robin, tomtit and blackbird) engage in crepuscular foraging (B.D. Lloyd, *pers. obs.*) when nocturnal arthropods are active. In addition, nocturnal arthropods that have ingested 1080 may become active in daytime as a result of 1080 intoxication (Hutcheson, 1989).

Proving that small free-living forest insectivores have succumbed to secondary poisoning following a 1080 operation will be difficult because insectivore gut retention times are generally less than the time between ingestion of 1080 and death. Gut-retention times for two avian insectivores [starlings (*Sturnus vulgaris* L.) and house wren (*Troglodytes aedon* L.)] are between 10 min and 2 h, with mean retention times of *c.* 50 min (Levey and Karasov, 1989, 1992, 1994; Dykstra and Karasov, 1992), whereas the time from ingestion of 1080 to death is 1.4–262 h (335 individuals, 33 species) (McIlroy, 1984). Thus, when individuals that have died of secondary poisoning are autopsied the 1080-contaminated arthropods will no longer be in the gut.

It should not be assumed that populations of insectivorous species are safe from poisoning during aerial 1080 operations, nor should it be assumed that any mortality of insectivores observed after 1080 operations is a result of primary poisoning caused by consumption of bait fragments. Where vulnerable populations of insectivores are present other methods of possum control such as trapping or the use of cyanide in bait stations should be considered.

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Addendum 1 The composition of pooled collections sent for 1080 assay

Taxonomic group	Lowest taxonomic unit	Collection No.		
		1	2	3
Cockroach (Blattaria)	Blattaria	1	1	0
Weta (Orthoptera)	Rhaphidophoridae	3	0	4
	Neonotus sp.	1	0	0
	Zealosandrus gracilis	1	0	1
	Weta sp.	1	4	1
Beetles & weevils (Coleoptera)	*Ctenognathus adamsi*	0	2	0
	Saphobius squamulosa	1	5	5
	"weevil"	1	0	0
Harvestmen (Phalangidae)	*Nuncia* sp.	4	1	1
	Pristobunus sp.	1	1	3
	Soerensenella rotara	0	1	0
	Pantopsalis sp.	0	1	0
Millipedes (Diplopoda)	*Dimerogonus* sp.	0	1	2
	Icosidesmus sp.	0	1	0
Centipedes (Chilopoda)	"orange centipedes"	1	2	1
Amphipod (Amphipoda)	Amphipods	0	4	1

Appendix 2:

Measuring mortality in short-tailed bats (*Mystacina tuberculata*) as they return from foraging after an aerial 1080 possum control operation

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ABSTRACT

Lesser short-tailed bats (*Mystacina tuberculata*) feed on arthropod taxa known to consume 1080 baits. Thus, they may be vulnerable to secondary poisoning after control operations for brushtail possum (*Trichosurus vulpecula*) using aerial broadcast 1080 baits. Short-tailed bat mortality was monitored during 11 days after 1080 baits were broadcast over their winter foraging area. Monitoring involved catching a sample of 269 bats as they arrived at a roost after foraging, then holding them in captivity for 48 hours. None of the captured bats displayed any symptoms of 1080 poisoning. Power analysis indicates that there was a ≥ 0.95 probability of detecting mortality when the actual mortality rate was above 11.1 deaths per thousand foraging flights. Uncertainties in assumptions about the bats' behaviour mean that the overall population mortality corresponding to this minimum detectable mortality rate may range from 5.4 to 28.4%, with a best estimate of 14.4%. Although it can be concluded that this 1080 operation probably did not cause major mortality of short-tailed bats, several replicate trials are required before a generalised conclusion can justifiably be drawn about the fate of short-tailed bats following aerial 1080 operations. More information about short-tailed bat population demography is required to assess the impact of 1080 operations on population viability.

INTRODUCTION

The lesser short-tailed bat (*Mystacina tuberculata*) is endemic to New Zealand and is the sole extant species of the family Mystacinidae. The species is ranked as a Category A threatened species, i.e., having the highest conservation priority in New Zealand (Molloy and Davis, 1994). Populations of lesser short-tailed bats occur in many of the extensive areas (i.e., >10 000 ha) of unlogged old-growth forest remaining on the North Island mainland (Lloyd, 2001). Only two populations are known on the South Island mainland (Lloyd, 2001), one in north-west Nelson, the other in Fiordland.

Since the 1970s there have been many operations to control brushtail possum (*Trichosurus vulpecula*), an introduced pest species, in forests throughout New Zealand. The principal control method is by aerial broadcast of carrot or grain-based baits impregnated with the toxin sodium monofluoroacetate, 1080 (Department of Conservation, 1994; Livingston, 1994). Daniel (1990) suggests that, because short-tailed bats are omnivorous and terrestrial, broadcast toxic baits may be hazardous to the species. However, the results of both bait acceptance trials with captive short-tailed bats, and a trial in which fluorescent dyed non-toxic baits were broadcast throughout an area inhabited by short-tailed bats, indicate that the short-tailed bats are unlikely to consume carrot or grain-based toxic baits (Lloyd, 1994). We measured concentrations of 1080 in arthropods collected from 1080 baits after a possum control operation (Lloyd and McQueen, 2000). The results of this study indicate that a short-tailed bat can receive a median lethal dose (LD_{50}) of 1080 from less than 0.7% of its daily food intake of arthropods that have fed on 1080 baits. Thus, short-tailed bats may be vulnerable to secondary poisoning via arthropods because they feed on many of the arthropod taxa (Lloyd, 2001) that feed on baits (Sherley *et al* 1999; S.M. McQueen and B.D. Lloyd, *unpubl.*).

Short-tailed bat's behaviour makes it difficult to measure 1080-induced mortality following aerial broadcast of 1080. During late winter, when most possum control operations are undertaken, their activity is variable, with periods of torpor lasting for up to 10 days interspersed with brief bursts of activity (Lloyd, 2001). While in torpor the bats are inaccessible, roosting deep within crevices in tree trunks. Thus, it is impossible to measure changes in the population level at the time of the 1080 operation.

Population levels were monitored during the summers before and after the 1080 operation. Treatment and non-treatment areas could not be established because individual bats are extremely mobile and there is no geographic partitioning of the population (B.D. Lloyd, *unpubl.*). The results of monitoring changes in summer population levels (to be published) can only provide weak inference about the levels of 1080 induced mortality. Monitoring radio-tagged bats was not attempted as experience with radio-tracking short-tailed bats indicates that the method could not provide conclusive evidence on the effect of 1080 operations, as poisoned bats are likely to die inside tree cavities where they would be inaccessible. When a radio-tag remains stationary inside a tree there is no way of telling what has happened: the radio-tag may have dropped off, the radio-tagged bat might be in torpor, or the bat might have died from poisoning or another cause.

This paper describes an attempt to measure the rate of 1080-induced mortality in a short-tailed bat population by monitoring the survival of samples of bats caught as they returned from foraging trips during an 11 day period immediately following a 1080 operation.

METHODS

Study area and population

The study was undertaken within a 10 000 ha continuous tract of unmodified old growth *Nothofagus* forest on the southern slopes of Mt Ruapehu, central North Island, New Zealand (39°23' S, 175°30' E; 700–1200 m a.s.l.) (Fig. 6.1). The forest extends from Ohakune Mountain Road in the west, to Karioi Pine Plantation in the east. It is bordered by pine plantations and farmland along southern lower edge and sub-alpine areas of Tongariro National Park in the north, and encompasses Rangataua Conservation Area and part of Tongariro National Park.

The forest is dominated by southern beeches *Nothofagus fusca* and *N. menziesii* throughout most of the area (Atkinson, 1981). At lower altitudes a variety of hardwoods and podocarps, especially rimu, *Dacrydium cupressinum*, are also present. At higher altitudes (> 1100 m a.s.l.), and on poorly drained soils in the west, the *N. fusca* and *N. menziesii* association gives way to a monotypic forest of *N. solandri* var. *cliffortioides*.

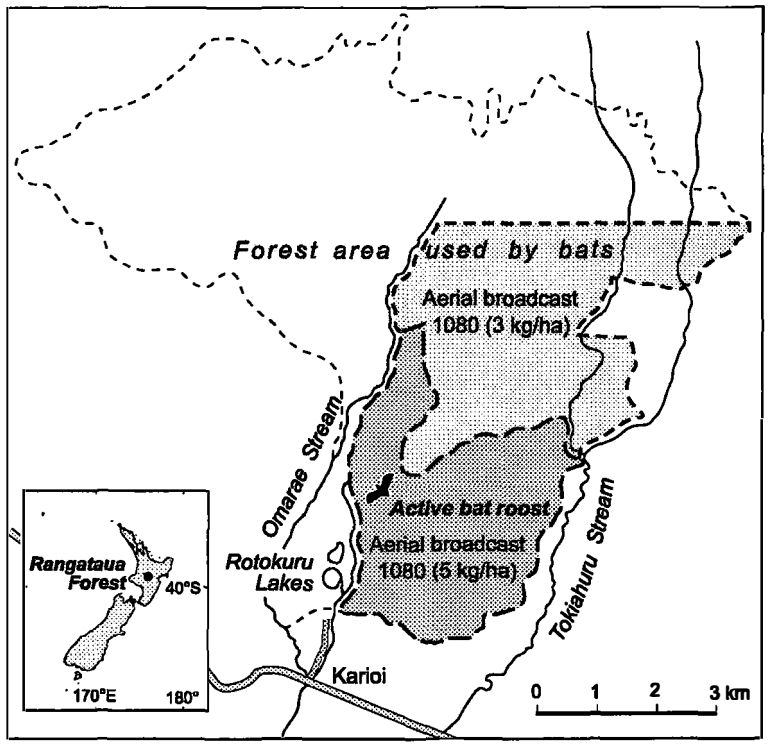

Figure 6.1 Map of the study area, showing the extent of the aerial 1080 operation

The results of simultaneous video counts of departures from all active colonial roosts during the summers of 1995–1999 indicate that a population of 6000–7000 short-tailed bats was resident in the area (Lloyd, 2001). Individual bats range widely throughout the entire forest area and there is no indication of any geographic or social partitioning within the population. In summer their activity extends into high-altitude montane forest, but during winter and early spring (i.e., June to September inclusive), when this study was undertaken, activity is restricted to low-altitude areas (< 900 m a.s.l.).

Field methods

The work was undertaken during a large-scale possum control operation that involved aerial broadcast of 1080 baits on 30 August 1997 in the south-east of Rangataua Conservation Area. Toxic baits were broadcast over 2500 ha, encompassing almost the entire winter range of the bat population. Bait sowing rates were 5 kg ha^{-1} for 1200 ha of lower altitude forest (< 900 m a.s.l.) and 3 kg ha^{-1} for the remainder (Fig. 6.1). The baits were Wanganui No. 7 pollard (i.e., grain based) baits from Pest Control Services,

Wanganui. The baits had a 0.15% toxic loading of 1080 and were cinnamon-lured and dyed green. Individual bait mass ranged from 4–6 g.

During the month before the poison operation, we identified sites within the proposed operational area where large numbers of bats could be caught easily (e.g., major flight paths or active colonial roosts). Areas with high levels of bat activity were sought by widespread survey with automatic bat monitoring systems (ABMS) (O'Donnell and Sedgeley, 1994). These systems can be left in place for several nights and will record echolocation calls from any short-tailed bat flying within 20 m. Active colonial roosts were sought both by tracking radio-tagged bats to their daytime roosts, and by inspecting known traditional colonial roosts. Nine bats were caught, radio-tagged, released, and radiotracked to their daytime roosts. The bats were caught in mist-nets at three sites in the operational area and released with miniature radiotransmitters (Holohil BD2A) attached to their backs using contact adhesive. Two days after being radiotagged, the bats' approximate daytime locations were determined by radiotracking from a Cessna 172 aircraft. The exact roost sites were found by radiotracking on the ground later on the same day. Crevices occupied by radiotagged bats, as well as all known traditional colonial roosts in the operational area, were monitored for bat activity by inspecting the roost entrances for fresh droppings and using hand-held bat detectors to detect activity within the roost during the late afternoon. Where there was any doubt whether the site was an active colonial roost it was monitored for a number of nights with either a night-time video surveillance system or an ABMS.

A target sample size of 500 bats was chosen using the power analysis method discussed below. The bats were caught as they returned from foraging during the period after the 1080 operation when toxin was available for consumption by the bats (i.e., the exposure period). They were caught both in mist-nets close to an active colonial roost, and using a harp trap at the roost entrance. Capture efforts began in the evening 90–120 minutes after the end of civil twilight, when most bats are returning to roost after foraging. Mist-nets were placed at two sites. A single rig with three 6 m long nets was 10 m from the roost, and two rigs, each with three 12 m long nets were *c.* 100 m from the roost. The harp trap was hung in front of the roost entrance, orientated radially to the main trunk of the tree. This is the best orientation for catching short-tailed bats entering a roost, as before entering, bats usually fly across the roost entrance several times. The harp-trap was modified to make it suitable for trapping large numbers of bats at a roost entrance. The trap is smaller (catch area 1 x 1.2 m) than standard harp-traps, making it

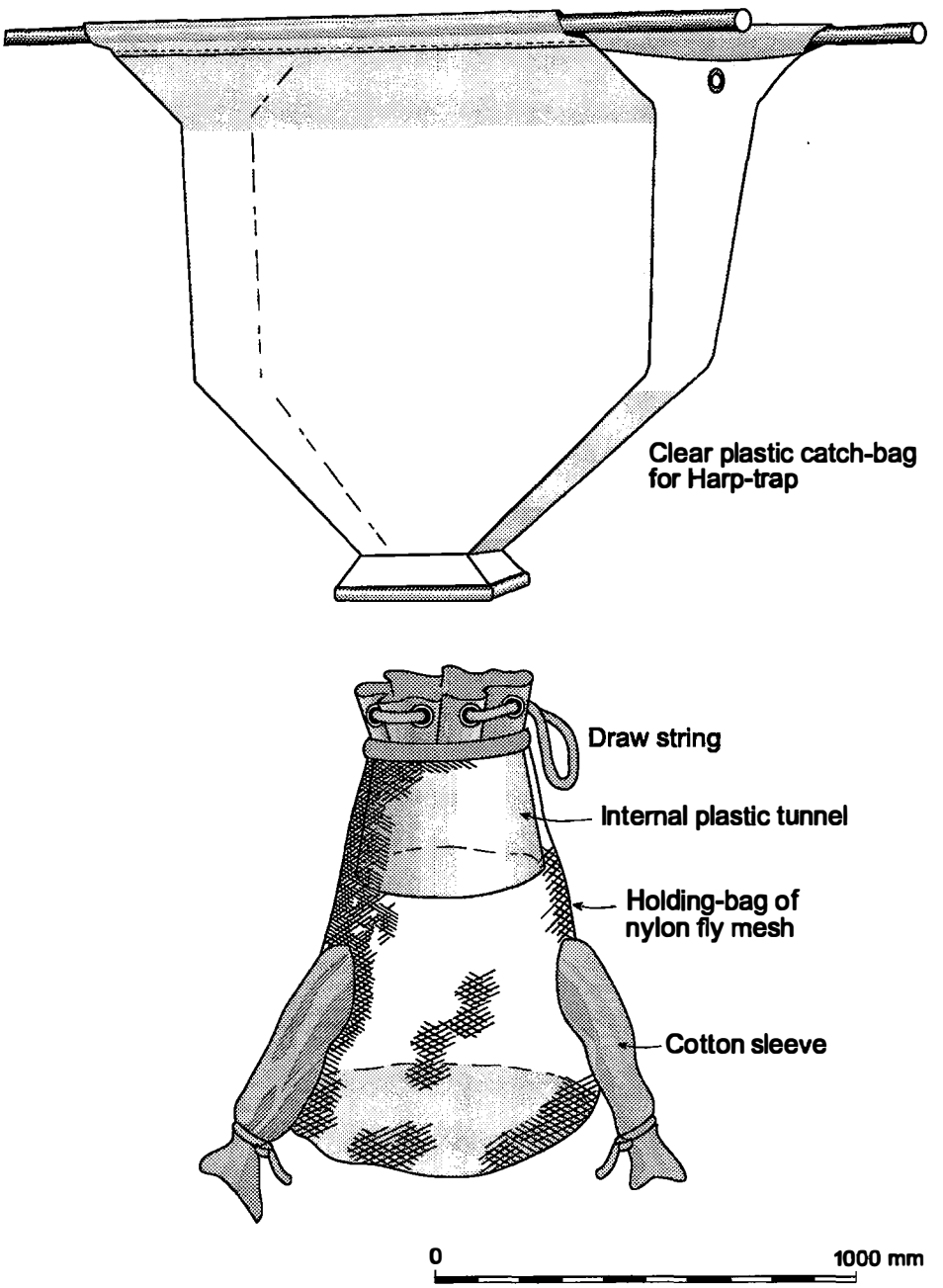

Figure 6.2 Modified catch-bag and holding-bag for a harp-trap, to allow continuous trapping at roost entrances

easier to haul, and the vertical tubes are bolted to the frame's base so that hanging does not affect the mono-filament tension. The catch-bag (Fig. 6.2) was designed to reduce handling time when removing bats. It is made from thick transparent plastic with the base sloping at 45° to a 100 mm diameter tube in the centre. Bats slide through the tube into a detachable holding-bag, which can be quickly replaced with minimal interference to trapping.

After capture, the bats were held for 48 h to monitor them for symptoms of 1080 poisoning. Two enclosures were used to keep bats caught on different nights separate. Each enclosure comprised a flight cage 1.2 m x 2.4 m and 2.15 m high with an adjoining roost area 1.2 m x 0.8 m and 2.15 m high. The walls and ceiling of the flight cages had internal linings of nylon fly-mesh to avoid damage to the bats' wings. Fresh food (mealworms, i.e., *Tenebrio* larvae, and 30% honey water solution) and water were provided each night. The bats were released into the enclosure at night, between 2 h and 5 h after capture. They were released individually so that each bat could be observed for unusual behaviour. The flight cage and the roost area were inspected on the following day for dead bats or bats behaving abnormally. On the second day the bats were recaptured, their individual details (sex, age, and reproductive status) recorded and each bat was marked with black hair-dye (Wella Bellalady) to identify the date of capture. The bats were released close to the original capture location on the evening of the second day, approximately 48 h after capture.

The incidence of 1080 poisoning in the sample of bats caught during the exposure period provides an estimate of the mortality rate per foraging flight. Overall mortality from 1080 poisoning depends on the number of foraging flights bats make during the exposure period. Overall mortality (d) was calculated from the mortality rate per foraging flight (m) using $d = 1 - (1 - m)^f$, where f is the average number of foraging flights by bats that survive the exposure period. Direct estimation of the number of foraging flights is difficult because of the bat's intermittent activity during the hibernal period (Lloyd, 2001) and was not undertaken. Indirect estimation was undertaken by determining the length of the exposure period and monitoring night-time weather conditions to identify nights suitable for foraging. The length of the exposure period was determined by measuring the concentrations of 1080 in samples of baits collected from the study area at intervals following the 1080 operation (Lloyd and McQueen, 2000). During their hibernal period bats will not forage when night-time temperatures drop to 0°C or lower, or during persistent rain. Temperatures were recorded with a temperature logger placed 1.5 m above the ground in a shady spot in the forest within the operational area. Rainfall was measured with a tipping bucket rain gauge and datalogger located in open farmland approximately 9 km west of the study area at a site with similar aspect and altitude to the operational area.

Data analysis

Power analysis was used both to determine the target sample size and as an aid to interpreting the results of the trial. Data from this trial were analysed using a one-tailed binomial test. The null hypothesis is that no bats died of 1080 poisoning, i.e., H_0: $m = 0$, where m is the 1080-induced mortality rate per foraging flight. The alternative hypothesis is H_A: $m > 0$. The α error is zero, because H_0 will never be rejected if it is true. The power of the trial (the probability of correctly rejecting H_0 when it is false) is the probability of catching one or more poisoned bat(s). This can be calculated as $Power = 1 - P(0)$, where $P(0)$ is the probability of not catching any poisoned bats. If n is the sample size, $P(0) = (1 - m)^n$ and $Power = 1 - (1 - m)^n$. Rearranged as $m = 1 - (1 - Power)^{1/n}$, the equation can be used to calculate minimum detectable mortality rates for different powers and sample sizes.

RESULTS

Eight days before the poison operation, 200 mm of snow fell. Although most of the snow-cover melted within four days, patches persisted in shady spots throughout the study area until three days after the operation. The implications of the snowfall are discussed later in this paper. The aerial broadcast was successful with good coverage throughout the operational area (John Luff, Department of Conservation, Ohakune, N.Z., *pers. comm.*).

At the time of the poison operation, large numbers of bats were using a traditional colonial roost tree within the operational area (Fig. 6.1). Monitoring with a night-time video surveillance system showed that when the roost was occupied and weather conditions were suitable around 200 bats left the roost during early evening and began returning about 1 h later. Radiotelemetry of radio-tagged bats indicated that over a period of several days the roost was visited by a much larger number of individuals. The identity of bats occupying the roost during the day varied from day to day, and on warm evenings bats using the roost during the day and others roosting elsewhere congregated at this roost after foraging. Radiotracking also demonstrated that bats using this roost dispersed over a wide area of forest, both for foraging and to use other daytime roosts. All trapping for the trial was undertaken at, or close to, this roost as bats

Plate 6.1 (Right) Short-tailed bat caught in a mist net

Plate 6.2 (Below) Short-tailed bat being inspected for unusual behaviour during the 48 h captive period

caught there are likely to be a representative sample of bats active in the operational area.

Trapping was undertaken on five of the first 11 nights after the poison operation. A total of 269 bats were caught and held in captivity for at least 48 h (Table 6.1). All 269 bats survived and were released without any unusual behaviour being noted. The estimated mortality rate is zero, with the 95% confidence interval enclosed by an upper limit of 11.06 deaths per thousand flights. The null hypothesis that no bats died of 1080 poisoning (H_0: $m = 0$) is not rejected, and the minimum detectable mortality rate is 11.07 deaths per thousand flights with $Power = 0.95$ (Table 6.2). The difference between the minimum detectable mortality rate and the 95% confidence limit arises from inaccuracies in the tables used to compute the confidence limit (Zar, 1984).

Table 6.1 Numbers of short-tailed bats caught during 11 days after the 1080 operation

Nights after the 1080 operation	Number of bats caught
1	52
2	90
4	68
9	0
11	65
Total	269

Two bats were released without details being recorded. Of the remaining 267 bats 64% were female and 36% male. Most (67%) of the females were nulliparous and were therefore young bats, probably less than three years old. Weighing individuals at capture was impracticable because of the large numbers of bats caught, but during initial handling all bats produced copious droppings which indicated they had already fed that evening. There was only one recapture: a parous female, originally caught on the second night and released on the fourth night, was recaptured on the eleventh night.

Table 6.2 Estimates of the detectable mortality rate and detectable overall mortality for actual (269) and target (500) sample sizes when the power of the trial is 0.95

Sample size	Detectable mortality rate (deaths per 1000 flights)	Detectable overall mortality (%) for different numbers of foraging flights (f)				
		$f = 5$	$f = 10$	$f = 14$	$f = 20$	$f = 30$
269	11.07	5.4	10.5	14.4	20.0	28.4
500	5.97	3.0	5.8	8.0	11.3	16.5

Levels of 1080 in the baits declined slowly from 0.156% at the time of broadcast to 0.07% 17 days after broadcast, when a period of sustained rain accelerated bait breakdown and detoxification (Lloyd and McQueen, 2000). High concentrations of 1080 can persist in arthropods for 4 days after they consume 1080 baits (Booth and Wickstrom, 1999; Eason *et al.*, 1993). The estimated exposure period to secondary consumption of 1080 via arthropods was therefore 21 nights.

There were only 14 nights during the 21 nights of the exposure period when weather conditions were suitable for foraging: activity was curtailed by low temperatures on six nights, and heavy rain on one night (Fig. 6.3). Echolocation call rates recorded on an ABMS 500 m from the active roost tree confirmed this pattern of activity for the first 12 nights of the exposure period (Fig. 6.3). Radiotelemetry shows that at this time of year (early spring) individual bats usually only leave their roosts to forage once a night. Thus, the best estimate of the average number of foraging flights by bats that survive the exposure period (f) is 14. With $f = 14$ the overall mortality level corresponding to the minimum detectable (*Power* = 0.95) mortality rate of 11.07 deaths per thousand flights is 14.4% (Table 6.2). Unfortunately, because of inadequate knowledge about the proportion of the population active and the number of foraging flights on each night, even the best estimate for f is not reliable. Values of f may range from 5 to 30, the corresponding overall mortality levels range from 5.4% to 28.4% (Table 6.2).

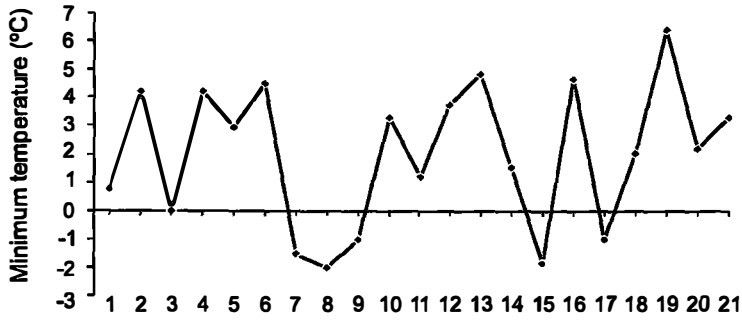

Figure 6.3a Minimum overnight forest temperatures during the 21 night exposure period following the poison operation

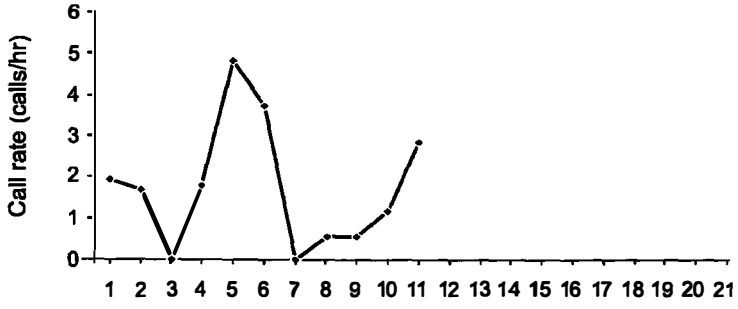

Figure 6.3b Short-tailed bat echolocation-calls per hour for the first 12 nights of the exposure period

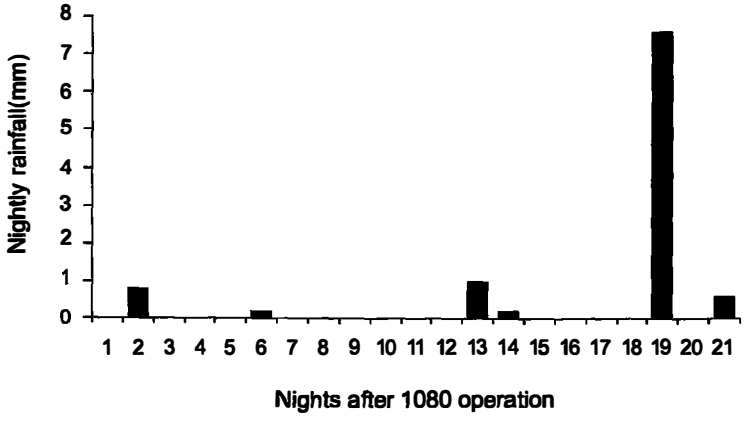

Figure 6.3c Total overnight rainfall during the 21 night exposure period following the poison operation

DISCUSSION

Failure to detect 1080 poisoning in any of the 269 bats caught during this trial is surprising. We have previously found strong, though inferential, evidence for the occurrence of secondary poisoning of short-tailed bats after aerial 1080 operations (Lloyd and McQueen, 2000). Before concluding that there was no 1080-induced mortality in the bat population in Rangataua Forest, it is necessary to consider the assumptions underlying the method as well as the power of the trial to detect 1080-induced mortality.

The most important assumption is that the latent period for 1080 (the time between ingestion of 1080 and the onset of symptoms) is long enough for bats to be captured at the roost after foraging, but less than the period the bats were held in captivity (48 h). Under this assumption bats would show symptoms or die during captivity but not during the period before capture. There are no estimates of the latent period for 1080 for any bat species. McIlroy (1986), in a comparison between major groups of animals, concludes that there is considerable inter- and intraspecific variability in latent periods for 1080. The best available predictor of short-tailed bat's latent period is the range of latent periods published for eutherian mammals, 0.4–48 h, derived from 250 individuals belonging to 22 species (McIlroy, 1981 1982a, b, 1983). These latent periods were determined using orally administered solution of 1080 in distilled water, and are likely to be shorter than the latent periods for 1080 ingested in arthropods because of faster absorption of 1080 from water than arthropod tissues. The results of video monitoring at the roost entrance and radiotelemetry indicate that individual short-tailed bats usually forage for about 1 h in the early evening before returning to roost. Thus, available information indicates that although the 48 h holding period is sufficient to identify any cases of poisoning in the captured bats, symptoms of 1080 poisoning may occasionally begin before poisoned bats return to the roost.

Failure to reject the null hypothesis, i.e., no bats died of 1080 poisoning, does not mean it should be accepted as true. In this trial the minimum detectable mortality rate (*Power* = 0.95) was 11.07 deaths per thousand flights. Thus, there is a 0.05 probability (i.e., the β error) of failing to detect any mortality when the true mortality rate was 11.07 deaths per thousand foraging flights. This corresponds to an overall mortality of about 14.4% (range 5.4–28.4%). The probability of failing to detect lower levels of mortality is greater than 0.05. If the target sample size of 500 had been achieved the magnitude of the minimum detectable mortality rate and corresponding overall mortality would have been halved (Table 6.2).

For conservation management purposes, the important question is not whether any bats died of 1080, but whether 1080 had a significant impact on the viability of the population. The results of this trial mean this question should be recast as: would a mortality level less than the minimum detectable mortality rate have a significant impact on the population's viability? The paucity of demographic data on short-tailed bats precludes rigorous treatment of this question. It seems likely that a single mortality episode of 14.4% (the best estimate for the minimum detectable overall mortality) will

not have a major impact on a viable population of short-tailed bats. Multiple mortality episodes of this magnitude resulting from repeated 1080 operations at short intervals could have consequences on the population's viability. A single episode of 28.4% mortality (the higher end of the range of overall mortality estimates) would be of concern. More information about short-tailed bat population demography is required for rigorous assessment of the impact of 1080 operations on population viability.

Although it is reasonable to conclude that this 1080 operation probably did not cause major mortality of short-tailed bats in Rangataua Forest, the trial was unreplicated. Several replicate trials would be required in a variety of circumstances before a generalised conclusion could be justifiably drawn about mortality of short-tailed bats during aerial 1080 operations. The results of this trial might be atypical as the heavy fall of snow 8 days before the poison operation reduced arthropod activity markedly for most of the trial period (S.M. McQueen and B.D. Lloyd, *unpubl.*).

The method described here provides a reliable method for measuring 1080-induced mortality rates in short-tailed bat populations when 1080 operations are undertaken during the bats' hibernal period. Larger sample sizes, and direct measurement of both the average number of foraging trips during the exposure period and the length of early-evening foraging sessions would provide a more robust basis for the method. When 1080 operations are undertaken outside of the hibernal period direct measurement of changes in the population by video counts of roost departures may be a more appropriate method.

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Addendum:

Addressing questions raised during the oral examination

THE LIMITATIONS OF USING A SINGLE MARKER

In this study, sequences from several mitochondrial genes were used. Mitochondrial DNA does not undergo recombination, therefore the entire mitochondrial genome shares the same pattern of common descent and constitutes a single locus (Wilson *et al.* 1985; Moritz & Hillis 1996). Because of selective forces operating on individual loci, as well as stochastic and demographic factors, gene trees inferred from a single locus can be expected to differ from the organismal phylogeny (Doyle 1992; Avise 1994). Evolutionary processes will leave a common signature across all neutral loci, thus, accurate inference of organismal phylogenies and population histories can best be achieved by combining information from multiple loci (Avise & Ball 1990; Slade *et al.* 1994; Moritz & Hillis 1996; Fu & Li 1999; Avise 2000; Hare 2001).

Although the need for information from multiple loci is widely acknowledged, many, or most, intraspecific phylogenetic and phylogeographic studies on animal taxa are based exclusively on mitochondrial DNA. Reliance on mitochondrial markers in studies of animal taxa reflects both the relative ease of technical procedures (Palumbi 1996), and the uniquely informative nature of data obtained from mitochondrial DNA (Ballard & Kreitman 1995; Avise 2000). Studies of mitochondrial DNA are technically considerably easier than comparable studies on nuclear DNA. The compact mitochondrial genome is robust and therefore not subject to degradation; it occurs in multiple copies in each cell, thereby providing high concentrations of template; and because it usually occurs in a haploid form, isolation of haplotypes is unnecessary (Palumbi 1996). In contrast, nuclear DNA is more prone to degradation, occurs as a single copy per cell, and usually occurs in a diploid form necessitating isolation of haplotypes. The most important feature of data obtained from mitochondrial DNA is that it is not subject to recombination. Recombination violates the fundamental assumption of phylogenetic analysis: that tree branches are non-reticulate (Schierup & Hein 2000). Mitochondrial DNA is well suited to the microevolutionary time scales for intraspecific studies (Avise 2000), because it has high rates of nucleotide substitution

and allele fixation (both within individuals and population). Maternal transmission of the mitochondrial genome means it is possible to disentangle gender specific transmission paths. Though conversely, it also means mitochondrial markers provide no information on patrilineal descent or male dispersal. Finally, the information on matrilineal history provided by mitochondrial markers is especially informative about population demography because recruitment of young depends on female reproductive success (Milligan *et al.* 1994; Avise 2000).

Although mitochondrial markers posses important advantages for intraspecific phylogenetic and phylogeographic studies, ideally information from them should be complemented by information from multiple nuclear loci (Avise 2000; Hare 2001). Shaw's (2002) observation that introgression of mitochondrial DNA can occur between related sympatric species reinforces this point. Absence of recombination means the mitochondrial genome is particularly susceptible to genetic hitchhiking accompanying selection at linked sites (Ballard & Kreitman 1995). This leads to errors in phylogenetic estimation as phylogenetic methods are generally based on the constant mutation rate neutral model of evolution (Kimura 1983).

Recent phylogeographic studies using nuclear loci indicate that technical and analytic problems that have limited their application are being overcome (Hare 2001). In future, information from cytoplasmic genes, such as animal mitochondrial DNA, may routinely be complemented by information from nuclear genes, to provide a more complete understanding of phylogeographic patterns (Hare 2001). Y-chromosome sequences probably provide the best nuclear DNA markers to complement mitochondrial markers, as they overcome some of the problems associated with nuclear DNA: isolation of haplotypes is unnecessary, and they provide direct information on patrilinear descent and male dispersal (VanlerBerghe *et al.* 1986; Avise 2000).

EVIDENCE FOR REFUGIA IN THE SOUTH ISLAND

A number of hypotheses could account for the observed distribution of genetic variation in the South Island. The hypothesis favoured in this thesis is that southern South Island populations of short-tailed bats originated by rapid range expansion from northern South Island refugia following the last glacial maximum. Alternative hypotheses are: colonisation from southern North Island refugia followed by southward range expansion in the South Island; or in-situ persistence of southern South Island

populations during the glacial maximum. In the case of in-situ persistence in the southern South Island, the current distribution could have resulted from either range expansion out of refugia, or fragmentation of a panmictic population. In considering these hypotheses evidence from a number of sources is considered including: the species behavioral ecology, the distribution of distribution of genetic variation in the species, paleo-climatology, and paleo-ecology.

There seems little evidence to support colonisation from the North Island after the last glacial maximum. With the exception of one individual in the northern South Island, divergences between short-tailed bats in the South Island and North Island extend back >0.5 Myr. The genotype of the aberrant individual diverged from North Island bat genotypes >100 kyrs. Additionally, although the existence of an extensive land connection between the North and South Islands until about 12 000 years ago has been theorised (e.g., Stevens *et al.* 1995), recent evidence indicates that there was no significant interchange of terrestrial fauna between the two islands throughout the late Pleistocene (Worthy & Holdaway 1994). Thus, any land connection between the two islands during this period was probably only limited and intermittent.

Low levels of genetic differentiation among South Island populations argues for high levels of gene flow among them, either by panmictic mixing or a recent expansion event. Given the high degree of differentiation among neighbouring populations in the North Island and the absence of observations of large-scale movements by short-tailed bats, panmictic mixing seems unlikely in this species.

Following range expansion from a single source population, highest levels of genetic diversity will usually be found in the source population (Avise 2000). Thus, it may be argued that the higher level of diversity found in the Codfish Island population is evidence of northward expansion from southern South Island. However, in this case it is more likely that higher diversity on Codfish Island reflects the sympatric occurrence of two distinct lineages with divergence extending back 0.5 Myrs. Divergences of that depth are unlikely to be retained in a single population, especially given the bottlenecks a population persisting through glacial maxima in the southern South Island would have been subject to. I therefore argue that sympatric lineages on Codfish Island are the result of secondary contact between lineages expanding from separate refugia.

Evidence from recent fossils (Daniel & Williams 1984; Daniel 1990; Worthy & Holdaway 1993; Worthy & Holdaway 1994; Worthy & Holdaway 1995; Worthy & Holdaway 1996) indicates that prior to human arrival short-tailed bats were widespread

throughout the South Island mainland, but as a result of the impact of human introduced species, and, or, widespread anthropogenic forest destruction (especially in areas with favoured microclimates), only two small isolated populations persist (Lloyd 2001). Thus, it is likely that bat populations no longer remain in areas that contained glacial refugia. Their absence renders attempts at identifying the locations of these refugia problematic.

To persist, populations of short-tailed bats require large mature trees (or possibly caves) for colonial roosts adjacent to extensive areas of forest for foraging. Thus, refugia for short-tailed bats during the last glacial maximum were probably in areas where extensive areas of old-growth forest persisted. During the last glacial maximum ice covered much of the South Island and most remaining areas were dominated by grasslands and shrublands. Extensive areas of forest only occurred in the north west of South Island, though small patches may have persisted in favourable microclimates elsewhere (McGlone *et al.* 1993; Stevens *et al.* 1995; McGlone *et al.* 1996).

Extant southern populations in coastal forest on Codfish Island and, until recently, the Titit Islands, appear to be close to the limits of the species climatic range. Indeed, they are probably only able to persist due to nutrient enrichment from large sea bird colonies. During glacial maxima mean annual temperatures in the South Island were 4.5-5°C lower than at present and drought, strong winds and polar air-masses (McGlone *et al.* 1993; McGlone *et al.* 1996) made condition even more inhospitable for bats.
In conclusion, although the persistence of short-tailed bat populations in southern South Island throughout the last glacial maximum can not be discounted, post-glacial range expansion from multiple refugia in northern South Island seems more plausible.

THE LIMITATIONS OF CURRENT PHYLOGENETIC TECHNIQUES FOR INTRASPECIFIC STUDIES

Studies of genetic variation within species frequently use traditional methods of phylogenetic reconstruction developed for estimating interspecific relationships (Hare 2001). Difference in the nature of evolutionary relationships above and below species levels lead to difficulties in the use of traditional phylogenetic methods for estimating genealogies within species (Posada & Crandall 2001). Traditional phylogenetic methods assume bifurcating trees with all extant haplotypes at tips of the tree. In contrast, intraspecific genealogies are often multifurcating with descendant and ancestral

haplotypes coexisting (Crandall & Templeton 1996; Posada & Crandall 2001). The application of traditional phylogenetic methods to intraspecific phylogenies is further compromised by lower levels of variation within species, the occurrence of reticulations (resulting from recombination, hybridisation, or homoplasy), and the large number of individuals typically used in intraspecific studies. Although many of these problems occur in recovering higher-level genealogies, they are more acute for recovering within species phylogenies. Two other features of the data used for single-species studies limit the accuracy of phylogenetic estimation. Because large numbers of individuals are used in most single-species studies, typically only small regions of a single gene are examined. Cummings *et al.* (1995) showed that analyses of individual mitochondrial gene sequences rarely gave the whole genome tree. Sequences approaching half the mitochondrial genome length were required to reliably obtain a tree identical to the whole genome tree. To obtain informative genetic markers for single-species studies, small regions with high recurrent mutation rates are chosen. Consequently, data sets are commonly homoplasic for the most polymorphic characters (Smouse 1998). The ensuing phylogenetic ambiguity confounds the choice of best trees from the frequently numerous alternative trees. Recently, a variety of networking approaches have been developed to accommodate the characteristics of data used in single-species studies (Templeton *et al.* 1992; Excoffier & Smousse 1994; Strimmer & von Haesseler 1996; Bandelt *et al.* 1999; Posada & Crandall 2001).

Phylogenetic studies of single-species commonly attempt to infer the demographic history of populations from the current geographic distribution of genealogical lineages (Avise 2000; Emerson *et al.* 2001). Because of the diversity of possible historic events (e.g. climatic, tectonic and biotic) and the stochastic nature of coalescent processes, such inferences are not easy. Methods that provide statistical frameworks for estimating historical demographic parameters assume unrealistically simple underlying population histories, whereas methods that consider realistically complex historical processes do not provide statistical framework for distinguishing among alternative hypotheses, or quantify uncertainty about conclusions (Knowles & Maddison 2002). An example of the latter is nested clade analysis (Templeton 1998), which has been promoted as a phylogenetic method able to infer complex historical processes within species. Simulation studies by Knowles & Maddison (2002) showed the method could not reliably distinguish between distinctive alternative processes.

A SUMMARY OF ATTEMPTS TO EXTRACT ANCIENT DNA FROM GREATER SHORT-TAILED BATS

Introduction

Dwyer (1962) described two subspecies of short tailed bat: *Mystacina tuberculata tuberculata*, found throughout much of New Zealand, and a larger subspecies *M. t. robusta*, restricted to the Titi Islands, off the coast of Stewart Island. Hill & Daniel (1985) subsequently elevated the subspecies to species status as the lesser short-tailed bat *Mystacina tuberculata* and the greater short-tailed bat *M. robusta*. The two species occurred sympatrically on the Titi Islands. There have been no confirmed sighting of greater short-tailed bats since 1967, when bats disappeared from the Titi islands following the arrival of ship rats *Rattus rattus* (Daniel 1990).

The species were defined on the basis of overall size and proportions of morphological characteristics measured on museum specimens, but separation between the species is not clear-cut. The sample sizes used in the comparisons were small and the data were presented without sample variances, or statistical analysis. There was no adjustment for the age or gender of the specimens. There are considerable overlaps in the values of many variables.

Methods

Tissue samples were obtained from nine specimens of *Mystacina* (DM 1083, 1553, 1554, 1555[1 & 2] and 1629[1–4]) held by the National Museum, Wellington, New Zealand. All specimens were collected on the Titi Islands between 1955 and 1965. Specimen DM 1083 is the holotype for *M. robusta* (Dwyer 1962). (Hill & Daniel 1985) identified specimen 1629[1] as southern lesser short-tailed bat *M. t. tuberculata*, and the other eight specimens as greater short-tailed bats, *M. robusta*. I believe Hill & Daniel (1985) were wrong in identifying specimen 1629[1] as belonging to a separate species. Although the specimen is smaller than the other specimens, development of the finger joints indicate the individual was a juvenile (Lloyd 2001), whereas the others were adults.

The specimens were in alcohol, but have probably been fixed in formalin. Before DNA extraction, 10-25 mg portions of the tissue samples were rinsed in phosphate-buffered saline (PBS). The samples were drained and placed in PBS for 2 hours before being drained again and then rinsed in ultrapure water. DNA was extracted using

DNeasy™ Tissue Kits (Qiagen). I attempted to amplified the four fragments (5′CR, 12S rRNA, 16S rRNA, and ND2) using the primers and reaction conditions described in Chapter 3. Because of the low concentrations of amplification products, it was necessary to reamplify the original amplification products following gel extraction and purification (QIAquick® Gel Extraction Kit; Qiagen). Both negative and positive controls were used throughout the procedures. Extraction and amplification procedures were repeated for each individual on three separate occasions.

Results

No amplification products were obtained for the ND2 fragment. Low yields of amplification products were obtained for the other three fragments from most individuals on at least one occasion. Sequences of these products were obtained following reamplification. Most sequences were of poor quality and were discarded. Remaining sequences were aligned with comparable lesser short-tailed bats sequences. The only features distinguishing the two sets of sequence were ambiguities in the "greater short-tailed bat" sequences. The ambiguities occurred at variable sites in the lesser short-tailed bat sequences. Close inspection of the chromatograms revealed multiple peaks at each of the ambiguous sites.

Conclusion

The "greater short-tailed bat" sequences obtained in this study were probably a result of multiple contamination with DNA from lesser short-tailed bats previously extracted and amplified in this laboratory. The methods used in this study do not meet the criteria for rigorous ancient DNA studies (Cooper 2000). To achieve a rigorous study the work should be undertaken in a physically isolated area, where other bat DNA has not been extracted and amplified. The procedures should be replicated both in the original laboratory and in a separate laboratory. Negative controls should be used throughout the procedure, but positive controls should not be used at any stage. Shorter DNA sequences (<500 base pair) should be targeted for amplification, as these are more likely to remain intact in ancient DNA.

THE EFFICACY OF CONTROL REGION SEQUENCES

The control region is the most rapidly evolving portion of the mammalian mitochondrial genome, with substitution rate estimates three to five times higher than for the remainder of the mitochondrial genome (Gemmel *et al.* 1996; Avise 2000). Resulting high levels of intraspecific polymorphism make control region sequences a powerful tool for intraspecific genetic studies. Consequently control region sequences, especially from the rapidly evolving 5'peripheral domain, or CR-left, have been widely used to examine intraspecific phylogenies and investigate population dispersal patterns in many mammal species (e.g., Worthington Wilmur *et al.* 1994; Palumbi 1996; Wilkinson & Fleming 1996; Petri *et al.* 1997; Avise 2000; Ingman *et al.* 2000). Control region sequences are considered especially useful for phylogenetic analyses over microevolutionary time scales of thousands or tens of thousands of years (Avise 2000).

Although the control region is a non-coding region, it is highly structured, containing the major regulatory elements for the replication and expression of the mitochondrial genome (Douzery & Randi 1997; Sbisa *et al.* 1997). Substitutions are not randomly distributed throughout the control region. Indeed substitution rates within the control region are extremely heterogeneous: a few hot spots evolve rapidly while most sites have very low rates of change (Excoffier & Yang 1999). Conserved elements such as the termination associated sequences (TAS) and conserved sequence blocks (CSBs) appear to be subject to strong selective constraints and have extremely low substitutions rate. Other sites, particularly in the peripheral domains, have high substitution rates; rapidly accumulating point mutations, indels and variable numbers of tandem repeats (Douzery & Randi 1997). Even within rapidly evolving regions individual substitutions are not independent, indicating that there is selective pressure to maintain secondary structure required for control region function (Howell *et al.* 1996).

Estimates for the rate evolution of the control region obtained using phylogenetic approaches (Pesole *et al.* 1992; Stoneking *et al.* 1992; Tamura & Nei 1993; Horai *et al.* 1995) range between 0.07 and 0.22 substitutions per site per million years (s/s/Myr). Genealogical approaches indicate control region may evolve considerably more rapidly with estimated substitution rates of 2.5 and 2.6 s/s/Myr in humans (Howell *et al.* 1996; Parsons *et al.* 1997). The disparity between substitution rate estimates from phylogenetic and genealogical methods may arise because genealogical methods measure movement through oscillating character states whereas phylogenetic methods measure the distance between initial and final character states, irrespective of the

number of intervening changes. Though, Parsons *et al.* (1997) indicate further explanation is required and suggests rapid sequence substitution in the short-term genealogical studies is masked by uncharacterised factors (drift or selection) when more highly diverged sequences are considered.

With the high substitution rates estimated by genealogical methods concentrated in a small number of "hot spots" high levels of homoplasy will arise rapidly. High levels of homoplasy among sequences can confound distance estimation and phylogenetic inference (Maddison *et al.* 1992; Tamura & Nei 1993; Ingman *et al.* 2000). The co-evolution of multiple substitutions to maintain secondary structure would exacerbate the problem. Given the high substitution rates reported by Parsons *et al.* (1997) and Howell *et al.* (1996) it seems likely that control region sequences may not be reliable even for divergences as recent as thousands or tens of thousands of years, though this has not been tested experimentally. Ingman *et al.* (2000) and the study reported in this thesis provide examples of intraspecific phylogenetic analyses where mitochondrial sequences from outside the control region provide more robust data than control region sequences. In the absence of suitable analytic models incorporating rate heterogeneity and co-evolution of sites within the control region sequences, sequences from mitochondrial protein coding regions should be preferred for intraspecific investigations where there is sufficient variation. Third codon sites, which have rapid substitution rates and relatively little rate variation, appear well suited to investigations of recent divergences.

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